A New Assay for the Microsomal Metabolism of Nitrosamines

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ABSTRACT

This report describes a new general assay for the microsomal oxidative dealkylation of nitrosamines. After precipitation of the microsomal proteins, aldehydes formed from nitrosamines are quantitated by high-pressure liquid chromatography as their 2,4-dinitrophenylhydrazones. This novel assay method offers some distinct advantages over the commonly used Nash reagent assay. Dimethylnitrosamine, diethylnitrosamine, and methylxylethyl nitrosamine were metabolized by microsomes from noninduced male Fischer rat liver, and the aldehydes produced by these reactions were examined. Two distinct kinetic lines were observed for reactions containing dimethyl nitrosamine or diethylnitrosamine. Similarly, two $K_m$'s were observed for the production of both formaldehyde and acetaldehyde produced by the metabolism of methylxylethyl nitrosamine.

INTRODUCTION

There is a great interest in the mechanism by which aliphatic nitrosamines exert their carcinogenic action. It is generally believed that the nitrosamines are acted upon by tissue-specific microsomal oxygenases to produce an intermediate activated compound (usually an aldehyde) and a reactive species which can be toxic, mutagenic, or carcinogenic (8, 9, 13). After Brouwers and Emmelot (3) demonstrated the formation of formaldehyde from DMN2 by liver microsomes, much work has been carried out on the oxidative dealkylation of this compound. Magour and Nievel (15), as well as Arcos et al. (1), have demonstrated that the microsomal oxidation of DEN produces acetaldehyde.

In general, however, little work has been carried out on other than demethylation or deethylation of nitrosamines because of a lack of adequate assay techniques, especially in the case of those compounds the metabolism of which might be expected to produce more than one aldehyde. A recent study by Chau et al. (4) on the microsomal metabolism of DMN, DEN, and MEN used a procedure (7) to specifically assay for formaldehyde and acetaldehyde, one in the presence of the other. The method, however, is not appropriate for the measurement of other aldehydes. To date, this is the only kinetic study which deals with both sides of a nonsymmetrical aliphatic nitrosamine. Chen et al. (5) used DNPH to assay the formation of 2-hydroxytetrahydrofuran formed from the microsomal oxidation of nitrosopyrrolidine. However, no precautions were taken to eliminate the extremely high backgrounds which are observed in the HPLC elution pattern in the area under which short-chain aldehyde derivatives elute. Thus, the method is not suitable to a general analysis of nitrosamine metabolism.

The method used in this report has its basis in the analytical procedure outlined by Selim (18) to measure the aldehydes produced by the microsomal oxidation of nitrosamines, and more importantly, is applicable to the study of a wide range of nitrosamines. The method takes advantage of the fact that 2,4-dinitrophenylhydrazones partition from an aqueous solution into isooctane, while DNPH does not. The derivatives can then be separated and quantitated by HPLC.

MATERIALS AND METHODS

DMN, DEN, and MEN were provided by Dr. W. Lijinsky and Dr. J. E. Saavedra of this institute. All 3 were distilled before use and were at least 99% pure as determined by HPLC.

Glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XV), NADP, Tris, zinc sulfate, and semicarbazide hydrochloride were obtained from Sigma Chemical Co. (St. Louis, Mo.). DNPH was a product of Eastman Kodak Co. (Rochester, N. Y.). Thymolphthalein, phenolphthalein, and standard HCl solution were obtained from Fisher Scientific Co. (Pittsburgh, Pa.). Acetaldehyde and sodium sulfite were purchased from Mallinkrodt, Inc. (St. Louis, Mo.). Paraformaldehyde was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Acetonitrile and isooctane were obtained from Burdick & Jackson Laboratories, Inc. (Muskegon, Mich.) and, before use, were redistilled from DNPH to remove possible traces of any interfering aldehydes and ketones. All water used in these experiments was redistilled from KMnO$_4$. Before addition to reaction mixtures, water was saturated with oxygen.

Microsomes were prepared from livers of noninduced male Fischer rats (10 to 15 weeks old, 200 to 250 g), obtained from the Frederick Cancer Research Center rat colony, as previously described (10). Immediately before use, the frozen microsomes were again centrifuged at 100,000 x g for 1 hr and resuspended in the reaction mixture. The protein content of reactions was determined by the method of Lowry et al. (14) on 5% trichloroacetic acid-precipitable material using bovine serum albumin as a standard.

Nitrosamine Assay. All assays were carried out at 37°C in a shaking water bath in 25-ml Erlemeyer flasks fitted with air-tight rubber septa. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), an NADPH-generating system (1 mM NADPH, 5 mM glucose 6-phosphate, glucose-6-phosphate dehydrogenase, 0.2 unit/ml), 5 mM semicarbazide-HCl, 1 mM EDTA, 1.4 to 2.0 mg microsomal protein, and the appropriate quantity of nitrosamine. The nitrosamines (in 0.1 ml) were placed in the flasks, which were then covered. The reactions were started by injection of the remainder of the reagents in 0.9 ml. After 20 min, the reactions were terminated by injecting 1 ml of saturated Ba(OH)$_2$ and 1 ml of ZnSO$_4$ solution. The 2 solutions were standardized according to the method of Somogyi (19) using phenolphthalein as an indicator so that 1 ml of the ZnSO$_4$...
solution would neutralize exactly 1 ml of the saturated Ba(OH)$_2$.
Control reactions either contained heat-inactivated microsomal
proteins or were carried out in the absence of nitrosamine. The
flasks were allowed to stand on ice for 1 hr, and the contents
were centrifuged to pellet the precipitated protein. The resulting
supernatants were used to assay for the presence of aldehydes
according to a modification of the method of Selim (18). Fifty-
m1 screw-capped test tubes containing Teflon liners were used
to carry out the reaction. An aliquot of the supernatant was
diluted to 10 ml with water. DNPH reagent (250 mg DNPH in
100 ml 6 N HCl) was filtered through glass wool, and 0.4 ml
was added to the tube. The aqueous layer was overlayed with
5 ml of isooctane. Both the water and isooctane had been
saturated with each other before use. The test tubes were
shaken horizontally for 30 min. Three ml of the isooctane layer
were removed, and DNPH derivatives contained therein were
removed by extraction with 1 ml of acetonitrile, which was
evaporated to dryness with a stream of N$_2$. The residue was
redissolved in acetonitrile, and aliquots were analyzed by HPLC
on a Waters Associates apparatus equipped with a Du Pont
Zorbax ODS reverse-phase column. Elution was carried out in
60% acetonitrile-water at 1.5 ml/min. Absorbance was moni-
tored at 340 nm. At this wavelength, the extinction of the
formaldehyde derivative is higher than that of acetaldehyde.
The concentrations of both derivatives were calculated from
the peak heights. Low concentrations of aldehydes were as-
sayed using a full-scale deflection on the recorder of 0.1
absorbance unit. This was changed to a full scale of 0.2 or 0.5
at higher concentrations. Under the conditions described here,
the metabolism of all 3 nitrosamines was linear for up to at least
30 min.
Standard curves were prepared by adding known amounts
of formaldehyde and acetaldehyde to tubes and assaying as
described above. Formaldehyde stock solution was prepared
from paraformaldehyde. The concentration of formaldehyde
was determined according to the method of Walker (20).

RESULTS

A typical HPLC pattern of a control reaction carried out in
the absence of nitrosamine is shown in Chart 1a. Arrows 1
and 2 indicate the elution positions of the DNPH derivatives
of formaldehyde and acetaldehyde, respectively. Arrow 3 re-
presents the elution position of a compound the mass spectrum of
which is identical with that of the DNPH derivative of acetone,
which is present in the reaction mixture. Peak 3 was used as
an internal standard against which the remaining peaks were
normalized. This peak remained constant throughout the
course of the reaction. All glassware must be scrupulously
clean, and all solvents must be freshly prepared as outlined in
"Materials and Methods." Deficiencies in either of these areas
will usually result in background patterns from HPLC which
will overwhelm those arising from metabolism. Standard curves
for the estimation of formaldehyde and acetaldehyde are illustrated
in Chart 2. Standards containing up to 8 nmol of aldehyde are
presented, although the method is linear up to the limit of
solubility of the derivative in isooctane (up to 4 mmol). When
standards were incubated in an active microsomal system and
analyzed as outlined in "Materials and Methods," the values
obtained were 100% of those determined from the standard
curves. Microsomal metabolism of the 3 nitrosamines, DMN, DEN,
and MEN, was carried out as described in "Materials and
Methods" over a concentration range of 2.5 to 100 mm. Chart
1, b to d, shows the HPLC patterns produced using 50 mm
concentrations of each nitrosamine after a 20-min reaction at
37°. Chart 1b depicts the results of DMN metabolism. The only increase is in the formaldehyde derivative. Chart 1c shows DEN metabolism in which only the acetaldehyde derivative increases from the blank. However, the metabolism of MEN yields both formaldehyde and acetaldehyde derivatives (Chart 1d). In no case does the acetone derivative increase significantly above control values.

The kinetics for the oxidative dealkylation of MEN to formaldehyde over a concentration range of 2.5 to 100 mM is shown in Chart 3, while Chart 4 records the kinetics of acetaldehyde formation over the same range of substrate concentrations. Neither plot exhibits simple Michaelis-Menten kinetics; each chart shows 2 distinct linear components. The metabolism of both DMN and DEN is similar to that of MEN, in that the double-reciprocal plot exhibits 2 linear components for the formation of formaldehyde and acetaldehyde, respectively (data not shown). The kinetic constants for all 3 compounds are summarized in Table 1.

**DISCUSSION**

To obtain meaningful results with this assay, it is essential that the precautions outlined in "Materials and Methods" are observed. When sufficient care is exercised, the only contaminants found are small quantities of formaldehyde, acetaldehyde, and acetone. The interfering carbonyl derivatives present in the controls can then be subtracted from experimental values. The method, therefore, is convenient for the assay of carbonyl groups which arise from the microsomal metabolism of nitrosamines, including those which are nonsymmetrical. The reproducibility and sensitivity of the method is excellent. For example, in the Nash (16) determination of formaldehyde, the molecular extinction of the adduct is 8,000. At 340 nm, the value for the DNP derivative of formaldehyde is 20,000. The limit of detection is greatly enhanced by chromatography on HPLC. This procedure can detect as little as 0.1 nmol of aldehyde. It has the advantage over other procedures in that it is applicable to the measurement of a wide variety of carbonyl compounds even in a complex mixture.

The fact that 2 separate kinetic curves exist for the oxidative demethylation and deethylation of the nitrosamines studied here offers suggestive evidence for the existence of at least 2 enzymes and is not unprecedented. Lake et al. (12), measuring the in vitro metabolism of DMN by liver microsomes from Wistar rats, found 3 separate $K_m$'s when formaldehyde was measured by the method of Nash (16). Their values ranged from 0.32 to 35 mM. At 129.2 mM were the values of Aroclor-induced animals. The values reported for $V_{max}$ were 0.9 and 2.35 nmol/hr/mg. Chau et al. (4) reported simple linear kinetics for the metabolism of DMN by enzymes from phenobarbital-induced Sprague-Dawley rats with a $K_m$ of 118 mM and a $V_{max}$ of 11 nmol/min/mg. For the same reaction, Kroeger-Koepke and...
Michejda (11) reported a $K_m$ of 1.48 mM and a $V_{max}$ of 2.26 nmol/min/mg for noninduced Long-Evans rats and a $K_m$ of 0.149 mM and a $V_{max}$ of 0.280 nmol/min/mg for noninduced Sprague-Dawley rats. In this study, formaldehyde was measured by the method of Nash (16).

Chau et al. (4) reported simple kinetics for the metabolism of DEN by phenobarbital-induced Sprague-Dawley rat microsomes with a $K_m$ of 8.1 mM and a $V_{max}$ of 4.6 nmol acetaldehyde per min per mg. The metabolism of MEN was reported in the same paper. The $K_m$ for the formation of formaldehyde was 48 mM, while that for acetaldehyde was 75 mM. The $V_{max}$‘s were 2.5 and 4.7 nmol/min/mg for the formation of the same aldehydes, respectively. However, in neither case did other than simple Michaelis-Menten kinetics apply.

It is obvious that the kinetic data for the metabolism of nitrosamine remain in a confused state at this time. A number of reasons exist which may be responsible for the confusion: (a) a large number of different strains of animals have been examined; (b) studies have been carried out on noninduced microsomes as well as on microsomes induced by either Aroclor or phenobarbital; (c) reaction conditions generally vary from laboratory to laboratory; (d) until this time, no single assay has been available for the study of the wide range of nitrosamines. Since it is possible to quantitate a large number of aldehydes by the method of Selim (18), the method reported here should provide a general method for examination of the kinetics of most dialkynitrosamines. It should find considerable utility since it is convenient, reliable, and highly reproducible, provided the aforementioned criteria of purity are rigorously observed.

REFERENCES


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