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Radiochemical Synthesis and Colorimetric Analysis of Hydroxyguanidine

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A procedure has been developed for the radiochemical synthesis of [^{14}C]hydroxyguanidine by reaction of [^{14}C]cyanamide and hydroxylamine. A colorimetric assay for hydroxyguanidine which can be used for its assay in biological systems has been developed. This assay was employed to evaluate a novel enzyme isolated from terminal instar larvae of the tobacco budworm, *Heliothis virescens* [Noctuidae], which catalyzes an NADH-dependent reduction of hydroxyguanidine to guanidine. © 1992 Academic Press, Inc.

L-Canavanine **(1)**, L-2-amino-4-(guanidinooxy)butyric acid, is a highly toxic nonprotein amino acid produced by leguminous plants (1, 2). Detoxification or catabolism of this deleterious arginine antimetabolite can involve the formation of hydroxyguanidine **(2)**. For example, a soil-borne pseudomonad has been isolated that hydrolyzes L-canavanine to L-homoserine **(3)** and hydroxyguanidine (3).

$$\underset{\text{L-canavanine}}{\underset{\textbf{(1)}}{H_2N{-}C(NH_2){=}N{-}O{-}CH_2{-}CH_2{-}CH(NH_2)COOH}} \Rightarrow \underset{\text{L-homoserine}}{\underset{\textbf{(3)}}{HO{-}CH_2{-}CH_2{-}CH(NH_2)COOH}} + \underset{\text{hydroxyguanidine}}{\underset{\textbf{(2)}}{H_2N{-}C(=NH){-}NHOH}}$$

Transamidinases obtained from such divergent sources as *Streptomyces griseus* (4), mammalian pancreatic and renal tissues (5, 6), and the liver of the chicken or frog (7) can transfer the formamidine group of arginine to hydroxylamine to produce hydroxyguanidine.

Because hydroxyguanidine shares structural features with hydroxyurea, a compound which elicits antitumor activity (6), hydroxyguanidine has also been tested as an antitumor drug. This substituted guanidine exhibits cytotoxic effects against mast cell P815, leukemia P388, leukemia L1210, and Walker carcinosarcoma 256 tumors; it extends significantly the median survival time of rats bearing these tumors relative to control animals (8). In a similar vein, Lape *et al.* (9) reported

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that hydroxyguanidine was one of the most efficacious, in terms of extended animal longevity, of a series of drugs used against L1210 leukemia developed in female B6D2F_1 mice.

The commonality in structural features between hydroxyguanidine and hydroxyurea accounts for their effectiveness in inhibiting DNA synthesis (10) and the catalytic action of urease (EC 3.5.1.5) (11). Hydroxyguanidine's adverse effect on DNA production results in part from curtailment of ribonucleoside diphosphate reductase (EC 1.17.4.1) activity (12).

Recent investigations of the tobacco budworm, *Heliothis virescens*, established that the highly destructive larvae have a remarkable resistance to canavanine (13). This herbivore relies upon a hydrolase to convert L-canavanine to L-homoserine and hydroxyguanidine. A second larval enzyme mediates an NADH-dependent reduction of hydroxyguanidine to guanidine. This is the first instance of this biotransformation observed in an animal.

The use of hydroxyguanidine in experimental and clinical studies is limited by the lack of a procedure for its radiochemical synthesis and the availability of a method for an accurate and reproducible assay exhibiting high specificity. This communication details efforts which overcome these deficiencies and documents its utility in the analysis of hydroxyguanidine reduction by *H. virescens*.

RESULTS AND DISCUSSION

Colorimetric Assay for Hydroxyguanidine

The hydroxyguanidine-containing sample (1.0 ml) is diluted with an equal volume of 200 mM sodium tricine (pH 7.00) and treated with 0.2 ml of 1% (w/v) potassium persulfate. Chromogen formation is initiated by addition of 0.2 ml of 1% (w/v) photoactivated trisodium pentacyanoammonioferrate (14). Exactly 5 min later, the hydroxyguanidine-PCAF chromogen complex is read at 447 nm. Chromogen complex formation obeys Lambert–Beers' law over a concentration range of 0.7 mM.

Formation of the hydroxyguanidine-PCAF chromogen occurs in the absence of persulfate but this oxidizing agent accelerates color formation and shortens significantly the assay time (Fig. 1). Chromogen formation is complete after 4 min in the presence of persulfate but there is a gradual reduction in color after 6 min (Fig. 1). It is convenient to initiate color development with three sets of samples at a 2-min interval and then to determine color development exactly 5 min later for each set.

The hydroxyguanidine-PCAF chromogen exhibits extinction maxima at 404 and 447 nm. The extinction maximum can vary between these two values but it is usually greater at 447 nm (Fig. 2). The pH of the colorimetric assay mixture must be monitored carefully. Color formation is pH dependent and deviation from neutrality of more than 0.5 unit will adversely affect hydroxyguanidine-PCAF chromogen formation (Fig. 3).

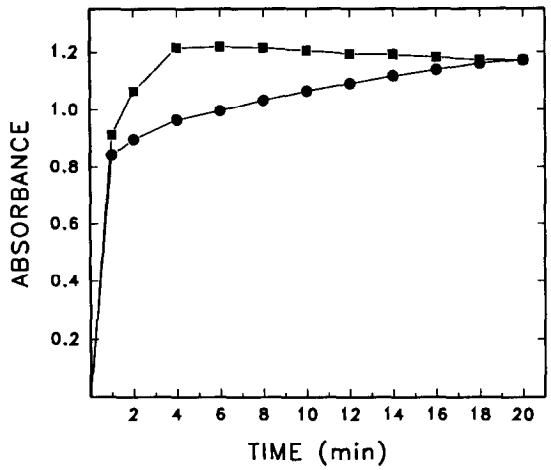

FIG. 1. The time course of hydroxyguanidine-PCAF chromogen formation. The hydroxyguanidine-PCAF chromogen was prepared in the presence (■) or absence (●) of potassium persulfate.

Specificity of the Assay

The PCAF assay is relatively specific for compounds containing a guanidinooxy moiety but it can react with such structurally disparate compounds as histidine (15). A very high degree of specificity can be achieved by passing a given sample at pH 3–4 through an appropriately sized column of Dowex-50 (NH_4^+) and then

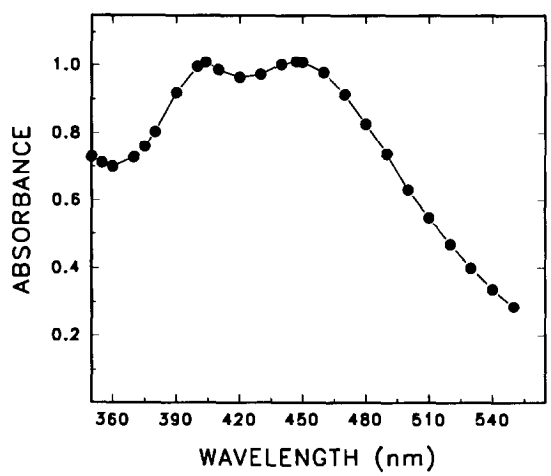

FIG. 2. The extinction of the hydroxyguanidine-PCAF chromogen as a function of wavelength. The extinction of the hydroxyguanidine-PCAF chromogen was determined with 0.6 mM hydroxyguanidine; chromogen formation was allowed to proceed for 4 min prior to monitoring the extinction with a Gilford "Response" automated recording spectrophotometer.

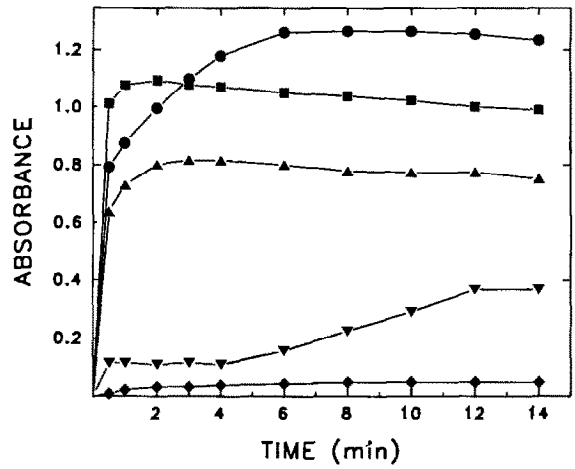

FIG. 3. The influence of buffer composition (200 mM) and pH on the formation of the hydroxyguandine-PCAF chromogen. Chromogen formation was evaluated with sodium tricine (pH 7.6) (●); potassium phosphate (pH 7.0) (■); sodium phosphate (pH 8.9) (▲); sodium acetate (pH 5.9) (▼); and glycylglycine (pH 8.9) (◆).

washing the resin with deionized water. Only substances that are at least moderately basic will be held to the resin (14). Column development is achieved with 50 mM ammonia; strongly basic substances such as arginine are held to the resin (16).

Hydroxyguanidine has a pK_a value of 7.89 (17) and its basicity is so close to canavanine that these two substances cannot be separated by gradient elution of the ion-exchange resin. The column effluent, however, can be treated with arginase, purified through the DEAE-cellulose step, to remove canavanine (18). The author is not aware of any natural product that can survive these procedures to vitiate a hydroxyguanidine-containing sample processed as above.

Addition of a known amount of hydroxyguanidine, containing radiolabeled [^{14}C]hydroxyguanidine, to a number of plant and animal extracts resulted in a hydroxyguanidine recovery that averaged 94%. The specific activity of the hydroxyguanidine added to a biological extract was unaffected by its being processed through the above procedures. This indicates that extraneous material, derived from the biological extract, fails to contribute to or otherwise affect the final hydroxyguanidine-PCAF chromogen.

Radiochemical Synthesis of [^{14}C]Hydroxyguanidine

One millimole of hydrogen cyanamide (42 mg) and 3.7 kBq of [^{14}C]cyanamide were reacted with 1.1 mM hydroxylamine sulfate for 7 days at 22°C after the procedure of Kalyankar *et al.* (3). Attempts to shorten the reaction time by elevating the temperature of the reaction mixture were futile due to hydroxyguanidine instability at elevated temperature. Radiolabeled hydrogen cyanamide was prepared from barium [^{14}C]cyanamide as described elsewhere (19).

The radioactive reaction mixture (pH 3.5) was applied to a 15 × 100-mm column of Dowex-50 (NH_4^+) and washed with deionized water. Unreacted radiolabeled cyanamide can be recovered fully in the first 25 ml of the aqueous wash. The column was developed with 50 mM ammonia and the carbon-14-bearing effluent concentrated by rotary evaporation *in vacuo* and the radiolabeled hydroxyguanidine stored at −60°C. The final yield was 12%.

The radiochemical purity of the [^{14}C]hydroxyguanidine was established by ion-exchange chromatography employing a Dionex D-300 automated amino acid analyzer. The column effluent was collected as 0.6-ml fractions at 2.0-min interval without being reacted with ninhydrin. Samples of the effluent were assayed for hydroxyguanidine colorimetrically and for carbon-14 by liquid scintillation spectroscopy. This procedure revealed that virtually all of the eluted carbon-14 of the sample exhibited the same column retention time as authentic hydroxyguanidine (column retention time = 132 min).

Preparation of Hydroxyguanidine Reductase

Heliothis virescens larvae, obtained from a continuous colony maintained at the University of Kentucky, were reared as described previously (20). All buffers used in the purification consisted of sodium tricine (pH 7.6) containing 1 mM dithiothreitol and 0.1% (v/v) 2-mercaptoethanol. Centrifugation was conducted at 12,000*g* for 15 min.

Preparation of the homogenate. *Heliothis virescens* larvae (n = 120), fresh weight 300 to 360 mg per larva, were submerged under crushed ice for at least 15 min. The chilled larvae were opened with a small scissors, the midgut was removed from the body cavity, and the gut contents were removed by forcing 50 mM buffer through the gut with a fine-needled syringe. The *clean* gut midguts were homogenized with 4 ml of 200 mM buffer and the turbid solution transferred to a centrifuge tube. A second 4 ml of buffer was employed to rinse the homogenizer. The combined buffered samples were centrifuged; the supernatant solution was removed carefully from the pellet and recentrifuged.

Ammonium sulfate precipitation. The clarified homogenate was taken to 50% (v/v) saturation with liquid ammonium sulfate and allowed to sit on ice for 90 min prior to centrifugation. The pellet was discarded and the supernatant solution taken to 85% (v/v) saturation and again allowed to sit on ice for 90 min prior to centrifugation. The pellet was dissolved in 35 mM buffer and ammonium sulfate removed by washing with this buffer via an Amicon Ultrafiltration unit equipped with a P-10 membrane.

DEAE-cellulose chromatography. The enzyme solution was applied to a 15 × 90-mm column of Whatman DEAE-cellulose (DE32) equilibrated with 35 mM buffer. The column was washed with 25 ml of the same buffer and then 50 ml of 200 mM buffer. The hydroxyguanidine-reducing activity was obtained by developing the washed column with a linear gradient consisting of 100 ml of 200 mM buffer and an equal volume of 200 mM glycylglycine buffer at pH 9.7. The effluent, at a flow rate of 1.5 ml min^{-1}, was collected at 2-min intervals. The three most active fractions were pooled and evaluated for enzymatic activity colorimetrically

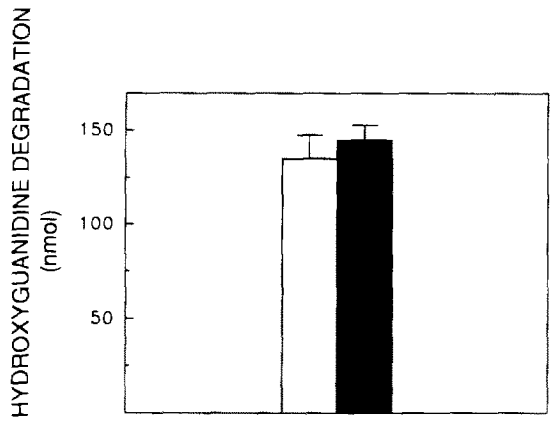

FIG. 4. Comparative evaluation of hydroxyguanidine degradation. Hydroxyguanidine biotransformation was measured colorimetrically (□) as described in the text. Substrate loss was also determined by monitoring the absorbance change at 340 nm resulting from loss of reduced coenzyme (■). Each value is the mean ±SEM of three independent determinations.

by the disappearance of hydroxyguanidine and spectrophotometrically by monitoring the oxidation of reduced NAD at 340 nm.

The assay system consisted of 15 mM hydroxyguanidine, 0.75 mM NADH, 150 mM sodium tricine buffer (pH 7.6), and no more than 0.3 mg of protein in a final volume of 1.0 ml. The assay was conducted at 37°C for 30 min and terminated by the addition of 0.5 ml of 27% (v/v) chilled perchloric acid. Five minutes later, the mixture was neutralized with 0.5 ml of 3.3 N KOH, allowed to sit on ice for 5 min, and then centrifuged. The supernatant solution was assayed for the disappearance of hydroxyguanidine. Zero time samples served as the control. All assays were conducted in triplicate.

Identification of the Reaction Product

The production of [^{14}C]guanidine from [^{14}C]hydroxyguanidine was established by isolation of the radiolabeled guanidine by ion-exchange chromatography as described previously (21). Authentic [^{14}C]guanidine was prepared by catalytic hydrogenolysis of hydroxyguanidine with palladium black (22).

Comparative Analysis of Hydroxyguanidine Reduction

The utility of the hydroxyguanidine colorimetric assay for monitoring *H. virescens* hydroxyguanidine reduction was evaluated by comparison with an assay predicated upon monitoring the oxidation of reduced NAD at 340 nm. Substrate conversion to product as determined by colorimetric analysis did not deviate significantly from the value secured by measuring the loss of reduced NAD (Fig. 4).

ACKNOWLEDGMENTS

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