Comparison of the in Vitro Metabolism of N-Nitrosohexamethylenimine by Rat Liver and Lung Microsomal Fractions

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ABSTRACT

The in vitro metabolism of N-nitrosohexamethylenimine by lung and liver microsomes and cytosol from uninduced male Fischer rats is described. Metabolites produced by both organs appeared to be identical. The liver subcellular fractions had a lower K_m (0.6 mm) than did lung fractions (3 mm) and metabolized 2.5 to 5 times as much nitrosamine per mg protein. Our results, together with those from our earlier studies, indicate that, as the size of the carbon ring increases from nitrosopyrrolidinone to nitrosohexamethylenimine, lung microsomes had an increased affinity for the cyclic nitrosamines; there was only a small effect with liver enzymes. This suggests that microsomal enzymes that metabolize cyclic nitrosamines in rat livers and lungs are not the same. The first stable α-hydroxylation product, 6-hydroxyhexanal, was not detected in reactions involving microsomes alone. Apparently, this compound is rapidly converted to 1,6-hexanediol by liver or lung microsomes. The presence of cytosol was needed for the full conversion of these metabolites to α-hydroxycaproate and maximal α-hydroxylation activity. α-Aminocaproate was always found in direct proportion to the hydroxyacid, suggesting that both acids arise from the same α-hydroxylation event by different breakdown mechanisms. β- and γ-hydroxy-N-nitrosamines were not metabolized significantly by rat liver enzymes and thus, in this species, may be "detoxification products" of N-nitrosohexamethylenimine.

INTRODUCTION

One of the more interesting aspects of nitrosamine carcinogenesis is the difference in organ specificities of structurally similar compounds. We have been engaged in studies of the metabolism of cyclic nitrosamines by both "target" and "non-target" organs (7–9, 18) in the hope that metabolic differences we observe may be related to the varying carcinogenic effects. In this paper, we report on the metabolism of NO-HEX2 which produces predominantly liver and esophageal tumors in 3 different strains of rat (4, 12, 13). N-Nitrosoheptamethylenimine which has only one more carbon atom in its ring causes tumors in both lung and esophagus (14, 20). By comparing the metabolism of these 2 compounds by subcellular fractions from uninduced rat liver and lung and incorporating the data we have obtained with NO-PYR, we hope to gain some insights on the initial events of nitrosamine carcinogenesis.

Information about the metabolism of NO-HEX can be found in several reports. Neunhoeffer et al. (16) demonstrated that acetone extracts of rat livers could produce small amounts of ε-aminocaprohydroxamic acid from NO-HEX. Grandjean (5) detected 14CO2 plus 11 different radioactive peaks in the urine of animals given [14C]NO-HEX by gavage. Four of these products were tentatively identified as hexamethylenimine, ε-aminocaproic acid, ε-caprolactam, and 6-aminocaprohydroxamic acid. Ross and Mirvish (17) gave convincing evidence that at least one of the adducts bound to RNA and possibly DNA after administration of labeled NO-HEX most probably resulted from active intermediates formed by decomposition of α-hydroxylated NO-HEX. The observation of Snyder et al. (18) confirmed the fact that, at low dose levels (~12 mg/kg), large percentages (43%) of NO-HEX labeled in the α-carbon atom were given off as CO2 within 24 hr; at higher dose levels, the percentage given off as CO2 was greatly reduced. Hecker and Saavedra (9) have reported on the occurrence of β-hydroxy-NO-HEX and γ-hydroxy-NO-HEX which account for one-third or more of the radioactive metabolites formed during the metabolism of NO-HEX by uninduced rat liver microsomes and cytosol (postmicrosomal supernatant).

MATERIALS AND METHODS

Instrumentation. Mass spectra were obtained from a VG Micromass ZAB-2F mass spectrometer equipped with a VG2025 data system. Gas chromatographic analyses were done on a Shimadzu Model 4-BM gas chromatograph equipped with a Hewlett-Packard 18652 A/D converter coupled to the recorder of a flame ionization detector or a Perkin-Elmer Model Sigma 3 gas chromatograph coupled to the mass spectrometer. The columns used in the Shimadzu gas chromatograph were 2 m x 2.6 mm and contained either Tenax G/C (Alltech Associates, Inc.), Deerfield, Ill.) or 3% SE-30 on Chromasorb W (Pierce Chemical Co., Rockford, Ill.). The silanized glass columns used on the Perkin-Elmer gas chromatograph were 6 ft x 2 mm and contained Tenax G/C, 3% SE-30 on Chromosorb W, or Ultrabond 20M (RFR Corp., Hope, R. I.).

HPLC was done on a Waters Associates, Inc., system using either 4.6 mm x 25 cm or 9.4 mm x 25-cm Whatman, Inc. (Clifton, N. J.) Partisil-10 ODS-2 columns. Radioactivity was monitored with a Packard Model 3225 liquid scintillation counter (8). Amino acid analysis was done on a Durrum DC-4A amino acid analyzer with a Durrum D500 column (48 mm x 1.75 mm) and Durrum Pico buffers for single column analyses (Dionex Corp., Sunnyvale, Calif.).

Chemicals. ε-Caprolactone, 1,6-hexanediol, and organic reagents were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). ε-Aminocaproic acid was purchased from Sigma Chemical Co. (St. Louis, Mo.). 6-Hydroxyhexanal was kindly supplied by Dr. Saavedra of our laboratory. ε-Caprolactone converted to sodium ε-hydroxycaproate by the methods of Marvel and Birkhimer (15). Diazomethane was generated by the procedures of deBaer and Backer (2). Nihydrin-dimethyl sulfoxide reagent was obtained from Pierce Chemical Co. O-P-Nitrobenzyl-N,N'-dialkylpropylsourea and OPT were obtained from Regis Chemical Co. (Morton Grove, Ill.). [14C]NO-HEX labeled in the α-carbon

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2 The abbreviations used are: NO-HEX, N-nitrosohexamethylenimine; NO-PYR, N-nitrosopyrrolidinone; β-hydroxy-NO-HEX, β-hydroxy-N-nitrosamines; γ-hydroxy-NO-HEX, γ-hydroxy-N-nitrosamines; HPCL, high-pressure liquid chromatography; OPT, o-phthalaldehyde; GC, gas chromatography; α-hydroxy-NO-HEX, α-hydroxy-N-nitrosamines; 2-oxo-NO-HEX, 2-oxo-N-nitrosamines.

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atoms was prepared as described previously (9, 18). Unlabeled NO-HEX was made by the procedure of Goodall et al. (4).

O-p-Nitrobenzyl-N,N'-diisopropylisourea derivatives of acids were made according to the instructions on p. 76 of the Regis catalog. Benzyol chloride derivatives of metabolites were made by dissolving 2 to 20 µg of compound in 50 to 100 µl acetonitrile, adding 1 µl of benzyol chloride and 3 µl of pyridine and then heating overnight at 60° in a sealed vial. After evaporation, the residue was resuspended in 0.5 to 1 ml of 2% sodium bicarbonate and extracted 3 times with ethyl acetate. The organic phase was washed twice with 0.1 N HCl, concentrated, and resuspended in ethanol. Standards were made in much the same manner except with higher concentrations of reactants and on a larger scale.

OPT derivatives of amino acids were made by modifying published methods (10). We have made OPT derivatives without using anaerobic conditions and β-mercaptoethanol. A stock solution of 1 mg OPT per ml was prepared in 0.4 M boric acid-KOH buffer (pH 10.4). Two volumes were mixed with one volume of amino acid solution (~1 mg/ml), and the reaction was allowed to continue for ~15 min at room temperature. The solution was then acidified with HCI to pH 1 to 2 and quickly extracted 2 or 3 times with ethyl acetate. The ethyl acetate is evaporated to dryness, and the derivative is dissolved in ~100 µl methanol and then reacted with an excess amount of diazomethane. The solution is evaporated again, and the final derivatized product may be dissolved in methanol, purified and/or analyzed by HPLC by monitoring absorbance at 254 nm, and analyzed by GC after purification.

Metabolism. Liver and lung microsomes and cytosol were prepared from male Fischer rats as described previously (7, 8). Optimal conditions for the metabolism of NO-HEX by liver microsomes have been published (9). Reactions with lung microsomes contained the following concentrations of reactants: [14C]NO-HEX (~1 µCi/ml; 0.1 to 3 mM); rat lung microsomes (~1.1 mg/ml); rat lung cytosol (~6. mg/ml); 50 mM Tris (pH 8.0); 1 mM EDTA; 5 mM glucose-6-phosphate; 1 mM NADP; 0.5 mM NADPH; and glucose-6-phosphate dehydrogenase (0.2 units/ml). Reactions (usually 100 µl) were assayed by either methylene chloride extraction (8) (this method was used to determine optimal conditions and K₅₅s) or BaSO₄ precipitation (19) followed by analysis on HPLC (7, 8). HPLC analyses were done on a Whatman ODS-2 column (9.4 mm × 25 cm) at 4.5 ml/min in 18% methanol-0.1 mM sodium acetate buffer, pH 5.5.

Large-scale reactions from which metabolites could be isolated containing either liver microsomes or liver microsomes + cytosol were processed as described previously (9). After chromatography on Sephadex G-10, 3 major fractions were isolated. The earliest eluting fraction (220 to 240 ml) contained both e-hydroxycaproate and ε-aminocaproate, the next major fraction eluting between 270 and 360 ml contained 1,6-hexanediol and 6-hydroxyhexanal, and hydroxylated nitrosamines eluted between 360 and 430 ml. ε-Aminocaproate and ε-hydroxycaproate were separated by Sephadex LH-20 chromatography (8). The amino acid was further purified on a Sephasorb HP column (1.8 mm × 98 cm) run in 2 mM sodium acetate buffer (pH 4.0) and finally on a 9.4 mm × 26 cm Whatman ODS-2 column on HPLC. The position of elution of all compounds on these C₁₈ columns may be determined by extrapolating the elution times shown in Chart 5 to other methanol concentrations. Analysis of the samples of liver microsomes + cytosol was performed by chromatography on ODS-2 columns in acetic acid buffer (pH 5.5) and 8% methanol, followed by chromatography in 18% methanol-1 mM HCl. In the HCI solution, the e-hydroxycaproic acid eluted at 13 to 15 min with a flow rate of 4.5 ml/min. The methyl ester of e-hydroxycaproic acid was purified by C₁₈ chromatography (elution time, 9 min) using a 35% methanol-water solution run at 4.5 ml/mm on a 9-mm × 25-cm ODS-2 column. 1,6-Hexanediol and 6-hydroxyhexanal were separated by chromatography on an ODS-2 column in 18% methanol-sodium acetate, pH 5.5. The separated peaks were collected and then chromatographed under the same conditions using water instead of buffer.

**RESULTS**

Identification of Metabolites. 14C-labeled metabolites of NO-HEX were purified from 30- or 60-ml reactions after precipitation with BaSO₄, and then ethanol. As described previously (7, 19), the concentrated extracts were chromatographed on a Sephadex G-10 column, which separated β-hydroxy-NO-HEX and γ-hydroxy-NO-HEX from the other metabolites. The remaining metabolites were further purified by chromatography on Sephadex LH-20, Sephasorb HP, and/or C₁₈ HPLC columns.

ε-Hydroxycaproate (Chart 4, Peak III). After purification, this metabolite was converted to the methylester with diazomethane. The mass spectra of the methylated metabolite and standard were also identical (Chart 1), but the molecular ion of m/z (146) was not apparent. Confirmation of identity was accomplished by making derivatives of the acid with O-p-nitrobenzyl-N,N'-diisopropylurea; derivatives of both the metabolite and standard coeluted at 10.5 min on Whatman ODS-2 columns (4.6 mm × 25 cm, 60% methanol-water, 1.5 ml/min). Upon acetylation of the hydroxyl group with acetyl chloride, both the radioactive metabolite and the standard cochromatographed at 9.7 min when they were run on an ODS-2 column in 70% methanol-water. ε-Hydroxycaproate was identified in in vitro reactions containing either microsomes or microsomes + cytosol.

ε-Aminocaproic Acid (Chart 4, Peak II). The purified metab-
Comparison of NO-HEX Metabolism by Rat Liver and Lung

olite was analyzed on a Durrum D500 column for amino acid analysis using a 115-min program. Both the metabolite and commercially supplied ε-aminocaproic acid eluted within 1 sec of each other at 65.6 min in an area of the chromatogram where none of the common α-amino acids eluted. This still did not prove that the radioactivity in this sample was associated with the amino acid. OPT derivatives of the metabolite and standard were purified by HPLC (see "Materials and Methods"). The peak of absorbance at 254 nm and the 14C radioactivity of the OPT metabolites obtained from either liver microsomal or microsome + cytosol reactions and the absorbance of the ε-aminocaproic acid derivative cochromatographed at 10.7 min on Whatman ODS-2 columns (4.6 mm x 25 cm, 60% methanol-water, 1.5 ml/min). Metabolite and known derivatives also coeluted at 9 min on SE-30 (2 m x 2.6 mm, 190°, 60 ml helium per min). Chart 2 indicates these OPT derivatives have identical mass spectra consistent with the structure shown in Chart 2A.

6-Hydroxyhexanal (Chart 4, Peak VIII). The identification of this metabolite is tentative. Both the metabolite and standard compound elute at 5.8 min on Tenax G/C (2.5 m x 2.6 mm, 60 ml helium per min, 200°). Although the mass spectra are somewhat similar, the small amount of material used and the lack of a molecular ion are not sufficient for identification. Both the radioactivity and absorbance at 254 nm of the benzoyl chloride derivative of the metabolite and the absorbance of the standard compound have retention times of approximately 7.5 min on ODS-2 columns (4.6 mm x 25 cm, 1.5 ml/min, 70% methanol-H2O). Furthermore, the GC retention times of the derivitized metabolite and standard on SE-30 (6 ft x 2 mm, 20 m helium per min, 190°) are similar as shown in Chart 3. Thus, this metabolite is most likely 6-hydroxyhexanal.

1,6-Hexanediol. After purification, this metabolite (Chart 4, Peak IX) eluted at 8 min on Tenax G/C (2.5 m x 2.5 mm, 60 ml helium per min, 200°) as was the case for a sample of 1,6-hexanediol. Mass spectra of the standard and metabolite were similar, but no molecular ion was apparent. The benzoyl chloride derivatives of the metabolite and known cochromatographed at 8 min on Whatman ODS-2 columns (87% methanol-water, 1.5 min ml/min) and on GC at 6.5 min using SE-30 columns (2 m x 2.6 mm, 60 ml helium per min, 225°). Identity was confirmed by high resolution mass spectra of both the metabolite and 1,6-hexanediol derivatives. The molecular ions had indistinguishable exact mass measurements (326.1510 versus 326.1508) with an elemental composition (C20H42O2) indicative of the dibenzoyl derivative of 1,6-hexanediol. These analyses were identical for metabolites obtained from reactions with either microsomes alone or microsomes + cytosol.

Metabolism. The activity of liver and lung subcellular fractions on [14C]NO-HEX was initially assayed by the methylene chloride extraction method we have described previously for NO-PYR (8). After methylene chloride extraction, most metabolites remain in the aqueous phase; these are the non-methylene chloride extraction products. In the case of NO-HEX, up to 20% of the less polar metabolites may be extracted into methylene chloride along with NO-HEX. This does not affect the validity of the assay because a constant proportion of all the radioactive metabolites always remains in the aqueous phase. As is the case for NO-PYR, the cytosol (postmicrosomal supernatant) itself metabolizes NO-HEX to only a small extent. Optimal results are observed only with combinations of microsomes + cytosol.

Reactions using liver subcellular fractions are optimal at pH 7.5 in the presence of Mg2+ and Mn2+, while the optimum activity for lung fractions is obtained at pH 8 in the absence of divalent cations. Using those conditions, the K_m for the liver fractions was ~0.6 mm as opposed to ~3 mm for the lung reactions. At NO-HEX concentrations of between 0.5 and 4 mm, liver subcellular fractions metabolize 2.5 to 3 times the


Chart 3. GC of the benzoyl chloride derivative of a metabolically produced 6-hydroxyhexanal. The top tracing is the HPLC purified benzoyl chloride derivative of the metabolite. The bottom tracing is of benzoyl chloride derivatives of 6-hydroxyhexanal and 1,6-hexanediol standards. Chromatography was done on a 6-ft x 2-mm, 3% SE-30 column at 190° and 20 ml helium flow per mm.
amount of nitrosamine as do equivalent lung systems. At lower substrate concentrations (0.1 mM), the liver fractions metabolize up to 5 times more NO-HEX. This may be seen by comparing Chart 4, A and C.

It is possible to study all the individual metabolites from microsomes + cytosol reactions on HPLC after precipitation of reaction mixtures with BaSO₄ (7, 18). The results of in vitro metabolism of 0.1 mM [¹⁴C]NO-HEX by liver and lung fractions are shown in Chart 4: A shows metabolism by liver microsomes and cytosol; B, liver microsomes; C, lung microsomes + cytosol; and D, lung microsomes. It is clear from this chart that both lung and liver subcellular fractions produce similar metabolites from NO-HEX. Identification of these products was carried out using liver microsomes + cytosol, since the liver produced a greater quantity of metabolites. In Chart 4A, the peaks are labeled I to IX. A general scheme for the metabolism of NO-HEX is presented in Chart 5, which was derived from the data listed below.

Peak II is ε-aminocaproic acid, and the majority of Peak III is ε-hydroxycaproic acid, both of which are products of α-hydroxylation. 6-Hydroxyhexanal, which would be the first stable product of α-hydroxylation, is present as a minor component in Peak VIII, and Peak IX contains mostly 1,6-hexanediol. Although all of these products should be derived from α-hydroxylation of NO-HEX, they do not have the same relationship to each other as the similar products from NO-PYR metabolism. We have shown for NO-PYR that microsomal reactions accumulate the hydroxyaldehyde and do not produce the dialcohol or the hydroxy acid (7, 8). When either liver or lung microsomes metabolize NO-HEX, 1,6-hexanediol is accumulated instead. Microsomes from both organs appear capable of converting 6-hydroxyhexanal to 1,6-hexanediol. This is in contrast to NO-PYR metabolism, where only the cytosolic enzymes convert 4-hydroxybutanal to 1,4-butanediol. Interestingly, we have not detected 6-hydroxyhexanal in large-scale liver microsomal reactions. This was confirmed using the Farrelly assay (3) in which products were extracted with methylene chloride instead of isooctane. This compound has not been detected in reactions with microsomes alone. 6-Hydroxyhexanal is only detected in small quantities (8 nmol/ml after a 4-hr incubation with 10 μmol NO-HEX per ml) when cytosol is present. If semicarbazide was included, 8 nmol of 6-hydroxyhexanal were
detected with microsomes alone, but ~15 nmol were found with microsomes + cytosol. In large-scale reactions, 6-hydroxyhexanal has been isolated only from microsome + cytosol reactions and was present only in amounts one-third that of 1,6-hexanediol. The relative accumulation of the diol is greater with lung microsomes and thus coincides with their lack of significant accumulation of 6-amino- and 6-hydroxycaproic acids (Chart 4D). In contrast, these acids do accumulate to a extent in liver microsomal reactions (Chart 4E). As would be expected, when either liver or lung cytosol is present as well, only a small amount of 1,6-hexanediol remained, and Peaks II and III increase (Chart 4, A and C). For both liver and lung reactions, the presence of cytosol increases the amount of \( \alpha \)-hydroxylation products (Peaks II, III, and IX) 55 to 65%.

The above data suggest that 1,6-hexanediol may be converted to the hydroxy acid. To test this hypothesis, 0.5 mm \([^{14}C]NO-HEX\) was incubated with liver microsomes for 4 hr, and then the extracted reaction mixture was chromatographed on HPLC as in Chart 4. Peak IX eluting at ~16 min was pooled, concentrated, and incubated with liver cytosol. Peak IX was converted to Peak III with time by liver cytosol. Liver microsomes also possessed this enzymatic activity. Therefore, liver and lung cytosols and liver microsomes are able to convert 1,6-hexanediol to \( \alpha \)-hydroxyprocaproic acid; the presursors of \( \alpha \)-aminocaproate and the reason for its parallel occurrence with \( \alpha \)-hydroxyprocaproate are unknown and will be discussed below.

Peaks IV and V are the \( \alpha \) and \( \gamma \) conformers of 6-hydroxy-NO-HEX (9). As we have noted previously, the ratio of conformers formed by liver and lung reactions differs, suggesting that, in the liver and lung, different enzymes perform similar functions. Upon addition of cytosol, the amount of \( \gamma \)-hydroxy-NO-HEX remains relatively constant, while the \( \alpha \)-hydroxylation products (Peak II + III + IX) increase. This suggests that enzymes that carry out \( \alpha \) and \( \gamma \)-hydroxylations may be different. Peak VIII contains the \( trans \) conformer of \( \beta \)-hydroxy-NO-HEX (9), although as noted previously, a small amount of 6-hydroxyhexanal is also present. Peak IX contains some of the \( cis \) conformer of \( \beta \)-hydroxy-NO-HEX. In liver reactions, the \( Z \) conformer of \( \beta \)-hydroxy-NO-HEX should be no more than 50% of the \( E \) conformer (9). Peaks VI and VII have not as yet been identified, but it should be noted that Peak VI is apparently formed only in the presence of cytosolic enzymes.

Peak I has not as yet been identified, but it is the product of metabolism of either \( \beta \)-hydroxy-NO-HEX and/or \( \gamma \)-hydroxy-NO-HEX. Neither \( \beta \)-hydroxy-NO-HEX nor \( \gamma \)-hydroxy-NO-HEX is metabolized significantly by uninduced or induced rat subcellular fractions. While liver microsomes + cytosol metabolize 40 to 50% of a 0.1 mm NO-HEX solution in 4 hr, only 1 to 2% of an equivalent concentration of \( \gamma \)-hydroxy-NO-HEX is metabolized within the same time period. The results with \( \beta \)-hydroxy-NO-HEX are similar.

**DISCUSSION**

Microsome + cytosol reactions from either uninduced rat livers or lungs produce similar products from NO-HEX, as is the case for NO-PYR (7). Although the \( K_m \) of liver microsomes + cytosol for NO-HEX and NO-PYR (0.6 mm versus 0.34 mm) are approximately the same, lung microsomes have a much lower \( K_m \) for NO-HEX (3 mm) than for NO-PYR (20 mm). As the size of the ring increases, lung microsomes apparently have more affinity for the substrate. Our results indicate that the rate of metabolism by liver microsomes + cytosol is up to 5 times greater than that for per mg of protein, which is consistent with the differences in \( K_m \). This supports our previous suggestions that the affinity of microsomes for nitrosamines as well as the total amount of hydroxylation are initially important in determining if a nitrosamine will be able to produce tumors in a given tissue (7, 8). The different \( K_m \)s for cyclic nitrosamines exhibited by liver and lung enzymes strongly suggest that hydroxylation of cyclic nitrosamines in these organs is carried out by either different proteins or similar proteins in different membrane environments.

Reactions containing microsomes + cytosol gave a higher rate of metabolism than those containing microsomes alone; similar results have been obtained with NO-PYR (8). From Chart 4, it may be determined that, in the absence of cytosol, \( \beta \) and \( \gamma \)-hydroxylation products form a greater percentage of total metabolites. These data are consistent with the supposition that microsomal enzymes involved in \( \beta \) and \( \gamma \)-hydroxylations are different than those which \( \alpha \)-hydroxylate NO-HEX in liver and lung.

Liver and lung microsomal reactions with NO-PYR accumulate 4-hydroxybutanal (6, 8), but the 6-carbon hydroxyaldehyde from NO-HEX microsomal metabolism is only found if cytosol is included in the reaction mixture. This is most probably due to the fact that, as soon as 6-hydroxyhexanal is formed by microsomes, it is immediately reduced to 1,6-hexanediol by either liver or lung microsomes; this activity is not found for the 4-carbon compound (7, 8). Since 6-hydroxyhexanal has been found only in reactions including cytosol, it is conceivable that it may be an intermediate in the conversion of 1,6-hexanediol to the hydroxy acid. Such aldehyde intermediates are metabolized to their respective aldehydes at different rates, and these compounds are subsequently reduced by the cytosolic enzymes of the liver and lung.
ates may, for the most part, be enzyme bound, allowing only small amounts to leak away from the active sites of the enzyme (see Ref. 11). It is apparent from our results that liver microsomes are capable of converting 1,6-hexanediol and/or 6-hydroxyhexanal to the hydroxy acid. Lung microsomes only have a trace of this activity and thus accumulate a greater relative amount of 1,6-hexanediol. In both cases (particularly the lung), the presence of cytosol is necessary for maximum production of \( \varepsilon \)-hydroxy-caproate and \( \varepsilon \)-aminocaproate.

The occurrence of \( \varepsilon \)-aminocaproate, a possible detoxification pathway for \( \alpha \)-hydroxylated nitrosamines, raises some interesting questions. The amount of amino acid formed is always in a constant proportion (50 to 67%) to the amount of hydroxy acid detected. This is true for both liver and lung reactions whether cytosol is present or not. This is also true for liver reactions with microsomes induced with phenobarbital or Aroclor 1254. Therefore, the appearance of the amino acid must be related to the formation of the hydroxy acid. Both metabolites must either arise from the same chemical intermediates or be produced by different mechanisms by the same enzyme.

Grandjean (5) has pointed out that \( \alpha \)-hydroxy-NO-HEX may either break down to the hydroxyaldehyde or it might form either 2-oxy-NO-HEX or \( \varepsilon \)-caprolactam instead, which would give rise to the amino acid by hydrolysis. Based on our data, it would appear that both the amino acid and the hydroxy acid (not detected by Grandjean) may be products of the \( \alpha \)-hydroxylated nitrosamine, regardless of its enzymatic origin. Experiments are currently underway to try to determine the mode of formation of \( \varepsilon \)-aminocaproate.

Although Grandjean (5) has detected \( \varepsilon \)-caprolactam in large amounts in the urine of animals fed NO-HEX and Cottrell et al. (1) have found low levels of pyrrolidine-2-one after administration of NO-PYR to rats, we have not yet identified \( \varepsilon \)-caprolactam in our reactions.

Neither \( \beta \)-hydroxy-NO-HEX nor \( \gamma \)-hydroxy-NO-HEX is metabolized to more than a small extent by rat liver subcellular fractions, and they may tentatively be classified as detoxification products in rat liver. This lack of metabolism is reflected by a lack of mutagenicity in the Ames assay of these 2 stable hydroxylated nitrosamines using rat liver S9 fractions.3

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