In Vitro Metabolism of Acrylonitrile to 2-Cyanoethylene Oxide, Reaction with Glutathione, and Irreversible Binding to Proteins and Nucleic Acids

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ABSTRACT

The metabolism of the suspected carcinogen acrylonitrile was studied using subcellular fractions isolated from rats and humans. Irreversible binding of radioactive label from [1-\(^{14}\)C]- or [2,3-\(^{14}\)C]acrylonitrile to protein and DNA was enhanced by reduced nicotinamide adenine dinucleotide phosphate in the presence of rat liver microsomes or a reconstituted cytochrome P-450 system. During the reduced nicotinamide adenine dinucleotide phosphate-dependent reaction, HCN was produced, and the heme of cytochrome P-450 was destroyed. Rat brain microsomes did not produce detectable levels of metabolites. Conclusive evidence of metabolically mediated binding of acrylonitrile to protein and DNA in human systems was not found. With rats, metabolism was induced by pretreatment of animals with either phenobarbital or 5,6-benzoﬂavone.

Labeled 2-cyanoethylene oxide was found to bind irreversibly to calf thymus DNA and microsomal protein. The extent of binding was greater in the case of 2,3-\(^{14}\)C-labeled than \(^{14}\)C-labeled material. The relative first-order rate of acrylonitrile binding to calf thymus DNA in rat liver microsomal systems was one to two orders of magnitude less than that for 1,1,2-trichloroethylene and three orders of magnitude less than that for vinyl chloride or vinyl bromide. Rat liver microsomes or a reconstituted cytochrome P-450 system catalyzed the mixed-function oxidation of acrylonitrile to 2-cyanoethylene oxide. 2-Cyanoethylene oxide has a half-life of about 2 hr in neutral buffer at 37°C. This epoxide was found to serve as a substrate for microsomal epoxide hydrolase. HCN was released during hydrolysis of 2-cyanoethylene oxide or reaction of the epoxide with reduced glutathione; however, HCN release in either case was not stoichiometric with epoxide disappearance. 2-Cyanoethylene oxide reacted less rapidly with reduced glutathione than did acrylonitrile. Rat liver cytosol preparations contained glutathione S-transferase activity towards acrylonitrile and greater activity with 2-cyanoethylene oxide as a substrate. Cytosol preparations from rat brain and human liver had no detectable glutathione S-transferase activity towards acrylonitrile but did exhibit some activity towards 2-cyanoethylene oxide.

These studies establish the basic pathways involved in the metabolism of acrylonitrile and should provide a basis for examination of the relevance of these individual steps and their roles in bioactivation and detoxication under more physiological situations.

INTRODUCTION

Acrylonitrile is used on a large scale in the manufacture of acrylic polymers. The acute toxicity of the compound has been recognized for some time (5). A recent epidemiological study suggested increased lung tumors in workers exposed to acrylonitrile when compared with a selected internal control but not when compared with the local or national population (26). Thus, questions exist relative to the induction of tumors in workers exposed to acrylonitrile. Exposure of rats to acrylonitrile by inhalation or in drinking water at dosages above 2 mg/kg/day for 2 years has been shown to cause tumors in the brain and Zymbal’s gland (32, 33). Maltoni et al. (25) have reported on studies in which rats were exposed to 2 mg/kg/day by gavage or 3 mg/kg/day by inhalation for 12 months and held for their remaining lifetimes; a significant increase in tumor incidence was not observed. More recently, it has been demonstrated that rats exposed to less than 1 mg acrylonitrile per kg body weight per day via drinking water for 2 years did not exhibit an increased tumor incidence. These findings are consistent with dose-response phenomena and suggest that a threshold may exist, either in terms of duration of exposure or level of exposure, below which there is no increase of risk in tumor induction. Duvenger et al. (6) reported that metabolic activation was required for mutagenicity in Salmonella test strains and postulated that radicals were involved as reactive metabolites. In contrast, positive results were obtained with Escherichia coli WP2 only in the absence of exogenous metabolic activation (39). No evidence of mutagenicity in intact mammals has been reported. Rabello-Gay and Ahmed (34) reported that there was not a significant increase in the frequency of cells with chromosomal abnormalities in bone marrow of either mice or rats following 16 to 30 days of exposure to 20 to 40 mg/kg/day. Leonard et al. (23) did not find any evidence of a dominant lethal mutagenic effect in mice receiving a single i.p. injection of 30 mg acrylonitrile per kg body weight.

The role of metabolism in the induction of tumors is presently unclear. Langvardt et al. (22) examined the urinary metabolites produced by rats and identified thiocyanate and N-acetyl-S-(2-cyanoethyl)cysteine. Abreu and Ahmed (1) demonstrated the oxidation of acrylonitrile to HCN in rat liver microsomes. The
latter 2 groups have proposed pathways in which acrylonitrile is converted to 2-cyanoethylen oxide (glycidenitrile), which was postulated to either hydrolyze or to react with GSH to release HCN.

The studies presented here were initiated with the goal of defining the major reactions and enzymatic pathways involved in the bioactivation and detoxication of acrylonitrile. Studies were carried out using subcellular fractions to provide a basis for further examination of relevant pathways in intact cells and in animals. Enzyme-mediated irreversible binding to protein and DNA was utilized as an index of bioactivation, and comparison of the activity of human and rat enzyme preparations was made.

MATERIALS AND METHODS

Chemicals. Acrylonitrile was purchased from the Aldrich Chemical Co., Milwaukee, Wis., and contained 35 ppm of p-methoxyphenol as a polymerization inhibitor. p-Benzoinone was also purchased from Aldrich and recrystallized from petroleum ether prior to use in the fluorimetric estimation of HCN. Radioactive acrylonitrile (1-14C) and (2,3,3-14C) was prepared by Pathfinder Chemical Co., St. Louis, Mo., and judged to be >99% chemically and radiochemically pure as judged by HPLC (see below). Stock materials were stored at ~70° either neat or as 0.5 M aqueous solutions. Aqueous solutions were routinely examined for purity before use; storage of aqueous solutions at 4° in the dark led to extensive decomposition within 21 days. 2-Cyanoethylen oxide was prepared by the Monsanto Co. 1,6-Ethenoadenosine and 3,6-ethenocytidine were purchased from P-L Biochemicals, Inc., Milwaukee, Wis.

Labeled 2-cyanoethylen oxide (1-14C) or (2,3,3-14C) was prepared from acrylonitrile using the general procedure of Payne et al. (30). In a typical synthesis, 0.15 ml of 70% aqueous tert-butyl hydroperoxide was stirred in 1.2 ml of benzene. To the stirred suspension, 76 µl of acrylonitrile and 35 µl of 1 M aqueous tetra(n-butyl)ammonium hydroxide were added in small aliquots over 30 min. Most of the residual water was removed with a syringe, and the reaction was stirred overnight at room temperature. The yield of epoxide was 60 to 70% as judged by quantitation using p-nitrobenzyl pyridine reagent (see below). Conversion of the nitrite group to the amide did not occur to the extent of more than 5% as judged by the amount of epoxide remaining after concentration of a reaction aliquot in a vacuum (35°, 10 mm Hg) (31). The synthetic material eluted from a HPLC column (Varian SI-5, 4 x 250 mm, hexane, retardation time = 5 min) in the same position as that of the incubation were added. Mixing was done on a vortex device, the layers were separated by centrifugation at 5000 x g for 5 min, and the organic phase was dried over MgSO4. An aliquot of the ethereal phase was chromatographed on a Varian SI-5 HPLC column using hexane to acetonitrile gradients. Aliquots of collected fractions were analyzed for activity by liquid scintillation counting and for 2-cyanoethylen oxide by reaction with p-nitrobenzyl pyridine (Amax = 240 nm). Complete incubation using [2,3-14C]acrylonitrile (2.8 mCi/mmol); B, complete incubation as in A in the presence of 1 mM 3,4-trichlorophenyle oxide; C, incubation as in B without NADPH; and D, complete incubation using [1-14C]acrylonitrile (0.066 mCi/mmol).

Chart 1. Conversion of acrylonitrile to 2-cyanoethylen oxide by rat liver microsomes in the presence of NADPH. Incubations contained 2 mg phenobarbital-induced rat liver microsomal protein per ml and 1 µM acrylonitrile. After 60 min, 0.2 µmol of authentic 2-cyanoethylen oxide and a volume of ether equal to that of the incubation were added. Mixing was done on a vortex device, the layers were separated by centrifugation at 5000 x g for 5 min, and the organic phase was dried over MgSO4. An aliquot of the ethereal phase was chromatographed on a Varian SI-5 HPLC column using hexane to acetonitrile gradients. Aliquots of collected fractions were analyzed for activity by liquid scintillation counting and for 2-cyanoethylen oxide by reaction with p-nitrobenzyl pyridine (Amax = 240 nm). Complete incubation using [2,3-14C]acrylonitrile (2.8 mCi/mmol); B, complete incubation as in A in the presence of 1 mM 3,3,3-trichloropropylene oxide; C, incubation as in B without NADPH; and D, complete incubation using [1-14C]acrylonitrile (0.066 mCi/mmol).
RESULTS

Formation and Hydrolysis of 2-Cyanoethylene Oxide. Preliminary experiments indicated that rat liver microsomes catalyze the mixed-function oxidation of acrylonitrile to 2-cyanoethylene oxide. The 2 compounds were readily separated by HPLC (Chart 1). Analysis of ether extracts of microsomal incubations indicated that label from either [1-14C]- or [2,3-14C]acrylonitrile with 5 mM GSH was monitored by spotting 10-μl aliquots of the reaction mixture onto filter paper. The spots were dried, allowing the unreacted acrylonitrile to evaporate, and removed to 1.0 ml of water in a scintillation vial. Scintillation cocktail (ACS) was added after 15 min, and the samples were counted. The reaction was linear for at least 15 min. GSH S-transferase activity towards 2-cyanoethylene oxide was estimated by monitoring the disappearance of GSH (36). Reactions contained 5 mM GSH, 44 mM 2-cyanoethylene oxide, and 1.4 mg protein per ml. Reactions were terminated by the addition of 5% (w/v) trichloroacetic acid after 3 min (rat liver) or 30 min (rat brain or human liver). Samples were centrifuged to remove precipitated protein, and the supernatants were assayed for GSH.

All results are presented as the mean ± S.D. of triplicate incubations unless noted otherwise.

The extent of accumulation of 2-cyanoethylene oxide, as estimated using this assay, was decreased by the presence of purified epoxide hydrolase.

2-Cyanoethylene oxide was found to have a half-life of about 2 hr in 0.1 M potassium phosphate buffer (pH 7.4) at 37°C. When a 0.5 mM solution of 2-cyanoethylene oxide was incubated with 30 μg purified epoxide hydrolase per ml, the epoxide was destroyed at the rate of 5.5 nmol/min. This rate may be compared to one of 1.7 nmol/min in the absence of epoxide hydrolase. 

HCN was released at the rate of 1.5 nmol/min during the action of epoxide hydrolase on 2-cyanoethylene oxide under the same conditions (the rate was 0.2 nmol/min in the absence of epoxide hydrolase). The lack of stoichiometry between HCN release and epoxide disappearance suggests that a finite level of the cyanohydrin exists in solution. In other experiments in which the rate of epoxide disappearance was measured colorimetrically with p-nitrobenzyl pyridine reagent at different concentrations of 2-cyanoethylene oxide, the K_m was estimated as 0.8 mM, and the V_max was estimated as 300 nmol/min/mg for the hydrolysis of 2-cyanoethylene oxide by purified rat liver microsomal epoxide hydrolase.

Mixed-Function Oxidation of Acrylonitrile to HCN and Irreversibly Bound Metabolites. Production of HCN, metabolites irreversibly bound to DNA, and metabolites not extracted by petroleum ether was significantly increased in all cases in microsomal incubations in the presence of NADPH. The time courses for production of nonextracted and DNA-bound metabolites were linear for about 60 min. The course of HCN
production showed a repeatable lag (10 to 15 min) before the linear phase. This lag phase would be expected for the accumulation of 2-cyanoethylen oxide to be hydrolyzed by epoxide hydrase due to the high Km value (18). Other experiments indicated that, using 1 to 2 mM acrylonitrile concentrations and incubation times of 60 min, metabolism in each case was proportional to concentrations of microsomal protein as high as 2 mg/ml. Variation of acrylonitrile concentrations yielded the following kinetic parameters for the production of the metabolites assayed: petroleum ether-insoluble metabolites, Km (3.2 mM) and Vmax (2.5 nmol/min/mg protein); HCN, Km (14 mM) and Vmax (1.1 nmol/min/mg protein); and DNA-bound metabolites, Km (17 mM) and Vmax (18 pmol/min/mg protein). While Duverger et al. (6) reported that exposure to visible light was not the metabolism of acrylonitrile to mutagens, we found no difference in the level of acrylonitrile metabolites irreversibly bound to DNA regardless of whether metabolism occurred in the presence or absence of light.

Conversion of acrylonitrile to metabolites irreversibly bound to protein and DNA was induced by pretreatment of rats with either phenobarbital or β-naphthoflavone (Table 1), when activity is expressed on the basis of microsomal protein. A substantial level of binding to microsomal protein, but not DNA, occurred in the absence of NADPH due to direct alkylation by acrylonitrile. In all cases, at least two-thirds of the protein binding was not the result of metabolism. The cyanide group was retained to a significant, but not stoichiometric, extent in the reaction of metabolites with protein or DNA. Binding was not enhanced by the presence of the epoxide hydrase inhibitor 3,3,3-trichloropropylene oxide nor inhibited by the addition of epoxide hydrase. (The increase in NADPH-dependent protein binding in the presence of epoxide hydrase may reflect the ability of that protein to bind activated metabolites.) Significant metabolism by rat brain microsomes could not be detected. A reconstituted enzyme system containing rat liver P-450 and NADPH-P-450 reductase catalyzed binding at rates which are consistent with the view that metabolism in microsomes is being carried out by these enzymes in phenobarbital-treated rat liver microsomes, as the rates where higher than observed with microsomes when expressed on a P-450 basis.

The heme of P-450 can be destroyed during the mixed-function oxidation of a number of olefins (13, 28). In the course of this work, we found that 28% of the P-450 heme in rat liver microsomes was destroyed in 60 min in the presence of acrylonitrile under typical incubation conditions. Evidence has been presented that intermediates other than epoxides are responsible for such heme degradation (13, 28).

A study of the preference of acrylonitrile metabolites to bind to various nucleic acids indicated that the extent of binding to RNA was significantly greater than DNA (Table 2), although the reason is not yet clear. The polynucleotides most readily alkylated were polyadenylic acid and polyuridylic acid. Polycytidylic acid was also alkylated to a level similar to that of yeast soluble RNA, and polyguanylic acid was the least preferred target.

Microsomes obtained from several human autopsy samples were also examined for ability to metabolize acrylonitrile (Table 3). Of the 6 samples, only 3 were shown to yield significant levels of metabolites not extracted with petroleum ether, one produced metabolites irreversibly bound to DNA (although the magnitude of the binding and the variation make this result questionable), and none produced detectable metabolites irreversibly bound to protein. These values were all substantially lower than those found with rat liver microsomes. Data obtained for the mixed-function oxidation of trichloroethylene to chloral

| Table 2 |
| Selectivity of irreversible binding of acrylonitrile metabolites to various polynucleotides |
| **Lever microsomes (2 mg protein per ml)** from phenobarbital-treated rats were incubated with 1 mM [2.3-^14C]acrylonitrile, an NADPH-generating system, and 1.5 mg calf thymus DNA per ml, yeast soluble RNA, or the indicated polynucleotide at 37°C for 60 min. Nucleic acids were isolated as described by Kildub et al. (19), and adduct formation was determined by measurement of radioactivity. Binding is expressed as nmol acrylonitrile adducts formed per mg protein. |
| **Nucleic acid** | **Adducts formed (nmol/mg protein/hr)** |
| | **Calf thymus DNA** | **Yeast soluble RNA** | **Polyadenylic acid** | **Polyadenylic acid** |
| | | | | | **0.49 ± 0.23** | **1.70 ± 0.07** | **1.73 ± 0.72** | **0.70 ± 0.18** | **0.18 ± 0.01** | **1.65 ± 0.63** |

| **Table 1** |
| Conversion of acrylonitrile to bound adducts by rat liver and brain microsomes and purified rat liver P-450 |
| Incubations were carried out at 37°C in the presence of 1 mM acrylonitrile for 60 min with microsomes or for 10 min with the purified and reconstituted P-450 system (used as described in Chart 2 with the addition of 2 mg bovine serum albumin per ml). Protein and DNA adducts were separated and quantitated as described under "Materials and Methods." All values are expressed per mg microsomal protein. Microsomal samples contained the following concentrations of P-450: untreated liver, 1.1 nmol/mg; phenobarbital-treated liver, 3.3 nmol/mg; β-naphthoflavone-treated liver, 2.1 nmol/mg; and untreated brain, 0.10 nmol/mg. |
| **Incubation system** | **Acrylonitrile label** | **Protein adducts (nmol/mg/hr)** | **DNA adducts (nmol/mg/hr)** |
| | | **+ NADPH** | **- NADPH** | **Net** | **+ NADPH** | **- NADPH** | **Net** |
| **Untreated liver microsomes** | 2.3^-14C | 4.5 ± 0.3^a | 3.8 ± 0.5 | ND | 0.207 ± 0.017 | 0.004 ± 0.002 | 0.20 |
| | 2.3^-14C | 7.1 ± 0.4 | 4.4 ± 0.2 | 2.7 | 0.356 ± 0.014 | 0.003 ± 0.002 | 0.35 |
| | 2.3^-14C | 7.0 ± 0.7 | 5.0 ± 0.5 | 2.0 | 0.320 ± 0.035 | 0.004 ± 0.005 | 0.32 |
| | 1^-14C | 7.8 ± 1.4 | 6.7 ± 0.4 | ND | 0.276 ± 0.037 | 0.005 ± 0.003 | 0.27 |
| | 2.3^-14C | 7.1 ± 0.3 | 4.0 ± 0.4 | 3.0 | 0.199 ± 0.012 | 0.003 ± 0.001 | 0.20 |
| | 3,3,3-Trichloropropylene oxide | 2.3^-14C | 11.7 ± 0.9 | 5.0 ± 0.6 | 6.7 | 0.409 ± 0.017 | 0.003 ± 0.001 | 0.41 |
| | 2^-14C | 4.3 ± 0.3 | 3.5 ± 0.7 | ND | 0.004 ± 0.001 | 0.003 ± 0.002 | ND |
| **Phenobarbital-induced liver microsomes** | 3.3,3-Trichloropropylene oxide | 2.3^-14C | 91.3 ± 22.2 | 18.2 ± 1.1 | 73.1 | 1.74 ± 0.52 | 0.012 ± 0.009 | 1.73 |

^a Mean ± S.D.  
^b ND, insignificant difference.
suggest that the lower values for acrylonitrile metabolism are not the result of general metabolic insufficiency in these preparations. Studies carried out with homogenates of the various human liver samples yielded values that reflected the microsomal data obtained for each patient, and in every case where metabolism was observed, the specific activity in the microsomal fraction was higher than in the crude homogenate.

**Reaction of Acrylonitrile and 2-Cyanoethylene Oxide with GSH.** When GSH (0.5 mM) was mixed with acrylonitrile (0.1 mM) at pH 7.7 (37°), the pseudo first-order rate constant for GSH disappearance was 0.28/min. In contrast, the rate constant found for GSH disappearance in the presence of 0.1 mM 2-cyanoethylene oxide was 0.11/min. The reaction of GSH with 2-cyanoethylene oxide was shown to liberate HCN, but the data presented in Chart 3 indicate that the level of HCN retained in GSH adducts. Langvardt et al. (22) administered labeled acrylonitrile to rats and examined the urinary metabolites. An N-acetylcysteine adduct was isolated that contained label derived from either [1-14C]- or [2,3-14C]acrylonitrile in that study. This adduct was tentatively identified as the cyclic compound 4-acetyl-3-carboxy-5-cyanotetrahydro-1,4-2H-thiazine (see below) on the basis of its mass spectrum. When 2-cyanoethylene oxide (0.2 mM) was reacted with N-acetylcysteine (0.1 mM) in 0.1 M potassium phosphate buffer (pH 7.7) for 60 min at 37° and derivatized in the manner described by Langvardt et al. (22), a compound was isolated with the identical methane chemical ionization mass spectrum, in support of the hypothesis that this compound is derived from the reaction of 2-cyanoethylene oxide with GSH in vivo. The spectrum showed major peaks at the following m/e values (relative abundance and assignment in parentheses): 257 (6, M + 29); 229 (51, M + 1); 215 (6, M − 13); 197 (15, M − 31); 188 (9); 197 (100, M − 42); 169 (17); 144 (9); 112 (3); and 102 (9).

GSH S-transferase activity of different cytosol preparations was tested with acrylonitrile and 2-cyanoethylene oxide as substrates (Table 4). All preparations showed activity towards 1-chloro-2,4-dinitrobenzene. In accordance with a previous report (4), rat liver cytosol also showed activity towards acrylonitrile. Kinetic analysis revealed that this activity was characterized by a K_m of 33 mM and a V_max of 57 nmol/min/mg protein. Other cytosolic preparations had no detectable activity toward acrylonitrile. Rat liver was active with 2-cyanoethylene oxide as a substrate, whereas rat brain and human liver showed more than an order of magnitude less activity towards the epoxide.

**Binding of 2-Cyanoethylene Oxide to Protein and DNA.** In order to further investigate the role of 2-cyanoethylene oxide, the kinetics of nonenzymatic binding of labeled 2-cyanoethylene oxide to protein and calf thymus DNA was examined. Kinetic data for the irreversible binding of 2-cyanoethylene oxide and acrylonitrile to DNA and microsomal protein are shown in Chart 4. Nonenzymatic binding of acrylonitrile to DNA was barely detectable, as expected from previous studies (Table 1). A significant level of binding of 2-cyanoethylene oxide to DNA occurred rapidly. The binding of 1-14C-labeled material to DNA was not as extensive as binding of 2-cyano-

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**Table 3**

<table>
<thead>
<tr>
<th>Metabolism of acrylonitrile by human liver microsomes</th>
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<tbody>
<tr>
<td>Incubations were carried out at 37° in the presence of 1 mM [2,3-14C]acrylonitrile for 60 min using 3 to 5 mg human liver microsomal protein per ml. Microsomes were incubated with 10 mM trichloroethylene for 10 min at 37° (1 mg protein per ml), and chloral was estimated in separate experiments. Data for rat liver acrylonitrile metabolism are presented from Table 1 for comparison.</td>
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<table>
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<tr>
<th>Total non-petroleum ether-extractable metabolites (nmol/mg/hr)</th>
<th>Protein adducts (nmol/mg/hr)</th>
<th>DNA adducts (nmol/mg/hr)</th>
<th>Chloral (nmol/mg/hr)</th>
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</thead>
<tbody>
<tr>
<td>+ NADPH − NADPH Net</td>
<td>+ NADPH − NADPH Net</td>
<td>+ NADPH − NADPH Net</td>
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</tr>
<tr>
<td>16</td>
<td>4.5 ± 0.3</td>
<td>3.8 ± 0.5 ND</td>
<td>0.207 ± 0.017</td>
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<tr>
<td>32</td>
<td>7.1 ± 0.4</td>
<td>4.4 ± 0.2 2.7</td>
<td>0.366 ± 0.014</td>
</tr>
<tr>
<td>Human No. 13</td>
<td>12.0 ± 0.4</td>
<td>10.8 ± 1.1 33</td>
<td>0.010 ± 0.004</td>
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<tr>
<td>Human No. 15</td>
<td>19.5 ± 0.9</td>
<td>11.5 ± 0.4 ND</td>
<td>0.022 ± 0.003</td>
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<tr>
<td>Human No. 16</td>
<td>10.4 ± 0.8</td>
<td>5.0 ± 0.4 33</td>
<td>0.025 ± 0.003</td>
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<tr>
<td>Human No. 175</td>
<td>14.3 ± 0.3</td>
<td>9.4 ± 0.4 33</td>
<td>0.036 ± 0.014</td>
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<tr>
<td>Human No. 19</td>
<td>13.9 ± 1.4</td>
<td>12.1 ± 0.1 ND</td>
<td>0.013 ± 0.006</td>
</tr>
<tr>
<td>Human No. 20</td>
<td>15.1 ± 0.4</td>
<td>15.2 ± 1.1 33</td>
<td>0.013 ± 0.006</td>
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</table>

Chart 3. Cyanide release during the reaction of GSH with 2-cyanoethylene oxide. 2-Cyanoethylene oxide (10 mM) was incubated in 0.1 M potassium phosphate buffer (pH 7.7) at 37° in the presence (●) or absence (○) of 0.5 mM GSH. Cyanide concentrations were estimated fluorimetrically (16). When GSH was present, levels of GSH were monitored (▲) as described under †Materials and Methods."
B) was determined as described under "Materials and Methods." were withdrawn, and irreversible binding of radioactivity to DNA and protein addition of acrylonitrile or 2-cyanoethylene oxide. At the indicated times, aliquots Teflon liners. The temperature was 37° and incubations were initiated by the (O), or 2-cyano-[2,3-14C]ethylene oxide (•) (all at 0.25 mM) in vials sealed withylene oxide to DMA and protein. Incubations contained 50 mM potassium phos- onitrile as substrate. Lower limit of detection was estimated to be about 4.0 nmol/mg/min with chloro-2,4-dinitrobenzene and 5 mM for the other reactions. All results have been corrected for the nonenzymatic reaction rates.

Table 4

<table>
<thead>
<tr>
<th>Cytosol preparation</th>
<th>Acrylonitrile</th>
<th>2-Cyanoethylene oxide</th>
<th>Rate of GSH conjugation (nmol/mg/min)</th>
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<tbody>
<tr>
<td>Rat liver</td>
<td>837 ± 18</td>
<td>31.9 ± 5.4</td>
<td>309 ± 24</td>
</tr>
<tr>
<td>Rat brain</td>
<td>123 ± 5</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.4 ± 3.1</td>
</tr>
<tr>
<td>Human Liver 13</td>
<td>460 ± 17</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Human Liver 15</td>
<td>861 ± 16</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human Liver 16</td>
<td>625 ± 27</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>Human Liver 175</td>
<td>692 ± 27</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.5 ± 3.3</td>
</tr>
<tr>
<td>Human Liver 19</td>
<td>480 ± 23</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.9 ± 4.4</td>
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<tr>
<td>Human Liver 20</td>
<td>450 ± 25</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.7 ± 1.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, insignificant rate above the blank.
<sup>b</sup> Lower limit of detection was estimated to be 3.5 nmol/mg/min with acryl-
nitrite as a substrate.
<sup>c</sup> Lower limit of detection was estimated to be about 4.0 nmol/mg/min with 2-cyanoethylene oxide as a substrate.

Acrylonitrile represents an interesting case of a postulated carcinogen which shows a high level of nonenzymatic binding to proteins and a low level of irreversible binding to nucleic acids after enzymatic activation. Moreover, one metabolite detected in subcellular in vitro systems is an epoxide with a reasonable degree of stability. Target tissues have little meta-

![Chart 4](chart4.png)

**DISCUSSION**

Acrylonitrile is oxidized by P-450S to form 2-cyanoethylene oxide. 2-Cyanoethylene oxide has several possible fates. The epoxide can react nonenzymatically with GSH or other sulphydryl compounds to form what are apparently multiple products. Release of HCN (Chart 6) would result from the attack of the sulphydryl groups on proteins. Presumably, 2-acetylaminaldehyde-reduced glutathione would also result. Such an attack of the [2,3-14C]ethylene oxide. This finding is consistent with the relative extent of binding of metabolites derived from [1-14C]- and [2,3-14C]acrylonitrile (Table 1) if the epoxide is a major acrylonitrile metabolite involved. About 5% of the epoxide was bound under these conditions. Label from 2-cyano-[2,3-

![Chart 5](chart5.png)
Metabolism of Acrylonitrile

Chart 5. UV and fluorescence spectra and HPLC chromatogram of the reaction products obtained by incubation of adenosine with acrylonitrile or 2-cyanoethylene oxide. Adenosine (Ad, 0.1 M) and either acrylonitrile (0.2 M), 2-cyanoethylene oxide (0.2 M), or no added nitrile were shaken at 37° for 40 hr in 0.1 M potassium phosphate buffer. The incubation mixtures were diluted into 200 parts of 0.1 M potassium phosphate buffer (pH 7.7) to obtain the UV (A) and fluorescence (B) spectra shown. In both cases, the cuvet path length was 10 mm. The fluorescence spectrum was obtained with an excitation wavelength of 300 nm using a Varian SF-550 instrument. The spectrum of an authentic 1,N6-ethenoadenosine (e-Ad) sample is shown with the broken line. Four μl of the incubation were injected onto a 0.2- x 50-cm Aminex A-7 HPLC column in C; the column was eluted with 0.4 M ammonium formate buffer (pH 4.50) at a rate of 30 ml/hr. In D, 2 μl of a 1 mM solution of authentic 1,N6-ethenoadenosine were chromatographed under conditions described for C.

Chart 6. Postulated scheme for metabolic transformation of acrylonitrile. See text for discussion.

Acrylonitrile is known to react nonenzymatically with the cysteine thiol groups present in proteins (8, 40). In an analogous reaction, both acrylonitrile and 2-cyanoethylene oxide underwent conjugation with GSH. The epoxide was less reactive than was cyanide (Chart 6). Binding of 2-cyanoethylene oxide to nucleic acids was more extensive than was the binding of acrylonitrile (Chart 4). The nature of these adducts is not yet known, and a variety of products was formed when 2-cyanoethylene oxide was incubated with ribonucleosides. One of the adducts formed under such conditions was 1,N6-ethenoadenosine (Chart 5), and a mechanism for formation of such residues can be drawn on the basis of other chemical studies (37). This modification is a major process in the alkylation of nucleic acids by metabolites of vinyl halides (29). The role of such an adduct is uncertain until such a product can be isolated from DNA.

Acrylonitrile is known to react nonenzymatically with the cysteine thiol groups present in proteins (8, 40). In an analogous reaction, both acrylonitrile and 2-cyanoethylene oxide underwent conjugation with GSH. The epoxide was less reactive than was the parent compound by a factor of about 2.5. Cytosol preparations exhibited GSH transferase activity towards these compounds, resulting in increased rates of reaction. 2-Cyanoethylene oxide was found to be a better substrate for these enzymes than was acrylonitrile. Rat liver cytosol exhibited at least an order of magnitude more activity than did either rat brain or human liver towards either substrate. Rat brain, but not liver, is a target organ in acrylonitrile tumorigenesis (32, 33). It is possible that brain is particularly sensitive to effects of acrylonitrile or its epoxide because of a lower capacity for detoxication through GSH conjugation. Purified epoxide hydrolase exhibited a Vmax of 300 nmol/min/mg for the hydrolysis of 2-cyanoethylene oxide, whereas crude cytosol preparations catalyzed GSH conjugation of this compound at
a similar rate. Thus, it is likely that GSH conjugation plays a more important role than does epoxide hydrolysis in the detoxification of any 2-cyanoethylene oxide which is generated in vivo.

The in vitro data can be compared with those obtained with vinyl halides. At saturating substrate concentrations, vinyl chloride is metabolized to protein-bound metabolites at a rate of 7.8 nmol/hr/mg protein and to calf thymus DNA-bound metabolites at a rate of 1.4 nmol/hr/mg protein in the presence of phenobarbital-treated rat liver microsomes (12). The work of Kappus et al. (20) indicates that a substrate concentration of 0.3 mm (based on total gaseous and dissolved) vinyl chloride is saturating. Under the same conditions of saturating substrate concentration, vinyl bromide is metabolized to protein-bound metabolites at a rate of 19.8 nmol/hr/mg protein and to DNA-bound adducts at a rate of 2.0 nmol/hr/mg protein (12). The observed \( K_m \) value is less than 0.1 mm. Thus, the first-order rate constants for the metabolism of vinyl chloride and vinyl bromide to DNA-bound metabolites would be on the order of 0.04 per hr per mg microsomal protein per ml. Direct data for comparison of 1,1,2-trichloroethylene are not available, but our own work suggests that the corresponding first-order constant for production of DNA-bound metabolites under similar conditions is roughly 0.002 per hr per mg protein per ml. The corresponding constants for metabolites irreversibly bound to protein are roughly an order of magnitude higher in both cases. From the kinetic data presented here, a first-order constant of \( 6 \times 10^{-9} \) per hr per mg protein per ml for production of DNA-bound metabolites can be calculated (i.e., \( V_{max} / K_m \)) (18). Again, the value for enzyme-mediated protein binding is an order of magnitude greater. These first-order rate constants predict the relative rates of conversion of compounds to DNA-bound adducts at low substrate concentrations, which are applicable in many chronic risk assessment situations. The rate for acrylonitrile is one to 2 orders of magnitude less than that predicted for trichloroethylene, a possible carcinogen, and 3 orders of magnitude less than that for vinyl chloride and vinyl bromide, for which more extensive evidence for carcinogenicity has been presented. Thus, the tumorigenicity observed in rats following acrylonitrile exposure may result from a mechanism other than DNA alkylation.

In conclusion, evidence has been presented that P-450s metabolize acrylonitrile to 2-cyanoethylene oxide, which can bind irreversibly to nucleic acids as well as protein, although acrylonitrile readily binds to protein without further metabolism. Epoxide hydrolyase and GSH S-transferase may have some protective roles. In general, all metabolic systems appear less active in humans than in rats. The relationship of these events to any tumor formation in extrahepatic tissues is presently unclear. 2-Cyanoethylene oxide is rather stable and could conceivably migrate from the liver, probably a major organ involved in its formation, to target tissues to alkylate DNA. Caution is needed in ascertaining the relevance of in vitro DNA binding produced in micosomal incubations (3). Nonenzymatic alkylation of proteins by acrylonitrile is extensive, and epigenetic mechanisms not involving bioactivation should be considered (35). In addition, tissue variation in rates of DNA repair needs to be taken into account, as repair in brain has been reported to be less rapid than in liver (21). These possibilities are being considered in further studies with more complex cellular systems. Nevertheless, the initial studies on the mechanisms of acrylonitrile metabolism in subcellular liver fractions are important in defining basic routes of biotransformation and in developing hypotheses to be tested in more complex systems.

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