Oxidative in vitro metabolism of N,N-dibutylnitrosamine (DBN) by 9000g supernatant fraction (S9 fraction) prepared from rat liver and oxidation of DBN by chemical model systems (Udenfriend and Fenton) were investigated. The products retaining the N-nitroso group of DBN were analyzed by gas-liquid chromatography. Incubation of DBN with the S9 fraction gave hydroxy derivatives of DBN at the \( \omega, \omega-1, \) and \( \omega-2 \) positions, while the chemical reaction of DBN in the Udenfriend and the Fenton model systems afforded compounds with an oxo group at the same positions. In all cases, \( \omega-1 \) oxidation was predominant, followed by \( \omega \) and then by \( \omega-2 \) oxidations, while \( \omega \) and \( \omega-1 \) oxidations were the principal metabolic pathways of DBN in vivo in the rat. The differences in the oxidative transformation of DBN among in vitro enzymatic, non-enzymatic and in vivo systems are discussed.

Key words: In vitro metabolism — Dibutylnitrosamine — S9 fraction — Fenton system — Udenfriend system

DBN\(^*\) is a potent carcinogen which induces tumors of the liver, esophagus, and urinary bladder in the rat when fed in the drinking water.\(^3\) Metabolic study of DBN in the rat has revealed that it undergoes transformation in vivo in at least three ways.\(^16\) The \( \omega \)-oxidation pathway, the major one, is responsible for the induction of urinary bladder tumors, while the \( \omega-1 \) and \( \omega-2 \) oxidation pathways are considered to be involved in the induction of liver tumors.

In vitro metabolism of DBN, on the other hand, has not been well elucidated. Blattmann and Preussmann\(^2\) identified butyraldehyde as a principal product together with other aldehydes after in vitro incubation of DBN with rat liver microsomes. Chemical model systems were also used for the oxidation of N-nitrosamines. Preussmann\(^12\) first used the Udenfriend system as a model for the metabolism of several N,N-dialkylnitrosamines including DBN, and later this system was employed by Rayman et al.\(^13\) and by Hsieh et al.\(^4\) for the oxidation of N-nitrosopiperidine and N,N-diethylnitrosamine, respectively. Oxidative conversion of N-nitrosomorpholine by the Fenton system was reported by Manson et al.\(^7\)

In connection with our studies on the metabolic fate of DBN and related compounds in relation to their organotropic carcinogenicity, we have further investigated the metabolism of DBN by rat liver S9 fraction and its oxidation by chemical model systems,
the Udenfriend and the Fenton oxidation systems, in order to elucidate the in vitro transformation of DBN and to compare it with that in vivo.

MATERIALS AND METHODS

Chemicals All the N-nitroso compounds used in the present experiment were synthesized according to the procedures described elsewhere. NADP was purchased from Oriental Yeast Co., Tokyo. Glucose 6-phosphate was obtained from Sigma Chemical Co., St. Louis. Salmonella typhimurium strain TA1535 was kindly supplied by Dr. B. N. Ames, University of California, Berkeley, Calif.

Preparation of Liver S9 Fraction Male Sprague-Dawley rats (Charles River Japan Inc.), 6 weeks old, weighing 140-180g, were given phenobarbital (0.1%) in their drinking water for 7 days before they were killed. PCB (Kanechlor-500) in corn oil was administered by a single intraperitoneal injection at a dosage of 500 mg/kg body weight 5 days before sacrifice. The supernatant fraction (S9) was obtained aseptically from the liver homogenate (5 ml of 0.15M KCl/g liver) by centrifugation at 9000 g for 10 min, as described previously. The S9 fraction was stored in sterile screw-capped tubes in 3 ml portions at -80°C until use. Protein content of the S9 fraction was 30-33 mg protein/ml as determined by the method of Lowry et al.

Oxidation of DBN by S9 Mix The S9 mix consisted of S9 fraction (3 ml) and 0.1M sodium phosphate buffer (pH 7.4, unless otherwise indicated), 8mM MgCl₂, 33mM KCl, and an NADPH generating system (4mM NADP, 5mM glucose 6-phosphate) to make a final volume of 10 ml. A mixture of DBN in dimethyl sulfoxide (300 µl) and S9 mix (5 ml) in a 25-ml brown test tube was incubated under shaking (150 strokes/min) at 37°C. After the incubation, 6N HCl (60 µl) was added to the reaction mixture and the whole was extracted with ethyl acetate (5 ml). The organic phase separated by centrifugation at 4°C was dried over anhydrous Na₂SO₄, and then analyzed for N-nitroso compounds by GLC.

b) Fenton system: DBN in acetone (500 µl) was added to 5 ml of a solution containing 50mM FeSO₄ and/or 50mM Cu(OAc)₂ in acetate buffer (pH 4.7, ionic strength 0.2) that had been saturated with nitrogen by bubbling. The headspace of the 25-ml brown test tube was flushed with nitrogen, and then 3% hydrogen peroxide (0.3 ml) was added to the solution. The mixture was treated in the same way as described above.

Chromatography GLC of N-nitroso compounds was carried out on a Shimadzu GC-4APG gas chromatograph equipped with a flame ionization detector. A glass column of 2% Silicone GE XF1105 coated on Chromosorb W (60 to 80 mesh, 2.5 m × 4 mm) was used. The column temperature was programmed at 80°C for 10 min and then 4°C/min to 180°C; injection port temperature 150°C, detector temperature 220°C; nitrogen carrier gas (30 ml/min). BCMN, BCPN, and BHCPN were analyzed as their methyl esters and BFPN as N-butyl-N-(4-oxoamyl)nitrosamine, after treatment of the ethyl acetate solution with diazomethane. The ethyl acetate extract (0.6 ml) was mixed with ethyl acetate solution containing 2.5-10mM PBN and 1.5mM methyl ester of BCPN (0.2 ml) as internal standards for the analysis of DBN and BHBN-3 or BOBN-3, respectively. On the other hand, the ethyl acetate extract (2.0 ml) was treated with diazomethane in ether for 1 hr at room temperature. The reaction mixture was concentrated in vacuo, then an ethyl acetate solution of 0.5mM PBN (0.2 ml) was added to the residue as an internal standard for the analysis of the other nitrosamines. Under the chromatographic conditions used, the retention times (min) of authentic specimens were as follows: PBN (18.6), DBN (22.4), BOPN (24.9), methyl ester of BCMN (25.4), BOBN-2 (27.6), BOBN-3 (28.2), BHBN-2 (29.2), BHBN-3 (30.1), N-butyl-N-(4-oxoamyl)nitrosamine (31.8), methyl ester of BCPN (32.1), BBN (33.0), methyl ester of BHCPN (35.4). The amounts of each product and remaining of authentic specimens were determined by comparison of their peak heights with those of standards. In order to obtain a calibration curve, authentic samples were added to the incubation mixture without preincubation, and in the case of the Fenton system, hydrogen peroxide was replaced by water.
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Mutation Test  The mutation test was carried out as described by Ames et al., with some modifications. DBN was dissolved in dimethyl sulfoxide (25 μl) and was preincubated with 0.5 ml of S9 mix and 0.1 ml of bacterial culture (1 × 10⁸ cells) at 37°C for 20 min.

RESULTS

Metabolism of DBN by S9 Mix Prepared from Liver of Phenobarbital-treated Rats  DBN (2mM, 4mM, and 8mM) was incubated for 1 to 5 hr with S9 mix prepared from the liver of phenobarbital-treated rats (hereafter referred to as PB-S9 mix). The ethyl acetate extract of the incubation mixture was analyzed by GLC, directly and after treatment with diazomethane (Fig. 1). BHBN-2, BHBN-3, BOBN-3, BBN and BCPN were identified as metabolites by comparing their GLC behavior with that of authentic specimens. No product was detected when DBN was omitted from the incubation mixture. BHBN-3 was the major metabolite, and its formation after incubation of various concentrations of DBN for 1 hr is shown in Fig. 2. The metabolites produced from DBN (8mM) and identified are indicated in Table I.

As shown in Fig. 2, the formation of BHBN-3 increased almost linearly with concentration of DBN up to 4mM. It also increased with incubation time up to 5 hr, as shown in Fig. 3. It was found further that the conversion of BHBN-3 to BOBN-3 under these experimental conditions also increased with incubation time, and the conversion was the highest when the concentration of DBN was 2mM. The formation of BBN, on the other hand, reached a maximum after 2 hr and its further conversion into BCPN increased grad-

![Fig. 1. Typical gas-liquid chromatogram of the metabolites after incubation of DBN with S9 mix prepared from the liver of phenobarbital-treated rats](image-url)

DBN was incubated with PB-S9 mix at 37°C for 1 hr. A: Ethyl acetate extract of the incubation mixture. I and VI were added as internal standards. B: After treatment of the ethyl acetate extract with diazomethane. I was added as an internal standard. I (PBN), II (DBN), III (BOBN-3), IV (BHBN-2), V (BHBN-3), VI (methyl ester of BCPN), VII (BBN).
The production of BHBN-2 increased slightly with incubation time, while its conversion to BOBN-2 could not be detected. Based on these findings we principally used 4mM DBN and a 1-hr incubation period for the metabolic study with S9 mix.

Formation of the principal metabolite BHBN-3 decreased considerably when S9 mix prepared from the liver of PCB-treated or untreated rats (hereafter referred to as PCB-S9 mix and untreated-S9 mix, respectively) was used instead of PB-S9 mix, as shown in Fig. 3. Moreover, a similar decrease in the formation of BHBN-3 was observed when the incubation was carried out at pH 6.5 instead of 7.4.

<table>
<thead>
<tr>
<th>Oxidation System</th>
<th>Incubation Time (hr)</th>
<th>Products (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S9 mix&lt;sup&gt;b)&lt;/sup&gt;</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Udenfriend</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>Fenton</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Percentage of DBN (8mM) used.<br>
<sup>b)</sup> Prepared from the liver of phenobarbital-treated rats.

Reactions of DBN in Chemical Oxidation Systems

a) Udenfriend system: DBN (4mM or 8mM) was incubated at 37° for 0.5 to 5 hr with the Udenfriend system. The ethyl acetate extract of the incubation mixture was analyzed by GLC, directly and after treatment with diazomethane. BHBN-2, BHBN-3,
BOBN-2, BOBN-3, BBN, BFPN, and BCPN were identified as products by comparison of their GLC behavior with that of authentic samples. No product formation was observed when DBN or FeSO₄ was omitted from the incubation mixture.

BOBN-3 was the major product and its formation was nearly doubled by doubling the concentration of DBN from 4 mM to 8 mM. Production of BOBN-3 increased with incubation time up to 5 hr, with a concomitant decrease of DBN content in the reaction mixture, as shown in Figs. 4a and 4b. The products formed after 5 hr from 8 mM DBN with the Udenfriend system are shown in Table I.

b) Fenton system: DBN (8 mM) was incubated for 0.5 to 2 hr with the Fenton system and modified Fenton systems with additional copper salt (Cu(OAc)₂) or with copper salt in place of the iron salt. The ethyl acetate extract of the incubation mixture was analyzed by GLC as described above. The reaction was completed within 1 hr and further incubation did not affect the formation of the products. The products after a 1-hr incubation of 8 mM DBN with the Fenton system are indicated in Table I. There was no substantial difference between the Udenfriend system and the Fenton system with respect to the products and their formation ratios.

BOBN-3 was again found to be the major product, followed by BHBN-3 and BFPN. No product could be detected in the absence of DBN in the reaction mixture. On the other hand, when hydrogen peroxide was omitted from the reaction mixture, slight formation of BOBN-3 and BFPN was detected. In the modified Fenton systems, the pattern of products was essentially the same, and the production rates of the above three principal products were decreased. These results are summarized in Table II.

**Mutagenicity of DBN** Fig. 5 illustrates the mutagenicity of DBN tested on *S. typhimurium* TA1535 in the presence of S9 mix. When preincubated with untreated-S9 mix,
Table II. Major Products Obtained from DBN (8mM) by Incubation (1 hr) with the Fenton and Modified Fenton Systems

<table>
<thead>
<tr>
<th>Metal salt</th>
<th>DBN remained (μmol)</th>
<th>Products (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BHBN-3</td>
</tr>
<tr>
<td>FeSO₄ (Fenton)</td>
<td>4.68</td>
<td>91</td>
</tr>
<tr>
<td>FeSO₄ + Cu(OAc)₂</td>
<td>5.58</td>
<td>38</td>
</tr>
<tr>
<td>Cu(OAc)₂</td>
<td>4.95</td>
<td>89</td>
</tr>
<tr>
<td>FeSO₄ b)</td>
<td>7.82</td>
<td>2</td>
</tr>
<tr>
<td>FeSO₄ + Cu(OAc)₂ b)</td>
<td>7.52</td>
<td>trace</td>
</tr>
</tbody>
</table>

a) Amount in 1 ml of the reaction mixture.
b) Hydrogen peroxide was omitted from the reaction mixture.

discussion

Incubation of DBN with the S9 mix prepared from liver of phenobarbital- or PCB-treated and untreated rats yielded BHBN-2, BHBN-3, BOBN-3, BBN and BCPN as metabolites (Fig. 6). The positions of oxidation on the butyl chain in these products and the extent of oxidation after incubation of DBN with the S9 mix for 1 hr are summarized in Table III. The major metabolites were hydroxy derivatives of DBN and the extent of oxidation was greatest at the ω-1 position, followed by the ω and then the ω-2 positions. As can be seen in Table III, treatment of rats with phenobarbital or PCB induced ω-1 oxidation selectively, while it had very little effect on ω and ω-2 hydroxylations.

Blattmann and Preussmann2) investigated the metabolism of DBN with rat liver microsomes in the presence of the necessary cofactors, but their primary intention was to identify butyraldehyde as a reaction product formed through ω-hydroxylation. Park and Archer11) identified N-propyl-N-(2-hydroxypropyl)nitrosamine (a product formed by ω-1 oxidation) in addition to propionaldehyde, n-propanol and isopropanol following incubation of N,N-dipropyl nitrosamine with a PB-S9 mix. We also made a preliminary study of the in vitro metabolism of N,N-dipropyl nitrosamine and N,N-diamyl nitrosamine with PB-S9 mix and found that ω-1 oxidation was the preferred metabolic pathway of these dialkynitrosamines.17)

In contrast to the in vivo metabolism of DBN in the rat (Fig. 6),16) further metabolic transformation of BCPN could not be demon-
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Table III. Positions of Oxidation of the Butyl Chain of DBN by Enzymic and Chemical Oxidation Systems

<table>
<thead>
<tr>
<th>Oxidation system</th>
<th>Treatment of rats</th>
<th>Concentration of DBN (mM)</th>
<th>Total oxidation product (nmol)</th>
<th>Product(%) oxidized at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\omega-2$</td>
</tr>
<tr>
<td>S9 mix</td>
<td>Phenobarbital</td>
<td>8</td>
<td>650 (8.1)</td>
<td>17 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>600 (15.0)</td>
<td>19 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>450 (22.5)</td>
<td>14 (3)</td>
</tr>
<tr>
<td></td>
<td>Phenobarbital</td>
<td>4</td>
<td>380 (9.5)</td>
<td>11 (3)</td>
</tr>
<tr>
<td></td>
<td>PCB</td>
<td>4</td>
<td>270 (6.8)</td>
<td>17 (6)</td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>4</td>
<td>230 (5.8)</td>
<td>14 (6)</td>
</tr>
<tr>
<td></td>
<td>Udenfriend</td>
<td>8</td>
<td>1180 (14.7)</td>
<td>120 (10)</td>
</tr>
<tr>
<td></td>
<td>Fenton</td>
<td>8</td>
<td>1010 (12.6)</td>
<td>104 (10)</td>
</tr>
</tbody>
</table>

$\omega-1$ $\omega-3$

$\omega-2$ $\omega-1$ $\omega-3$

$\omega-2$ $\omega-3$

$\omega-1$ $\omega-3$

$\omega-2$ $\omega-3$

a) Percentage of DBN incubated in parentheses.
b) Amount in 1 ml of the incubation or reaction mixture.
c) Percentage of total oxidation product in parentheses.
d) BHBN-2 + BOBN-2
e) BHBN-3 + BOBN-3
f) BBN + BFPN + BCPN
g) Incubation was performed at pH 6.5 instead of 7.4.

Fig. 6. In vitro oxidative transformations of DBN

a) Conversion of BFPPN to N-butyl-N-(4-oxopentyl)nitrosamine for determination by GLC.
b) Further metabolic transformation of BCPN in vivo.16

strated in the in vitro study using the S9 mix. Thus BCPN, the principal urinary metabolite of DBN in the rat,16 could be identified in the present in vitro work as a product, but BHCPN, BCMN and BOPN, which are subsequent transformation products in vivo of BCPN by $\beta$-oxidation according to the Knoop mechanism, could not be detected under the present in vitro experimental conditions. This indicates that the in vitro system was unable to oxidize DBN extensively.

Reaction of DBN in the non-enzymic oxidation systems (Udenfriend and Fenton) yielded compounds oxidized at the $\omega$, $\omega-1$, $\omega-2$.
and ω-2 positions of the butyl chain, i.e., BHBN-2, BOBN-2, BHBN-3, BOBN-3, BBN, BFPN and BCPN. The positions oxidized in these products and their yields are also given in Table III. The major products were oxo derivatives of DBN, in contrast to hydroxy derivatives of DBN obtained with the S9 mix. The preferred position of the oxidation was again ω-1, followed by ω and then ω-2. This pattern of oxidation is similar to that observed in the in vitro system, suggesting that these chemical systems are good models for the oxidation of dialkylnitrosamines with respect to the position to be oxidized.

The obligatory intermediary metabolite BFPN, which is situated between BBN and BCPN in the ω-oxidation pathway of DBN, could not be identified in the in vivo experiment, while it was the principal ω-oxidation product of DBN in the chemical model systems (Table I). This is possibly due to rapid and effective conversion of BFPN to BCPN in vivo. BFPN has been synthesized chemically,14) and its determination was carried out by GLC after conversion to N-butyl-N-(4-oxoamyl)nitrosamine by treatment with diazomethane (Fig. 6).

Oxidation of several N,N-dialkylnitrosamines including DBN by the chemical model systems was first investigated by Preussmann,12) who observed no reaction with the Fenton system, though several products were formed from DBN in the Udenfriend system as determined by thin-layer chromatography (these products were not identified, however). Rayman et al.,13) on the other hand, investigated the oxidation of N-nitrosopiperidine using the Udenfriend system, and the result was compared with that obtained in an in vitro metabolic study of the cyclic nitrosamine with rat liver microsomes. The principal products identified in these studies were N-nitroso-4-piperidone and N-nitroso-4-hydroxypiperidine in the Udenfriend and the microsomal systems, respectively. This correlates well with the present result that the chemical model system mainly gave ketones and the liver fraction yielded hydroxylated compounds as the principal products.

Hsieh et al.4) worked on N,N-diethylnitrosamine with the Udenfriend system and isolated N-ethyl-N-(2-hydroxyethyl)nitrosamine as the major product. They also demonstrated the formation of N-nitroso-4-piperidone by a similar treatment of N-nitrosopiperidine. Manson et al.7) compared the oxidation of N-nitrosomorpholine with rat liver microsomes and the Fenton system, and observed its conversion into N-nitroso-2-hydroxymorpholine by both systems.

Preferential hydroxylation at the ω-1 position of the butyl chain was observed with compounds other than nitrosamine: e.g., 1-n-butyl-5,5-diethylbarbituric acid undergoes hydroxylation preferentially at the ω-1 position of the butyl group in in vivo metabolism and with the postmitochondrial fraction of liver in the rat.20)

Incubation of DBN with PB-S9 mix at pH 6.5 in 0.1M phosphate buffer did not affect the relative ratio of the products, but it considerably reduced the total amount of oxidation products compared to that obtained at pH 7.4, as shown in Table III. Lotlikar et al.5) reported, on the other hand, that the optimum pH for liver microsomal fraction was 6.5 in the oxidation of N,N-dimethylnitrosamine.

Mutagenic and non-mutagenic effects on S. typhimurium strain TA1535 of DBN in the presence and absence of the S9 mix have already been reported.8,22) In the present work, we have investigated the effect of inducers on the mutagenic activity of DBN in relation to its in vitro metabolic conversion. We found no correlation between the mutagenic potency and the amount of the oxidation products retaining the N-nitroso function. The formation of the principal products oxidized at the ω-1 position was much higher with PB-S9 mix than with PCB-S9 mix, and no marked difference in the formation of oxidation products was observed between PCB-S9 mix and untreated-S9 mix (Table
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III). On the other hand, PB- and PCB-S9 mix had similar extents of metabolic activation of DBN to a mutagen (or mutagens) in S. typhimurium TA1535, and untreated-S9 mix was inactive in this metabolic activation (Fig. 5). These results suggest that oxidation at the remaining position of the butyl chain, i.e. the α-position, is involved in the induction of mutation by DBN. This will be the subject of the following paper.

The principal products formed from DBN in the in vitro system and chemical model systems are BHBN-3 and BOBN-3, respectively, which are derived by ω-1 oxidation of the butyl chain, while ω and ω-1 oxidations (both being about equal in importance) are the principal metabolic pathways in vivo of DBN in the rat, yielding BCPN and BHBN-3 (including its glucuronide) as major urinary metabolites. A possible explanation for this discrepancy is that the end product BCPN resulting from the ω-oxidation is sufficiently soluble in water for easy excretion into urine, while the ketone, a further oxidation product of the hydroxy compound, still has high lipophilicity and can undergo further metabolic degradation. In fact, neither ketone (BOBN-3 or BOBN-2) could be detected among the urinary metabolites of DBN. As the biliary excretion of DBN metabolites has not been determined, there is also a possibility that ω-1 oxidation may be the principal metabolic pathway of DBN in vivo as demonstrated in vitro, and that the glucuronide of BHBN-3 is excreted preferentially into bile, thus resulting in a reduced urinary excretion of BHBN-3 as glucuronide.

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