Letter to the Editor

In Vitro Detection of Nitrosamines and Other Indirect Alkylating Agents by Reaction with 3,4-Dichlorothiophenol in the Presence of Rat Liver Microsomes

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SUMMARY

A new method for the detection of indirect alkylating agents, such as the nitrosamines, using 3,4-dichlorothiophenol as trapping agent in the presence of rat liver microsomes is reported. 3,4-Dichlorophenyl alkyl thioethers thus formed during the incubation are detected by the use of a gas chromatography-mass spectrometry combination unit and are quantified by use of external standards. A series of methylalkynitrosamines, used in the present experiments, predominantly give rise to 3,4-dichlorophenyl methyl thioether, as compared with other alkyl thioethers. Branching in the α position of the alkyl chain of the nitrosamines reduces the amount of the alkyl thioethers formed. Symmetrical dialkylnitrosamines, when incubated as above, preferentially give rise to methyl thioether as compared with the corresponding alkyl thioethers. The results show a good relationship between carcinogenicity and alkylating capacity of the nitrosamines. These findings are also in agreement with the in vivo studies on alkylation of nucleic acids with different nitrosamines. This method can be used as a rapid screening technique for detection of indirect alkylating agents.

INTRODUCTION

Most chemical carcinogens and mutagens require in vivo activation, mainly by so-called “drug-metabolizing enzymes” to form reactive electrophilic (“alkylating”) intermediates (9, 10). In some instances, the reaction products of such “ultimate” carcinogens with biopolymers, mainly with nucleic acids, have been identified in in vivo experiments (9). The inherent difficulties in such types of alkylation experimentation prompted us to look for a simple technique to detect indirect alkylating agents.

We report here our 1st results on the use of 3,4-dichlorothiophenol to trap the electrophiles that had been formed in vitro from dialkynitrosamines in the presence of rat liver microsomes. The reagent has recently been used by Göthe et al. (4) in studying the metabolism of vinyl chloride. These authors report that 3,4-dichlorothiophenol is not metabolized itself and does not decrease the activity of liver microsomal preparations. Furthermore, chlorine atoms in the reagent facilitate the interpretation of formed alkyl thioethers by GC and mass spectrometry.

MATERIALS AND METHODS

The nitrosamines used were synthesized by known methods and purified by repeated fractional distillation. Their purity was checked by GC using a very sensitive N-specific detector system (14). Methylethylnitrosamine contained traces of dimethylnitrosamine (100 ppb, 0.01%) while diethyl- and methylisopropylnitrosamines were contaminated to the extent of 40 ppb (0.004%) of dimethylnitrosamine. All other nitrosamines were chromatographically pure to a detection limit of 1 to 10 ppb. 1,2-Dimethylhydrazine and 3,3-dimethyl-1-phenyltriazene also did not contain detectable impurities.

Preparation of Rat Liver Homogenate “S-9” Fraction (3). Female Sprague-Dawley rats (200 to 250 g) on a standard pelleted diet received 0.1% sodium phenobarbital in the drinking water for 3 days. They were killed by cervical dislocation and the livers were removed immediately. All further steps in the preparation were performed at 0-4°C. After being rinsed with 0.15 M KCl, the livers were minced in 15 ml of O₂-saturated phosphate buffer, pH 7.4, per 5 g liver, wet weight, and homogenized with a Teflon pestle in a Potter-Elvehjem apparatus. The homogenate was centrifuged for 10 min at 9000 x g and the supernatant S-9 fraction was used for such incubations. Preparations from 20 to 30 rats were pooled and stored in 15-ml portions at -80°C until they were used.

Incubation Experiments. A 15-ml portion of the S-9 fraction that contained an equivalent to 5 g of wet liver was used for every incubation and fortified with the necessary NADPH-generating system (7.5 mg NADP⁺, 58 mg glucose 6-phosphate, 75 mg nicotinamide, 322.0 mg MgCl₂, and 25 µl glucose-6-phosphate dehydrogenase; Boehringer,
Formation of 3,4-dichlorophenyl alkyl thioethers from 50 μmoles of substrate after 90 min incubation in the presence of rat liver microsomes, NADPH-generating system (pH 7.4 at 37°C), and 3,4-dichlorothiophenol (500 μmoles) as trapping reagent.

Results given are the average of a minimum of 3 experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Formula</th>
<th>Alkyl 3,4-dichlorophenyl thioether formed (μmoles)</th>
<th>Quantity of thioether formed (μmoles)</th>
<th>% of theoretical yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl-NA</td>
<td>CH₃</td>
<td>Methyl</td>
<td>2.0</td>
<td>4.1</td>
</tr>
<tr>
<td>Methylethyl-NA</td>
<td>CH₃</td>
<td>Methyl</td>
<td>1.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Methyl-n-propyl-NA</td>
<td>CH₃</td>
<td>Methyl</td>
<td>4.8</td>
<td>9.6</td>
</tr>
<tr>
<td>Methyl-isopropyl-NA</td>
<td>CH₃</td>
<td>Methyl</td>
<td>2.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Methyl-n-butyl-NA</td>
<td>CH₃</td>
<td>Methyl</td>
<td>2.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Methyl-isobutyI-NA</td>
<td>CH₃</td>
<td>Methyl</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Diethyl-NA</td>
<td>C₂H₅</td>
<td>Methyl</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Di-n-propyl-NA</td>
<td>C₃H₇</td>
<td>Methyl</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Di-n-butyI-NA</td>
<td>C₄H₉</td>
<td>Methyl</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Methyl-tert-butyl-NA</td>
<td>CH₃</td>
<td>Methyl</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,2-Dimethylhydrazine</td>
<td>CH₅-NH-NH-CH₃</td>
<td>Methyl</td>
<td>1.3</td>
<td>2.6</td>
</tr>
<tr>
<td>3,3-Dimethyl-1-phenyltriazene</td>
<td>C₆H₅- N=N-N(CH₃)₂</td>
<td>Methyl</td>
<td>0.6</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* NA, nitrosamine.
Mannheim, Germany) in 5 ml of phosphate buffer, pH 7.4. To this mixture were further added 50 μmoles of the substrate in 1 ml of buffer and 500 μmoles of 3,4-dichlorothiophenol in 1 ml of dimethyl sulfoxide. The mixture was incubated at 37° in a closed vessel and shaken in an atmosphere of oxygen. After 90 min, ether (25 ml) was added, proteins were precipitated with methanol (5 ml), and the mixture was centrifuged for 15 min at 6000 × g. The organic layer was separated and the aqueous phase was reextracted 4 times with ether (20 ml). The combined ether extracts were dried with Na₂SO₄, and reduced to 10 ml.

**Detection of 3,4-Dichlorophenyl Alkyl Thioether.** For this detection a GC-mass spectrometry combination system (LKB-9000) was used. Thioethers were separated on GC (3-m x 2.2-mm glass column filled with 3% OV 1 on 100/120 mesh Gaschrom Q. Flash heater and separator were held at 220°, the column was at 180°, the carrier gas (helium) flow was 20 ml/min), and the derivatives were identified by specific ion detection of the M⁺ ions. Pure reference compounds were used to check retention times (methyl thioether, 255 min; ethyl, 305 min, n-propyl, 337 min, and n-butyl, 410) and quantification. Results were corrected for a small but constant background level (0.1 to 0.2 μmole) of apparent methyl thioether formation in the blank experiments carried out without carcinogen addition. This value was constant for each pool of microsomal preparations. Incubations with 3,4-dichlorothiophenol and nitrosamines, under the same conditions but without microsomal fraction, did not show alkyl thioether formation.

**RESULTS AND DISCUSSION**

The results obtained with 10 nitrosamines, 1,2-dimethylhydrazine, and 3,3-dimethylphenyltriazene are summarized in Table 1. The 2 latter compounds are also known to be indirect alkylating agents with potent carcinogenic activity (12). All carcinogenic nitrosamines presently tested formed alkylating intermediates that could be detected as the corresponding 3,4-dichlorophenyl alkyl thioethers. It was remarkable that the nitrosamines carrying at least 1 methyl group and another longer alkyl chain preferentially formed methyl thioether, while the longer alkyl chain was transferred to an alkylating moiety to a much lesser degree. Magee and Lee (8) reported that the administration of methyl-n-butylnitrosamine in vivo also resulted in the preferential formation of N-7-methylguanine.

The results show that chain branching at the α position decreases methylation of the reagent. The noncarcinogenic methyl-tert-butyl nitrosamine does not alkylate under the reaction conditions used. These results indicate good correlation between carcinogenicity (2) and alkylating capacity of nitrosamines. Amidopyrine as a substrate, which is easily demethylated with liver microsomal fraction, does not give evidence of methylation.

Methyl-n-propylnitrosamine gave maximum yield of the methyl thioether. Determination of unreacted carcinogen after incubation, however, showed that 80% of the added nitrosamine could be recovered. A level of 50% methylation is therefore found if the amount of nitrosamine metabolized is used as a basis for calculation instead of the actual amount added, as in Table 1.

The symmetrical diethyl-, di-n-propyl-, and di-n-butylnitrosamines also form preferentially methyl thioether in the present experiments. These findings are in agreement with in vivo experiments with the latter 2 compounds demonstrating 7-methylguanine as a preferential reaction product with nucleic acids (6). However, they are not in accordance with observations in vivo in the case of diethylnitrosamine (8). Alkyl chain degradation (1, 11) has also been demonstrated in metabolic studies using di-n-butyl nitrosamine. We further found ethylation, propylation, and butylation with the corresponding nitrosamines, although the yields of the alkyl thioethers were low. These findings were also in agreement with in vivo results (6).

It has been suggested that 7-methylguanine formation in vivo after administration of diethylnitrosamine might be due to contamination with dimethylnitrosamine (13). The present experiments excluded this possibility since all of the nitrosamines used as substrates were checked for contamination of dimethylnitrosamine or methylalkyl nitrosamines. Impurity was detected only in 3 nitrosamines, but the amount of contamination was too low to account for the quantities of methyl thioethers formed. These results can be explained by assuming that the nitrosamines are metabolized to alkylating species which then react with the thiol to give the corresponding alkyl thioethers. We intend to check whether long-chain alkyl thioethers are degraded to shorter-chain alkyl thioethers, such as methyl thioether, in the presence of liver microsomes.

Finally, the methylating activity of 1,2-dimethylhydrazine and 1-phenyl-3,3-dimethyltriazene demonstrate the possible versatility of the test for the detection of indirect alkylating agents. These results show good correlation with results in intact animals of DNA alkylation (5, 7). In view of our present results we think that 3,4-dichlorothiophenol may be a useful reagent for in vitro identification of indirect alkylating agents in a simple screening system.

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**REFERENCES**


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