significantly depress body temperature suggest that nitroprusside causes hypothermia, in part, through an action on the CNS. Conceivably, the drug may cause nonspecific depression of thermoregulatory control centrally. This action could explain the absence of compensatory shivering and increased muscle tone, although nitroprusside interference at the effector level could account for these absences.

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[x] To whom inquiries should be directed.

Application of ^{13}C-NMR Spectroscopy to *In Vitro* Analysis of Enzyme Kinetics

H. L. JOHNSON [x], D. W. THOMAS, M. ELLIS, L. CARY, and J. I. DeGRAW

Abstract □ The conversion of D,L-α-^{13}C-histidine to similarly labeled α-^{13}C-histamine by bacterial and mammalian histidine decarboxylase was studied by ^{13}C-NMR spectroscopy and GLC–mass spectrometry. The results obtained with the partially purified bacterial enzyme were in essentially perfect agreement with results obtained simultaneously with a standard radioisotopic method using carboxyl-labeled-^{14}C-L-histidine. For a crude tissue preparation of the mammalian enzyme, the radioisotopic method indicated an activity three times that based on ^{13}C-NMR measurement of α-^{13}C-histamine. The difference in results was accountable in terms of additional ^{13}C-NMR signals attributable to products other than histamine due in part to enzymatic degradation of the latter.

Keyphrases □ D,L-α-^{13}C-Histidine—conversion to histamine by histidine decarboxylase, ^{13}C-NMR and GLC–mass spectral study □ D,L-α-^{13}C-Histamine—conversion from histidine by histidine decarboxylase, ^{13}C-NMR and GLC–mass spectral study □ Enzyme activity—histidine decarboxylase, conversion of D,L-α-^{13}C-histidine to histamine, ^{13}C-NMR and GLC–mass spectral study □ ^{13}C-NMR spectroscopy—study of conversion of D,L-α-^{13}C-histidine to histamine by histidine decarboxylase □ GLC–mass spectrometry—study of conversion of D,L-α-^{13}C-histidine to histamine by histidine decarboxylase

Many *in vitro* assays of enzyme activity are relatively inconvenient, time consuming, or indirect in that measurements are based on cofactors or coproducts rather than the major product. Furthermore, compounds are frequently assayed for inhibitory activity using crude tissue preparations where the primary product may be exposed to further enzymatic transformations that may or may not be affected by the test compound. An example is the assay of specific histidine decarboxylase (EC 4.1.1.22) in minced tissue or crude extracts of mammalian tissues (1). One method of assay measures the ^{14}C-carbon dioxide evolved from carboxyl-labeled ^{14}C-histidine (2), and other methods are based on the measurement of the histamine produced (1).

The increasing availability of stable isotopic intermediates suggested exploration of NMR spectra using synthetic isotopic-enriched substrates as a convenient means of quantitatively and directly determining the kinetics of formation of multiple products in a crude enzyme assay. For initial exploration, ^{13}C-enriched histidine (3) was used; ^{13}C-NMR results were compared with a standard radioisotopic method (2) using histidine decarboxylase of both bacterial and mammalian origin. With the bacterial enzyme, mass spectrometry served as an additional comparison.

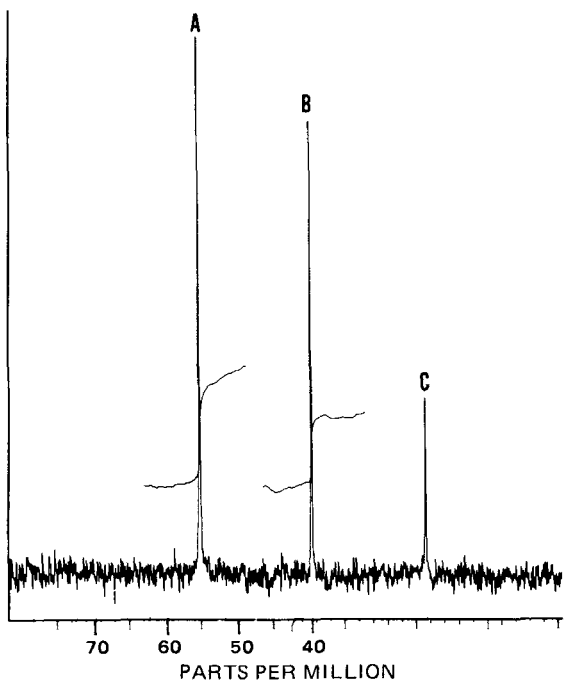

Figure 1—*^{13}C-NMR spectrum of bacterial histidine decarboxylase reaction mixture [conversion of D,L-α-^{13}C-histidine (M + 4) to α-^{13}C-histamine (M + 4)]. Protein was removed by heat precipitation. Key: A, α-^{13}C-histidine; B, α-^{13}C-histamine; and C, reference ^{13}C-acetate.*

EXPERIMENTAL

Carboxyl-labeled-^{14}C-L-histidine (13 mCi/mmole) was obtained commercially[1]. Nonradioactive isotopic D,L-histidine (2,5-^{2}H,3-^{15}N,α-^{13}C-labeled) was synthesized with 80% enrichment in M + 4 and 20% M

[1] New England Nuclear Corp., Boston, Mass.

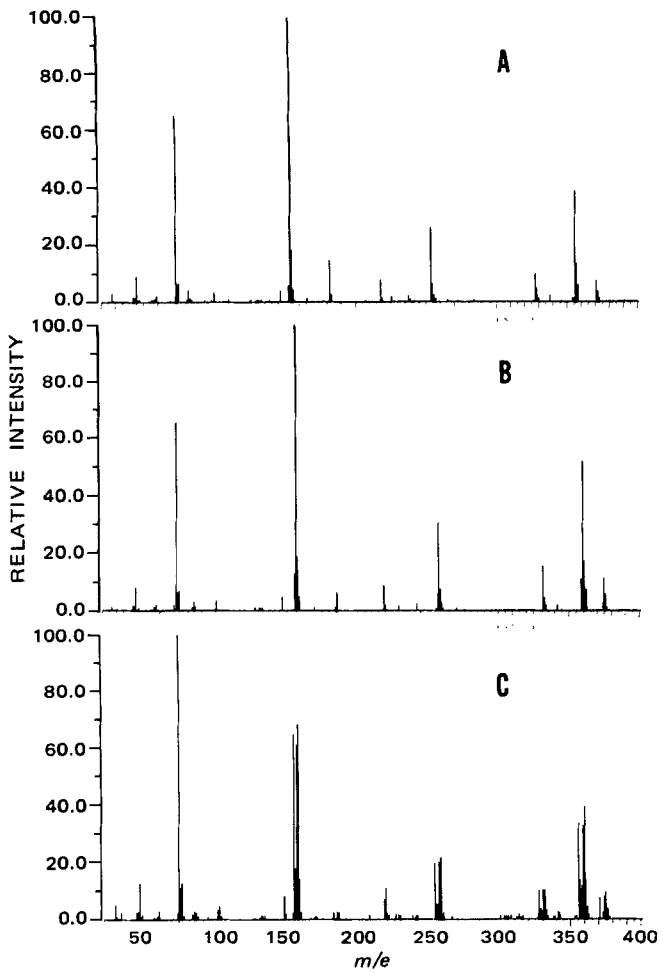

Figure 2—*Mass spectra of trimethylsilyl-derivatized histidine. Key: A, reference histidine (M); B, M + 4 histidine; and C, M and M + 4 histidine from the decarboxylase reaction mixture of Fig. 1.*

+ 3 (3). Bacterial histidine decarboxylase (*Clostridium welchii*) was obtained as the acetone powder (Type I[2], 0.015 unit/mg), which was used as a lightly homogenized suspension (2 mg/ml) in 0.2 *M* ammonium acetate buffer (pH 4.8). Mammalian histidine decarboxylase was obtained as a crude pyloric stomach extract (clear supernate) from male Sprague–Dawley rats [1:2.5 homogenization in 0.05 *M* phosphate buffer (pH 7.2) centrifuged at 5000×*g* for 30 min at 0°].

The bacterial enzyme was incubated at 37° with ^{14}C-L-histidine (0.033 μCi/ml) and D,L-α-^{13}C-histidine (M + 4) (0.33 mg/ml) at pH 4.8 (4). The mammalian enzyme was incubated under nitrogen at 37° (for 1.5 and 3 hr) with L-^{14}C-histidine (0.038 μCi/ml), D,L-α-^{13}C-histidine (M + 4) (0.2 mg/ml), streptomycin sulfate (0.05 mg/ml), and pyridoxal 5-phosphate (2×10^{-5} *M*; 10-min preincubation before addition of substrate) at pH 7.2 (5). The radioisotopic assay (^{14}C-carbon dioxide) was a modification of the Levine and Watts (2) method, with termination of the reaction by citric acid addition. ^{14}C-Carbon dioxide was absorbed on 10 × 20-mm folded strips of filter paper[3] dipped in quaternary ammonium hydroxide[4] and allowed to drip dry.

For ^{13}C-NMR and mass spectrometry, the reaction was stopped by heat treatment (100° for 10 min). Protein was removed by centrifugation prior to analysis. Samples (10.0 ml) were lyophilized and reconstituted in 2.0 ml of water containing 10% D_2O prior to NMR analysis of mammalian enzyme activity. A 2-ml aliquot of the bacterial enzyme samples was analyzed directly by NMR spectroscopy after addition of 10% D_2O. For mass spectrometry, nonisotopic histidine and histamine were added as standards, and samples were evaporated to dryness before treatment with trimethylsilyl chloride.

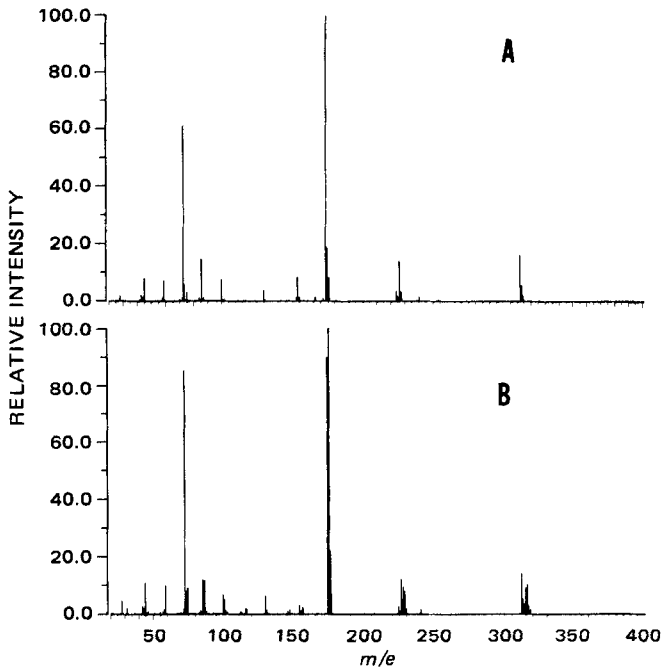

Figure 3—*Mass spectra of trimethylsilyl-derivatized histamine. Key: A, reference histamine (M); and B, M and M + 4 histamine from the decarboxylase reaction mixture of Fig. 1.*

Mass spectral analysis was performed on a combination gas chromatograph–mass spectrometer[5] using an OV-17 column and multiple ion detection. Measurements by ^{13}C-NMR spectroscopy utilized a Fourier transform spectrometer[6] (50,000 transients) with wide band proton decoupling (12-mm tube). Adequate signal-to-noise ratios were obtained. Radioisotopic assays used a scintillation spectrometer[7] with a counting efficiency of 93% (*p*-bis[2-(5-phenyloxazolyl)]benzene and 2,5-diphenyloxazole) in toluene with 1.0 ml of ethanol.

RESULTS AND DISCUSSION

Bacterial enzyme was used for establishing the accuracies and repro-

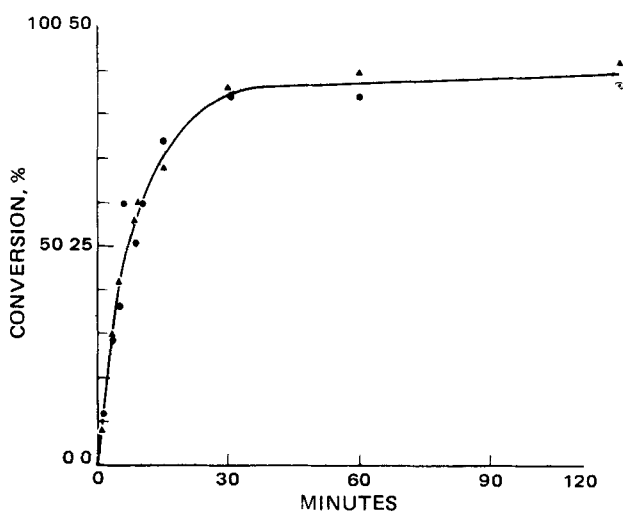

Figure 4—*Conversion by bacterial histidine decarboxylase of* L-^{14}C-*histidine to histamine plus* ^{14}C-*carbon dioxide (●) (0–100% scale) versus conversion of* D,L-α-^{13}C,3-^{15}N,2,5-^{2}H-*histidine (M + 4) to* α-^{13}C,3-^{15}N,2,5-^{2}H-*histamine (M + 4) (0–50% scale) as measured by* ^{13}C-*NMR (▲) and GLC–mass spectrometry (□).*

[2] Sigma Chemical Co., St. Louis, Mo.
[3] Whatman 3MM.
[4] Hyamine.

[5] LKB 9000, LKB Instruments, Rockville, Md.
[6] XL-100, Varian Instrument Division, Palo Alto, Calif.
[7] Mark III, Searle Analytic, Des Plaines, Ill.

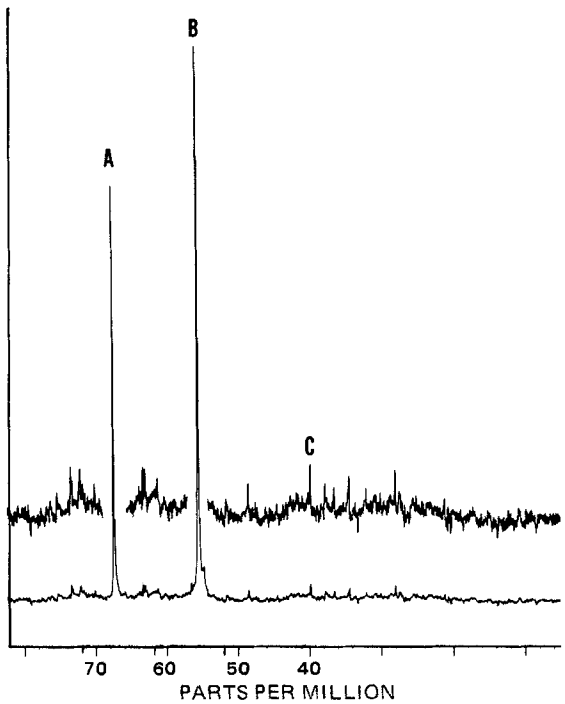

Figure 5—*^{13}C-NMR spectrum of mammalian histidine decarboxylase reaction mixture [rat pyloric stomach extract; conversion of D,L-α-^{13}C-histidine (M + 4) to α-^{13}C-histamine (M + 4)]. Protein was removed by heat precipitation. Key: A, reference dioxane; B, α-^{13}C-histidine; and C, α-^{13}C-histamine.*

ducibilities of the methods. A typical NMR spectrum is illustrated in Fig. 1 (bacterial enzyme sample, 2 hr), showing 43% α-^{13}C-histamine and 57% α-^{13}C-histidine. The maximum theoretical conversion was 50% because of the use of D,L-α-^{13}C-histidine. Added α-^{13}C-acetate served as an additional quantitation standard.

Typical mass spectra for histidine, M + 4 histidine, and the mixture of the two obtained from the 2-hr bacterial enzyme sample are shown in Fig. 2. Similarly, Fig. 3 shows the mass spectra of histamine and the mixture of histamine and M + 4 histamine obtained from the enzyme sample. Based on the amounts of histidine and histamine added to the sample before analysis, M + 4 histamine was 46% and M + 4 histidine was 54%. Included in the measurements are the M + 3 and M + 2 peaks of isotopic histidine. These were increased in the enzyme sample at the expense of the M + 4 peak because of some exchange loss of deuterium during enzymatic conversion (Fig. 2).

Typical radioisotopic results for the bacterial enzyme (^{14}C-carbon dioxide counts as a percent of initial ^{14}C-histidine counts) are plotted as a function of incubation time in Fig. 4. A common curve could be drawn through these data points and those obtained by ^{13}C-NMR spectroscopy and GLC–mass spectrometry. Radioisotopic results were based on a theoretical maximum conversion of 100% of L-^{14}C-histidine.

Typical results obtained with the mammalian enzyme preparation are shown in Fig. 5. In addition to the small amount of α-^{13}C-histamine formed (~3.3%), other ^{13}C-signals of comparable intensity are evident. One of these was tentatively identified as methylhistamine, formed by the enzymatic methylation of histamine on imidazole nitrogen (6); others are presumably direct metabolites of histidine and other metabolites of histamine. Results of the radioisotopic assay indicated 10% decarboxylation of α-^{13}C-histidine.

The radioisotopic carbon dioxide method is presumed to provide a reliable estimate of decarboxylase activity but gives no information concerning further metabolism. Methods based on measurement of product histamine may yield erroneously low estimates of activity due to such metabolism (1). These points are well illustrated by the present results, confirming the accuracy and reliability of the radioisotopic carbon dioxide method.

Crude enzyme preparations can be useful for purposes of *in vitro* drug screening where actions on various enzymes concerned with synthesis and metabolism of a given substrate are of interest. The increasing sensitivity of NMR instrumentation provides the potential for conveniently assessing such drug effects simultaneously in a single assay on a routine basis. Appropriate nonradioactive isotopic labeling of substrates introduces the additional advantage of further quantitative identification of metabolites by GLC–mass spectrometry through the simple expedient of using nonlabeled samples of presumed or suspected metabolites as carrier-standards.

Assays involving TLC frequently leave doubt because of superimposed spots. The NMR methodology, particularly when coupled with GLC–mass spectrometry, eliminates the need for extensive separation procedures and simplifies product identification when adequate UV, fluorometric, or other quantitative characterization techniques are not available.

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[x] To whom inquiries should be directed.