BIOORGANIC CHEMISTRY 8, 227–235 (1979)

The Nonmicrosomal Production of *N*-(4-Chlorophenyl)-glycolhydroxamic Acid from 4-Chloronitrosobenzene by Rat Liver Homogenates

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Received September 7, 1978

The incubation of 4-chloronitrosobenzene (4-CNB) with subcellular fractions of rat liver resulted in the formation of a previously unknown type of hydroxamic acid metabolite for mammals. This new metabolite, *N*-(4-chlorophenyl)glycolhydroxamic acid (Gl-CHA), is most likely formed through the action of liver transketolase on the substrate 4-CNB. Gl-CHA was produced only by the 10 000*g* and 105 000*g* supernatant fractions, and required glucose-6-phosphate as an energy source. No hydroxamic acid metabolites were produced in detectable quantities by the microsomal fraction of the rat liver homogenate. Gl-CHA was positively identified by isolation and comparison to an authentic sample of Gl-CHA. Authentic Gl-CHA was prepared by the condensation of 4-chlorophenylhydroxylamine with glycolic acid in the presence of dicylohexylcarbodiimide. The highest observed conversion of 4-CNB to Gl-CHA was 18%, which occurred at the lowest concentration of 4-CNB incubated with the 105 000*g* supernatant. Gl-CHA was not produced by C-hydroxylation of the corresponding acetyl-derived hydroxamic acid, since none of the subcellular fractions of rat liver would effect this conversion. The incubation of 4-chloroaniline under identical conditions failed to result in the production of Gl-CHA; however, such an observation is probably not important to the possibility that Gl-CHA might be a significant metabolite *in vivo*.

INTRODUCTION

It is well established that the toxicity or carcinogenicity of many aromatic amides, amines, and nitro compounds is mediated by the conversion of such common functional groups to arylhydroxylamines and hydroxamic acids (*1*). The tendency of an organ or cell type to effect such conversions is of great importance to our understanding of toxicity by any compound. The major biochemical pathway for the production of hydroxamic acids involves N-oxidation by microsomal oxidases; however, other pathways to hydroxamic acid production *in vivo* might be important, especially in cell types that are deficient or lacking in microsomal oxidases. We have been interested in such nonmicrosomal pathways to hydroxamate production, and have succeeded in demonstrating that alternate biochemical mechanisms do exist (*2–4*).

The alternate pathways are the result of the interaction of thiamine-dependent enzymes, such as pyruvate decarboxylase and transketolase, with arylnitroso compounds (Fig. 1). Two structural classes of hydroxamic acids can result, depending upon the function of the thiamine-dependent enzyme. *N*-Acetylhydroxylamines (e.g.,

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Ac-CHA) are produced by pyruvate-metabolizing enzymes (*3*); while the *N*-glycolylhydroxylamines (e.g., Gl-CHA) are produced by transketolases (*4*). Most recently, we reported that both of these thiamine-dependent conversions are operative in an intact microbial species (*5*).

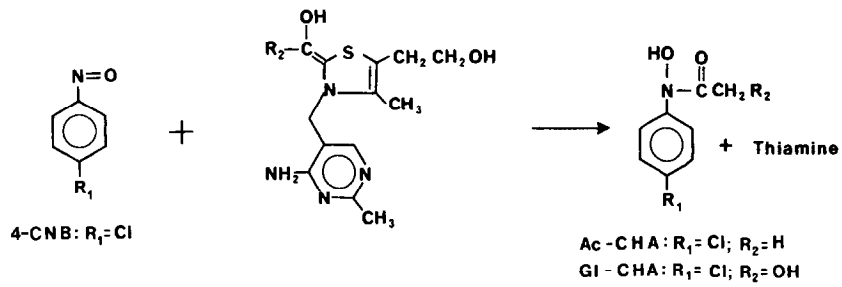

FIG. 1. Thiamine-mediated conversion of arylnitroso compounds to hydroxamic acids.

We now report that the transketolase-derived hydroxamic acid (Gl-CHA) is produced from 4-CNB[1] by rat liver homogenates. Such an *N*-glycolylhydroxylamine has never been reported as a mammalian metabolite, and nothing is known concerning the toxicity of this new structural group.

EXPERIMENTAL

4-CNB was prepared according to a previously described method (*6*) from 4-chloronitrobenzene obtained from Eastman Chemical Company (Rochester, N.Y.). Purification of 4-CNB was achieved by codistillation with ethanol, followed by precipitation when H_2O was added to the dark blue distillate. Recrystallization from ethanol gave pure 4-CNB (mp 87–88°C) which is indefinately stable when stored in the dark at −20°C. Ac-CHA was prepared according to the method of Smissman and Corbett (*7*). Infrared spectra were obtained on a Perkin–Elmer Model 180 spectrophotometer. The uv-visible spectrophotometric measurements were obtained on a Beckman Model 24 spectrophotometer. NMR spectra were conducted on a Jeol C-60 HL nuclear magnetic resonance spectrometer. Melting points were obtained on a calibrated Thomas Hoover melting point apparatus. Thin-layer chromatography (tlc) was conducted on 5 × 20-cm EM silica gel 60 F-254 plates obtained from Brinkman Instruments (Westbury, N.Y.). Elemental analyses were performed by Galbraith Laboratories (Knoxville, Tenn.). All biochemical reagents and enzymes were purchased from Sigma Chemical Company (St. Louis, Mo.).

Synthesis of Gl-CHA. Dicyclohexylcarbodiimide (8.3 g, 0.04 mol) dissolved in 25 ml of anhydrous ether was added to a solution of 4-chlorophenylhydroxylamine (2.9 g, 0.02 mol) in 50 ml of anhydrous ether contained in a 250 ml R.B. flask and cooled in an ice bath. Immediately following this addition, glycolic acid (3.0 g, 0.04 mol) dissolved in 10 ml of dimethyl formamide was added in the course of 15 min with continued cooling

[1] Abbreviations used: 4-CNB, 4-chloronitrosobenzene; Ac-CHA, *N*-(4-chlorophenyl)acetohydroxamic acid; Gl-CHA, *N*-(4-chlorophenyl)glycolhydroxamic acid.

and stirring by means of a magnetic stir bar. After 20 min additional reaction time, the suspension was filtered and the dicyclohexylurea was washed with 20 ml of ether. The combined ether solution was extracted thoroughly with 1.6 g (0.04 mol) of NaOH in 30 ml of H_2O. The ether layer was extracted with 10 ml of H_2O and the aqueous portion was combined with the aqueous base extract, which was then acidified (pH 5) with 5% HCl. The cloudy solution was extracted twice with 40 ml of ethyl acetate, and the combined organic portions were dried (Na_2SO_4) and evaporated *in vacuo* to give an oil. Trituration of the oil with 50 ml of H_2O induced solidification of Gl-CHA, which was recrystallized from aqueous ethanol to give 2.2 g (55%) of Gl-CHA as a white crystalline powder (mp 124–125°C). *Anal.* Calcd. for $C_8H_8NO_3Cl$: C, 47.64; H, 4.00; N, 6.95. *Found:* C, 48.11; H, 4.24; N, 6.85. Infrared and nuclear magnetic resonance spectra of the product were consistent with the chemical structure for Gl-CHA.

Preparation of subcellular fractions. The livers of three male Sprague–Dawley rats weighing 42 g were homogenized for 30 sec in 0.10 *M*, pH 7.4, Trizma–HCl buffer at 4°C by use of a Virtis 45 homogenizer at 45,000 rpm. The homogenate was centrifuged at 10 000*g* for 30 min by use of a Sorvall RC-5 centrifuge to give 100 ml of 10 000*g* supernatant. An 80-ml portion of this supernatant was centrifuged at 105 000*g* for 1 h to give the 105 000*g* supernatant and a microsomal pellet which was resuspended in 80 ml of buffer to give the microsomal fraction. The three subcellular fractions were kept in ice and used within 2 h after preparation. Protein was determined by the method of Bradford (*8*) employing crystalline bovine serum albumin as the standard. Transketolase activity of the 105 000*g* supernatant fraction was determined by a spectrophotometric method (*9*) at 25°C.

Incubation procedure. Individual incubations were carried out in 50-ml flasks by adding 20.0 ml of 0.1 *M*, pH 7.4, Trizma–HCl buffer containing 56.4 mg (0.2 mmol) of glucose-6-phosphate (monosodium salt), 3.0 mg (0.03 mmol) of $MgCl_2$, and 0.96 mg (2.1 μmol) of thiamine pyrophosphate to each flask. After the temperature of the

TABLE I

CONVERSION OF 4-CNB TO Gl-CHA BY SUBCELLULAR FRACTIONS OF RAT LIVER[a]

Fraction	Amount of 4-CNB (μmol)	Amount of Gl-CHA produced (μmol)	Percentage conversion to Gl-CHA
10 000*g* supernatant	40	2.5 ± 0.5	6 ± 1
10 000*g* supernatant	20	1.4 ± 0.3	7 ± 1
Microsomal	40	0	0
105 000*g* supernatant	40	4.1 ± 0.8	10 ± 2
105 000*g* supernatant	20	3.0 ± 0.6	15 ± 3
105 000*g* supernatant	10	1.8 ± 0.3	18 ± 3

[a] Incubation conditions and methods for the quantitative determination of Gl-CHA are described in the experimental section. Each value for Gl-CHA production is the average of five individual incubations, and the precision for each value is expressed as standard deviation (±SD).

incubation media had equilibrated to 37°C, 2.0 ml of the subcellular fraction or control was added and the mixture was preincubated in a New Brunswick Model G24 incubator shaker for 1 min. 4-Chloronitrosobenzene was then added in a total volume of 200 μl of 95% EtOH, the flask was stoppered, and incubation was conducted for 30 min at 37°C with an agitation rate of 200 rpm. For the incubations conducted on microsomal pellets the above incubation method was employed, but with the addition of 25 units of glucose-6-phosphate dehydrogenase and 7 mg (0.01 mmol) of NADP to each incubate (*10*). The quantities of 4-CNB utilized in individual incubations are given in Table 1. The incubations of authentic Ac-CHA and Gl-CHA with the 105 000*g* supernatant were conducted in an identical manner, except that single substrate concentrations were employed by the addition of 5.0 μmol of each to separate flasks. Incubations containing 80 μmol of 4-chloroaniline were conducted in an identical manner employing the 10 000*g* supernatant. Controls were run with each subcellular fraction and consisted of 2.0 ml of the respective fraction that had been gently boiled for 1 min. Incubations were also conducted in which glucose-6-phosphate was absent from the incubation media. All controls were conducted at the highest concentration of 4-CNB only.

Quantitative analysis of Gl-CHA production. At the end of the 30-min incubation period each incubate was treated with 5 g of NaCl and extracted twice with EtOAc. Any emulsions that formed were readily broken by a short period of low-speed centrifugation. The EtOAc was dried (Na_2SO_4) and evaporated to give a solid residue. The residue was dissolved in 0.50 ml of $CHCl_3$/MeOH (1 : 1), from which solution 2-μl aliquots were chromatographed on silica gel tlc plates (5% MeOH/$CHCl_3$) along with a series of concentrations of authentic Gl-CHA ranging from 0.2 to 4.0 mg/ml. The silica gel plates were sprayed with 1% $FeCl_3 \cdot 6H_2O$ in acidified MeOH (0.12 *N* HCl) and the amount of Gl-CHA produced in each incubate was then determined by visual comparison of color intensities. A second quantitative determination of Gl-CHA production was made by evaporation of a 200-μl aliquot of the solution employed for tlc analysis. The residue was dissolved in 1.0 ml of MeOH and treated with 1.0 ml of the 1% $FeCl_3 \cdot 6H_2O$ in MeOH, and the optical absorbance at 535 nm was determined by use of a Beckman Model 24 spectrophotometer. Comparison of the absorbance of the ferric complex of Gl-CHA with a standard curve prepared from authentic Gl-CHA gave quantitative values of Gl-CHA production, which agreed closely with those obtained by quantitative tlc analysis.

Isolation of Gl-CHA from incubation product. A large-scale incubation was performed with the 105 000*g* supernatant and highest 4-CNB concentration as described above, except that the final incubation volume was 220 ml. After 40-min incubation time the incubate was saturated with NaCl, filtered through a glass wool pad, and extracted once with 200 ml of EtOAc. The organic layer was dried (Na_2SO_4) and evaporated *in vacuo* to give a yellow residue. The residue was applied to a 25 × 1.4-cm bed of EM silica gel 60 (70–230 mesh) and eluted with $CHCl_3$ to give 3 mg of 4,4′-dichloroazoxybenzene. Elution with 10% MeOH/$CHCl_3$ gave a hydroxamic acid fraction that appeared to be pure by tlc analysis. Recrystallization from aqueous ethanol gave 4 mg (5% theoretical) of product, which was identical in mp, infrared, and tlc characteristics to authentic Gl-CHA. The tlc analysis of other chromatographic fractions failed to indicate the presence of Ac-CHA in the incubation product.

RESULTS

The incubation of 4-CNB with both the 10 000*g* and 105 000*g* supernatants obtained from a rat liver homogenate resulted in the production of Gl-CHA. The identification of this hydroxamic acid product was achieved by previously described chromatographic methods (*4*) and by isolation of the product in pure form from a large-scale incubation with the 105 000*g* supernatant. The product possessed an identical melting point and infrared spectrum with authentic Gl-CHA. The authentic Gl-CHA was obtained by the condensation of 4-chlorophenylhydroxylamine with glycolic acid in the presence of dicyclohexylcarbodiimide.

No Ac-CHA was detected in any of the incubations conducted in the present study, although a trace of Ac-CHA had been detected in an earlier preliminary study with rat liver homogenates. 4,4′-Dichloroazoxybenzene was also identified as a product of these incubations, although no attempts were made to quantitate the extent to which it was produced. Unreacted 4-CNB was not present in detectable quantities in any of the incubate products.

The percentage conversion of 4-CNB to Gl-CHA was dependent both upon the particular subcellular fraction that was employed and upon the amount of 4-CNB present in the incubate (Table 1). The highest conversion of 18% occurred with the 105 000*g* supernatant at the lowest concentration of 4-CNB. Substantially lower conversions occurred with the 10 000*g* supernatant, which is probably the result of the higher protein concentration of this fraction (Table 2). The rates of Gl-CHA production reported in Table 2 are for comparison between the three fractions and are likely much lower than maximal velocities. Both the complexity of the incubation mixtures and the analytical method for Gl-CHA prevented the determination of maximal velocities. Most significant is the failure to observe any production of Gl-CHA by the microsomal fraction.

Preliminary investigations on the accuracy of the analytical determination for Gl-CHA indicated that about 90% of the Gl-CHA is recovered from spiked incubates. The results presented in Table 1 indicate a modest precision of about 20%. Attempts to develop a simpler, more precise analytical determination of Gl-CHA employing high-pressure liquid chromatography have been unsuccessful.

The incubation of either Gl-CHA or Ac-CHA with the subcellular fractions resulted in the recovery of the substrate without any observed interconversion between the two

TABLE 2

PROTEIN CONTENT AND RELATIVE RATES OF CONVERSION OF 4-CNB TO Gl-CHA BY RAT LIVER SUBCELLULAR FRACTIONS[a]

Fraction	Protein (mg/ml)	Rate of Gl-CHA production (μmol/mg) (30 min)
10 000*g* supernatant	30.0	0.08
Microsome	4.2	0
105 000*g* supernatant	16.9	0.24

[a] The rate of Gl-CHA production was determined at the highest concentration of 4-CNB employed.

hydroxamic acids. The incubation of 4-chloroaniline with the subcellular fractions did not result in the production of any observed hydroxamic acid, even with prolonged 2-hr incubation periods. The exclusion of glucose-6-phosphate from incubates that normally produced Gl-CHA resulted in no observation of hydroxamic acid production. As expected, the use of heat-denatured fractions failed to result in hydroxamic acid production.

Incubations conducted with 4-CNB resulted in a gradual formation of a precipitate which was quite extensive by the end of the incubation period. No such precipitation was noted when 4-chloroaniline, Gl-CHA, or Ac-CHA were employed as substrates. Transketolase activity was found to be present in the 105 000*g* supernatant to the extent of 0.017 units/mg protein for a total of 0.6 units of transketolase activity measured at 25°C in each incubation conducted with the 105 000*g* supernatant. One unit of transketolase activity is that amount that results in the conversion of 1.0 μmol of xylulose-5-phosphate per minute under defined conditions (*9*). After 30-min incubation with 4-CNB no transketolase activity was present, which indicates enzyme denaturation by 4-CNB.

DISCUSSION

The conversion of 4-CNB to Gl-CHA by the 105 000*g* supernatant of rat liver and not by the microsomal fraction is highly significant of several reasons. This is a non-microsomal conversion that could account for hydroxamic acid production in cells that are low or devoid of microsomal oxidases, in addition to providing an alternate pathway to hydroxamic acids in cells that do possess microsomal oxidases. Furthermore, Gl-CHA is a glycolic acid-derived hydroxamic acid and represents a previously unknown type of mammalian metabolite.

Gl-CHA was not produced by hydroxylation of the alpha carbon of Ac-CHA. This was clearly demonstrated by our failure to observe any conversion of Ac-CHA to Gl-CHA by either the 105 000*g* supernatant or by the microsomal fraction. Kiese has reported alpha hydroxylation of aromatic amides by rabbit liver microsomes (*11*, *12*) but the conversion of acetyl-derived hydroxamates to glycolyl-derived hydroxamates has never been reported. Thus, the production of Gl-CHA is not related mechanistically to the known production of glycolamides.

The most obvious explanation for the conversion of 4-CNB to Gl-CHA is the adventitious interaction of 4-CNB with transketolase, which is known to be present in the cytoplasm of liver cells in appreciable quantities (*13*). We detected the presence of transketolase in the 105 000*g* supernatant fraction that was employed in these studies. Thus, we conclude that it is transketolase activity that gives rise to Gl-CHA, especially in view of our earlier results employing purified yeast transketolase (*4*). A probable chemical mechanism to account for this conversion has been proposed (*4*) although an alternate mechanism to explain the interaction of nitroso compounds with transketolase is equally possible (*5*).

Before the production of metabolites, such as Gl-CHA, could be expected to occur within a cell, a source of the nitroso compound must exist. Although the arylnitroso functional group is not present in pharmaceuticals, food additives, or significant

environmental pollutants, this functional group is a well-known intermediary metabolite of the much more common xenobiotics, namely the arylamines and nitroaromatics. All known conversions of arylamines to nitrosoaromatics in mammals require an initial oxidation by a microsomal oxidase; however, the location of such microsomal oxidases need not be in the same tissue that eventually effects a thiamine-mediated conversion. This is obvious from the fact that nitrosoaromatics are well-known metabolites present in the circulatory system following the ingestion of arylamines (*14–16*). Hepatic oxidation of an arylamine to the corresponding arylhydroxylamine, which then enters the circulatory system is the initial biochemical event leading to methemoglobin production. Arylhydroxylamines are oxidized spontaneously in solution (*17*, *18*) or by oxyhemoglobin to nitrosoaromatics, a process which converts the oxyhemoglobin to methemoglobin (*16*). In turn the resulting nitroso compound can be reduced back to the hydroxylamine level by NADH-dependent enzymes and then subsequently reoxidized to the nitroso group. This cyclic process maintains a dose-dependent level of the nitrosoaromatic which slowly decreases as side reactions occur that serve to remove the two cycling reactants. In view of our present results, one of these side reactions is quite possibly the interaction with thiamine-dependent enzymes. That any cell type could be involved is obvious from the extent of blood flow throughout all tissues, and the ubiquitous distribution of thiamine-dependent enzymes.

Very recently, we demonstrated that a certain peroxidatic enzyme efficiently catalyzes the oxidation of arylamines to arylnitroso compounds without any detectable side products (*19*). This is a great contrast to the extensive studies on arylamine oxidations by horseradish peroxidase, which leads to polymerization with virtually undetectable levels of nitrosoaromatics being produced (*20*). Should such peculiar peroxidases be present in specialized cell types, then another source for nitroso compounds would exist within that cell, even though the cell might be devoid of microsomal oxidases.

The observed conversion of 4-CNB to Gl-CHA was as high as 18%, which is surprising in view of the extreme reactivity of arylnitroso compounds under such conditions. Arylnitroso compounds are known to react with protein thiol groups, which results in the reduction of the nitroso group to the hydroxylamine oxidation level (*21*). The simultaneous presence of both the nitroso and hydroxylamine compounds can result in a nonenzymatic production of the azoxy compound (*22*), especially as substrate concentrations are increased. Indeed, we did observe the presence of 4,4′-dichloroazoxybenzene in our incubation products. Covalent binding of nitroso compounds to protein is also well known (*23*). Our observation of extensive protein denaturation by the end of the incubation period of 4-CNB, but not for Gl-CHA, Ac-CHA, or 4-chloroaniline is most likely a result of the covalent binding of 4-CNB to protein. Nitrosoarenes are also known to react with unsaturated fatty acids under physiological conditions (*24*). With all the possible side reactions available to 4-CNB, our observed 18% conversion to Gl-CHA could well be considered a major biochemical process. Even though high concentrations of arylnitroso compounds are not expected *in vivo*, an 18% conversion of what might be present could lead to very significant amounts of glycolic acid-derived hydroxamates through the action of transketolase. Likewise, tissues with pyruvic acid dehydrogenase as the major thiamine-

dependent enzyme might be expected to yield significant quantities of acetyl-derived hydroxamates.

The fact that glycolic acid-derived hydroxamates, such as Gl-CHA, have never previously been observed as mammalian metabolites is by no means an argument against their actual production *in vivo*. From our present results it is obvious that any *in vitro* study employing microsomal fractions alone is insufficient as a model for actual total *in vivo* production of hydroxamic acid metabolites. Even whole liver homogenates are inadequate since liver microsomes do not produce appreciable quantities of nitroso compounds from arylamines (*18*). This is much more a property of oxyhemoglobin. Thus, our failure to observe conversion of 4-chloroaniline to Gl-CHA is not surprising, since the substrate for transketolase, 4-CNB, is not produced in significant quantities by rat liver alone. Unfortunately, *in vivo* studies rarely result in a complete elucidation of the biochemical conversions of a xenobiotic. This is particularly apparent for aromatic amines and related compounds since the extremely important hydroxylamine and hydroxamic acid metabolites undergo further conjugation reactions and binding to macromolecules (*1*). Since the glycolic acid-derived hydroxamates possess two functional groups, their potential for binding to macromolecules could be expected to be greater than that for the acetic acid-derived hydroxamates. Thus, the probability that the glycolic acid-derived hydroxamates might go undetected in whole animal experiments is great. We must conclude that although the biochemical pathways for the production of compounds, such as Gl-CHA, do exist, very carefully designed *in vivo* studies will be necessary to establish their significance.

ACKNOWLEDGMENT

This work was supported by National Cancer Institute, DHEW Grants CA 21668 and CA 21992.

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