Table 1. Bradykinin (BK) potentiating action on guinea-pig ileum and inhibition of human plasma ACE by captopril, MK-422 and α-casein fractions

Substance	PU* (μg/ml)	I_{50} ACE† (μg/ml)	Maximal potentiation BK‡
Captopril	$(8.1 \pm 3.6) \times 10^{-4}$§	$(6.0 \pm 0.12) \times 10^{-3}$	7.2 ± 0.6
MK-422	$(2.3 \pm 0.4) \times 10^{-3}$	$(4.3 \pm 0.15) \times 10^{-3}$	5.2 ± 0.12
Tryptic hydrolysate	95.0 ± 4.0	81.0 ± 1.05	2.0 ± 0.18
Sephadex G-25 F active fractions	2.0 ± 0.09	23.0 ± 1.15	13.3 ± 0.3

* PU—potentiating unit: amount of peptide per milliliter able to double the action of a single dose of BK on the isolated guinea-pig ileum.
† I_{50} ACE—50% inhibition of angiotensin converting enzyme.
‡ For definition, see text.
§ Standard errors with two degrees of freedom.

*Department of Biochemistry and Immunology and ‡Department of Physiology Institute of Biological Sciences, University of Minas Gerais, Belo Horizonte, MG, Brasil

OLGA B. HENRIQUES*†
ROSANGELA B. DE DEUS*
ROBSON A. S. SANTOS‡

† Address correspondence to: Prof. Olga B. Henriques, Departamento de Bioquímica, Instituto Butantan, Av. Dr. Vital Brasil, 1500, 05504 - São Paulo - SP, Brasil.

REFERENCES

1. O. B. Henriques, L. F. Ferreira, H. M. Flash and E. B. Oliveira, *Adv. exp. Med. Biol.* **156**A, 621 (1983).
2. T. L. McMeekin, in *The Proteins* (Eds. H. Neurath and K. Bailey), Vol. 2A, p. 389. Academic Press, New York (1954).
3. S. H. Ferreira, D. C. Bartelt and J. L. Greene, *Biochemistry* **9**, 2583 (1970).
4. D. W. Cushman and H. S. Cheung, *Biochim. biophys. Acta* **250B**, 261 (1971).
5. R. A. S. Santos, E. M. Krieger and J. L. Greene, *Hypertension* **7**, 244 (1985).

Biochemical Pharmacology, Vol. 36, No. 1, pp. 184–187, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

Evidence for the microsomal metabolism of glycolonitrile

(*Received* 26 *March* 1986; *accepted* 26 *June* 1986)

Saturated aliphatic nitriles are commercially important chemicals used in the manufacture of plastics, pharmaceuticals, pesticides and synthetic fibers [1]. The acute toxicity of the saturated nitriles has been attributed to a metabolite, CN^-* [2].

The mechanisms of metabolism of saturated nitriles to CN^- have not been well characterized. Ohkawa *et al.* [3] hypothesized that the initial step in nitrile metabolism is a mixed-function oxidase-dependent oxidation at the alpha-carbon, resulting in the formation of a cyanohydrin, which may be unstable and breakdown non-enzymatically to HCN and an aldehyde. Based upon data which indicated that 4-methylpyrazole and ethanol delayed the time to death in mice administered succinonitrile, Doherty *et al.* [4] suggested that alcohol dehydrogenase may catalyze the liberation of CN^- from the cyanohydrin of succinonitrile and, further, that the metabolism of other nitriles may also involve two enzymatic steps. Ethanol and pyrazole-compounds are also inhibitors of certain hepatic mixed-function oxidase systems [5, 6], however, and these chemicals may delay toxicity by inhibiting the initial oxidation of the parent nitrile. In our research on the metabolism of MeCN [7], we observed and report here that HCHO was not a metabolite of this nitrile. The present studies were thus initiated to study the liberation of CN^- from GCN, the cyanohydrin of MeCN. The data presented here indicate that a microsomal enzyme, probably cytochrome P-450, may catalyze the metabolism of this cyanohydrin to CN^- via a reaction which does not involve the release of HCHO.

* Abbreviations: MeCN, acetonitrile; CN^-, cyanide; HCHO, formaldehyde; and GCN, glycolonitrile.

Materials and methods

Chemicals. MeCN (99+%) and GCN (70%) were purchased from the Aldrich Chemical Co. (Milwaukee, WI) and Alfa Products (Danvers, MA) respectively. Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD) and NADP were obtained from the Sigma Chemical Co. (St. Louis, MO). Cobaltic protoporphyrin 9-chloride (cobalt heme) was purchased from Porphyrin Products (Logan, UT). All other chemicals were of the highest grade commercially available. All buffers and water were passed through Chelex 100 (Bio-Rad Laboratories, Richmond, CA) prior to use to reduce the concentrations of contaminating trace metals.

Microsomal preparations. Female Sprague–Dawley rats, obtained from the Charles River Breeding Laboratories (Lakeview Facility, NJ) and weighing 170–260 g, were used in these studies. The animals were dosed with either a 25% (v/v) aqueous solution of acetone (1960 mg/kg, p.o.) or water (10.0 ml/kg, p.o.). The rats were killed (+24 hr), and the livers were collected, pooled by dosage group, and homogenized in 0.1 M sodium phosphate buffer containing 0.15 M KCl, pH 7.4. Microsomes were prepared by differential centrifugation, washed with 0.15 M KCl, resus-

pended in the phosphate buffer and stored at −70° until use. Groups of rats were also treated with cobalt heme (90 μmoles/5 ml/kg, s.c.) or phosphate-buffered saline (PBS) (5 ml/kg, s.c.) and killed 48 hr later. Hepatic microsomes were prepared as described above.

Enzyme assays. The reactions were carried out at 37° in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.15 M KCl with a final assay volume of 2.0 ml. The incubation mixtures contained microsomal protein (0.5 to 4.0 mg/ml), substrate, and an NADPH-generating system consisting of 0.5 mM NADP, 4.0 mM G6P, 2.5 units of G6PD, and 25 mM $MgCl_2$. All reactions were initiated with substrate and terminated with 0.4 ml of 100% (w/v) trichloroacetic acid. Inhibitors were incubated with microsomes for 10 min prior to initiation of the reaction.

Analytical methods. Microsomal protein content was determined by the method of Lowry *et al.* [8]. Cyanide was analyzed colorimetrically [9] following purification by microdiffusion [10]. Formaldehyde was determined by the method of Kleeberg and Klinger [11]. Cytochrome P-450 was determined using the method of Omura and Sato [12].

Statistical analyses. Experiments involving comparisons of data from several groups were analyzed by analysis of variance using a SAS program. Experiments in which data from two treatment groups were compared were analyzed using Student's *t*-test.

Results and discussion

The hypothesis of Ohkawa *et al.* [3] predicts that the metabolism of nitriles should result in the liberation of CN^- and an aldehyde containing one less carbon atom than the parent nitrile. In the present studies, however, HCHO was not identified as a metabolite of MeCN (Table 1). Results of extensive recovery studies indicated that neither MeCN nor NaCN interfered with either the recovery or the colorimetric determination of HCHO, and HCHO concentrations of 10–100 μM could be readily measured. These data suggested that either a cyanohydrin (GCN) was not formed as an intermediate of MeCN metabolism, or that the cyanohydrin was further metabolized. Therefore, the degradation of GCN to CN was studied. The degradation of GCN to CN^- and HCHO occurred readily under slightly alkaline conditions (Table 1). The presence of microsomes resulted in a 5-fold increase in CN^- liberation without a similar increase in HCHO formation. These results suggest that the liberation of CN^- from GCN may occur enzymatically. Because HCHO does not appear to be a metabolite of either MeCN or GCN, the possible formation of GCN during the metabolism of MeCN cannot be ruled out. It must be recognized, however, that the cyanohydrin has not been identified as a metabolite of the nitrile, and it is possible that GCN and MeCN may be metabolized by different pathways, neither of which result in the production of HCHO.

The microsomal metabolism of GCN to CN^- was linear with time (Fig. 1), and dependent upon the microsomal protein content in the incubation mixtures (Table 2). Neither bovine serum albumin nor heat-inactivated microsomes catalyzed the liberation of CN^- from GCN (Table 2). The microsomal metabolism of GCN to CN^- also appeared to be NADPH (Table 2) and oxygen (Table 3) dependent. These data might be interpreted to suggest catalysis by a mixed-function oxidase.

Metyrapone and SFK-525A significantly inhibited the metabolism of GCN to CN^- (Table 3). These compounds also inhibit the metabolism of MeCN to CN^-.* The metabolism of both MeCN and GCN was decreased markedly in microsomes from rats treated with cobalt heme which decreased hepatic cytochrome P-450 (Table 4). These data suggest that cytochrome P-450 may be involved in the

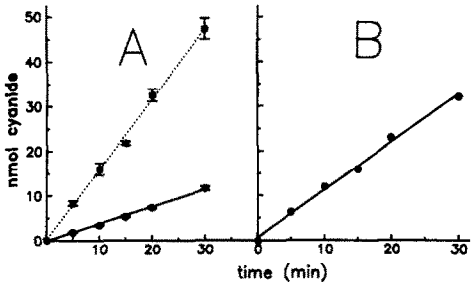

Fig. 1. Time course of microsomal metabolism of glycolonitrile to cyanide. Reaction mixtures contained 200 μM GCN, 1.0 mg/ml microsomal protein, and an NADPH-generating system. Microsomes were omitted from one-half of the reactions. Following a 3-min preincubation at 37°, the reactions were initiated with GCN. (A) Time courses in the complete microsomal system (dotted line) and in the same system without microsomes (solid line). Data are graphed as the mean ± SD. (B) Time course attributable to enzymatic CN production, calculated as the difference between the two lines shown in panel A. Cyanide determinations were performed in quadruplicate.

* J. J. Freeman and E. P. Hayes, manuscript submitted for publication.

Table 1. Stoichiometry of acetonitrile and glycolonitrile metabolism by rat liver microsomes

Assay conditions*†	Product formation‡ Cyanide (total nmoles)	Formaldehyde (total nmoles)
Microsomes + MeCN (150 mM)	202.2 ± 3.1	0
Water (pH 4.2) + GCN (2.0 mM)	3.2 ± 0.1§	3.4 ± 2.3§
Buffer (pH 7.4) + GCN (2.0 mM)	51.6 ± 10.1‖	77.4 ± 2.3‖
Microsomes + GCN (2.0 mM)	259.4 ± 24.5¶	47.5 ± 1.2¶

* Microsomes were isolated from acetone-pretreated (-24 hr) rats (1960 mg/kg, p.o.). All reactions were initiated with substrate and incubated at 37° for 30 min. The assay system contained substrate, 2.0 mg/ml protein, and an NADPH-generating system in 0.1 M sodium phosphate buffer with 0.15 M KCl, pH 7.4.

† Pretreatment of rats with acetone results in a 4-fold increase in the metabolism of MeCN to CN^- *in vitro* [7]. In the present studies, this procedure facilitated metabolite indentification.

‡ Results are expressed as the mean ± SD of quadruplicate determinations.

§–¶ The means of groups with different superscripts are significantly ($P < 0.05$) different.

Table 2. Protein- and NADPH-dependency of microsomal metabolism of glycolonitrile to cyanide

Assay conditions*	Cyanide production† (nmoles/20 min)
No protein	365 ± 8‡
+ Bovine serum albumin	371 ± 11‡
+ Heat-inactivated microsomes	440 ± 4§
+ Microsomes (0.5 mg/ml)	554 ± 5‖
+ Microsomes (1.0 mg/ml)	663 ± 5¶
+ Microsomes (2.0 mg/ml)	856 ± 21**
+ Microsomes (4.0 mg/ml)	1130 ± 41††
− NADP	456 ± 8§

* Microsomes were isolated from water-pretreated rats (10.0 ml/g, p.o., -24 hr). All reactions were initiated with GCN (10 mM) and conducted at 37° for 20 min in 0.1 M sodium phosphate buffer containing 0.15 M KCl, pH 7.4. All reaction mixtures contained an NADPH-generating system and 1.0 mg protein/ml (unless noted otherwise).

† Results are expressed as the mean ± SD of quadruplicate determinations.

‡–†† The means of groups with different superscripts are significantly ($P < 0.05$) different.

metabolism of GCN. Pretreatment of rats with acetone, which induces a specific isoenzyme of cytochrome P-450 [13], and induces the metabolism of MeCN to CN^- [7], enhanced the microsomal metabolism of GCN to CN^- (Table 4).

Since the cyanohydrin is also an alcohol, pathways of metabolism distinct from monooxygenation might also be considered. For example, catalase- and free radical-dependent routes of metabolism may be involved, as has been reported for ethanol [14, 15]. The data reported here do not support the hypothesis that alcohol dehydrogenase may be involved in the metabolism of GCN, which was NADPH dependent and occurred in microsomes without the production of HCHO. Although further research is needed to clarify the pathways of nitrile and cyanohydrin metabolism, the results of the present studies may be consistent with a mechanism of nitrile metabolism involving two sequential enzymatic steps.

In summary, the evidence presented suggests that the liberation of CN^- from GCN may be catalyzed by a microsomal system dependent on cytochrome P-450. This metabolism was linear with time and NADPH dependent. Formaldehyde was not produced during the enzyme-catalyzed liberation of CN^- from either MeCN or GCN.

Acknowledgements—This work was supported, in part, by a Spill Fund Fellowship to J. J. F. from the New Jersey Department of Environmental Protection.

Table 3. Effect of inhibitors on the metabolism of glyconitrile to cyanide in rat liver microsomes*

Inhibitor	Cyanide production (nmoles/min/mg protein)	% of control
Control	7.50 ± 0.38‡	100
Metyrapone (2 mM)	5.72 ± 0.67§	76.3
SKF-525A (1 mM)	6.53 ± 0.28§	87.1
Nitrogen¶	2.62 ± 0.84‖	34.9

All reactions were performed for 20 min at 37° in 0.1 M sodium phosphate buffer with 0.15 M KCl, pH 7.4. All reaction mixtures contained glycolonitrile (10 mM), microsomal protein (1 mg/ml), and an NADPH-generating system. Following preincubation for 10 min with inhibitor, the reaction was initiated by addition of NADP + G6P and substrate. Values are mean ± SD, N = 4.

‡–‖ Means of groups with different superscripts are significantly ($P < 0.05$) different.

¶ Nitrogen was bubbled through the microsomal suspension for 1.5 min, and sealed vials containing the suspension were preincubated for 10 min followed by initiation of the reaction as above.

Table 4. Effect of treatment with acetone or cobalt heme on the metabolism of glyconitrile and acetonitrile to cyanide by rat liver microsomes*

Treatment	Cytochrome P-450 (nmoles/mg protein)	Acetonitrile metabolism to cyanide	Glycolonitrile metabolism to cyanide
Water, 10 ml/kg, p.o.	1.05	4.64 ± 0.35†	7.41 ± 0.18‡
Acetone, 1960 mg/10 ml/g, p.o.	1.57	20.56 ± 3.02§	13.44 ± 0.71§
PBS, 5 ml/kg, s.c.	1.08	4.64 ± 0.21	8.33 ± 0.46
Cobalt heme, 90 μmoles/5 ml/kg, s.c.	0.576	1.74 ± 0.40§	3.99 ± 0.37§

* All reactions were performed for 20 min at 37° in 0.1 M sodium phosphate buffer with 0.15 M KCl, pH 7.4. Reaction mixtures contained microsomal protein (1 mg/ml), an NADPH-generating system, and glycolonitrile (20 mM) or acetonitrile (160 mM). Following preincubation for 3 min, the reactions were initiated by addition of NADP + G6P and substrate.

† Cyanide production from acetonitrile is expressed as nmoles/mg protein/20 min (mean + SD, N = 4).

‡ Cyanide production from glycolonitrile is expressed as nmoles/mg protein/min (mean + SD, N = 4).

§ The mean of this group is significantly different ($P < 0.001$) from the mean of the respective control group.

Department of Environmental and Community Medicine,
University of Medicine and Dentistry of New Jersey—Rutgers Medical School and the Joint Graduate Program in Toxicology,
Rutgers, The State University
Piscataway, NJ 08854, U.S.A.

JAMES J. FREEMAN*
EILEEN P. HAYES†

REFERENCES

1. NIOSH, *Criteria for a Recommended Standard for Occupational Exposure to Nitriles.* U.S. Government Printing Office, Washington DC (1978).
2. C. C. Willhite and R. P. Smith, *Toxic. appl. Pharmac.* **59**, 589 (1981).
3. H. Ohkawa, R. Ohkawa, I. Yamamoto and J. E. Casida, *Pestic. Biochem. Physiol.* **2**, 95 (1972).
4. P. A. Doherty, R. P. Smith and V. H. Ferm, *J. Pharmac. exp. Ther.* **223**, 635 (1982).
5. R. Peng, Y. Y. Tu and C. S. Yang, *Carcinogenesis* **3**, 1457 (1982).
6. A. I. Cederbaum and L. Berl, *Archs Biochem. Biophys.* **216**, 530 (1982).
7. J. J. Freeman and E. P. Hayes, *Toxicologist* **5**, 246 (1985).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 267 (1951).
9. R. V. Blanke, in *Fundamentals of Clinical Chemistry* (Ed. N. W. Tietz), p. 1117. W. B. Saunders, Philadelphia (1976).
10. M. Feldstein and N. C. Klendshoj, *J. Lab. clin. Med.* **44**, 166 (1954).
11. U. Kleeberg and W. Klinger, *J. pharmac. Meth.* **8**, 19 (1982).
12. T. Omura and R. Sato, *J. biol. Chem.* **221**, 253 (1965).
13. D. R. Koop and J. P. Casazza, *Fedn Proc.* **44**, 474 (1985).
14. D. Keilin and E. F. Hartree, *Biochem. J.* **39**, 293 (1945).
15. A. I. Cederbaum, E. Dicker and G. Cohen, *Biochem. biophys. Res. Commun.* **78**, 1254 (1977).

* Current address: Exxon Corporation, Research and Environmental Health Division, P.O. Box 235, East Millstone, NJ 08873.

† Address all correspondence to: Eileen P. Hayes, Sc.D., Department of Environmental and Community Medicine, UMDNJ – Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854.