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Purification and Characterization from *Chromobacterium violaceum* of an Enzyme Catalyzing the Synthesis of γ-Cyano-α-aminobutyric Acid and Thiocyanate†

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ABSTRACT: A cell-free extract of *Chromobacterium violaceum* D 341, a strain that vigorously produces cyanide and converts it into γ-cyanoaminobutyric acid, has been shown to catalyze the synthesis of this cyanoamino acid. An enzyme, γ-cyanoaminobutyric acid (γ-CNabu) synthase, has been purified 270-fold from the extract. It is an acidic protein with a molecular weight near 130,000. It is activated by simple thiols and generally inactivated by disulfides. Pyridoxal 5′-phosphate can serve as its cofactor. In the presence of the enzyme homocystine and cyanide react to give γ-CNabu and thiocyanate. As a possible intermediate for this reaction γ-thiocyanoaminobutyric acid (*S*-cyanohomocysteine), a new sulfur amino acid, has been synthesized. It has been shown to form by a nonenzymatic cyanolysis of homocystine and to serve as a good substrate in place of homocystine for the enzymatic synthesis of γ-CNabu and thiocyanate.

A recent contribution from this laboratory described the isolation and identification of γ-cyano-α-aminobutyric acid as a new product of cyanide assimilation from *Chromobacterium violaceum* strain D 341 (Brysk and Ressler, 1970). This report describes the purification from the same organism of an enzyme that catalyzes the synthesis of γ-CNabu[1] and some of its properties. In the presence of this isolated enzyme, cyanide and a four-carbon chain of homocystine are utilized to form γ-CNabu, and sulfur from homocystine is converted into thiocyanate (reaction 1). Although roles for homocysteine in transmethylation, transsulfuration, and desulfurase reactions are well established, the corresponding disulfide, homocystine, has not previously been implicated in a synthetic aspect of metabolism.

$$\text{homocystine} + 2CN^- \xrightarrow[\text{enzyme}]{} \gamma\text{-cyanoaminobutyric acid} + SCN^- + \text{homocysteine} \quad (1)$$

In a preliminary consideration of a possible route for the enzymatic utilization of homocystine, it has been found that homocystine and cyanide react nonenzymatically to form γ-thiocyano-α-aminobutyric acid and that the enzyme herein described catalyzes the reaction of this amino acid with cyanide to give γ-CNabu and thiocyanate. The enzyme is provisionally named γ-thiocyanoaminobutyric acid thiocyano-lyase (adding CN) (EC 4.4.1) and for convenience is referred to as γ-CNabu-synthase.

Experimental Section

Synthesis of γ-Thiocyano-α-L-aminobutyric Acid. L-Hcy was treated with cyanogen halide as for acetylcysteine and cysteine (Aldridge, 1951; Catsimpoolas and Wood, 1964).

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[1] Abbreviations used are: ME, 2-mercaptoethanol; Hcy, homocysteine; Cm-Hcy, carboxymethylhomocysteine; Cm-HcySH, carboxymethylthiohomocysteine; γ-CNabu, γ-cyano-α-aminobutyric acid; γ-SCNabu, γ-thiocyano-α-aminobutyric acid; β-CNala, β-cyanoalanine; pyridoxal-P, pyridoxal 5′-phosphate.

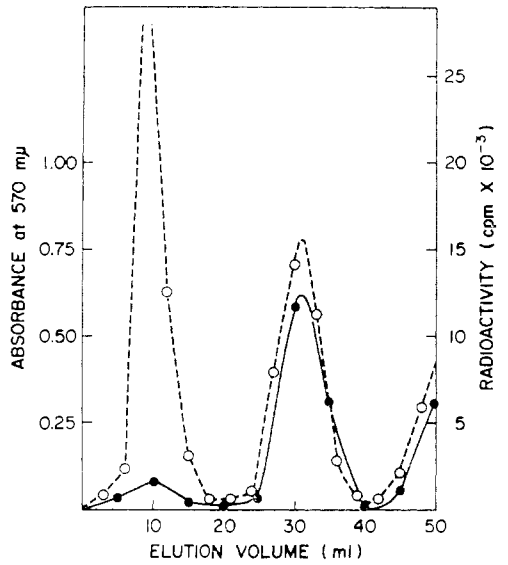

FIGURE 1: Elution patterns in the (●) chromatographic ninhydrin assay and the (○) chromatographic radioassay for γ-CNabu-synthase (see text). The specific activity was 0.029. γ-CNabu is eluted between 25 and 38 ml.

The use of cyanide and a lowered pH increased the yield. A solution of 7.36 g (150 mmol) of NaCN in 160 ml of water was adjusted to pH 5.5 with 4 N acetic acid. L-Hcy, 2.66 g (76% SH; 15 mmol), was added with magnetic stirring, followed by 60 ml of AG 1-X4 resin (CN^- cycle, 100–200 mesh). The pH was promptly readjusted and 2.1 g (19.8 mmol) of cyanogen bromide was added in portions in the course of 15 min until the nitroprusside test for sulfhydryl (Toennies and Kolb, 1951) became negative. The mixture was then stoppered and stirred for 30 min. The resin was filtered off and washed well with water. The filtrate was evacuated for 45 min and then was concentrated to dryness. The residue was suspended in 15 ml of water, adjusted to pH 2.2 with 6 N HCl, and applied to a 3.5 × 45 cm column of Dowex 50-X4 resin (H^+ cycle, 200–400 mesh). The column was washed with 295 ml of water and then was eluted with 1 M pyridine acetate buffer (pH 4.0); 4-ml fractions were collected. Fractions 108–133, R_F 0.57 (thin-layer chromatography, tlc), were taken to dryness and further dried over P_2O_5; wt 3.1 g, mp 125–129°. Some homocystine, R_F 0.17, was present in fractions 140–170. The chromatographed material was dissolved in 125 ml of water and was decolorized with activated C. After concentration to 10 ml and storage overnight in the cold, the crystalline product separated and was collected by centrifugal filtration in the cold; wt 1.56 g, mp 158–161°; 0.37 g, mp 150–161°. Recrystallization from water–tetrahydrofuran gave 1.3 g (54%) of mica-like plates, mp 165° dec. The analytical sample was from water and was homogeneous on amino acid analysis and tlc; mp 167° dec, $[\alpha]_D^{25}$ +27.6° (*c* 1, 1 N acetic acid); infrared (ir) bands (KBr) cm^{-1} 3330–2500 (NH_3^+, COOH), 2170 (SCN), 2070 (NH_3^+), and 1670 (COO^-, NH_3^+); nuclear magnetic resonance (nmr) (D_2O) δ 2.3 (2 H, m, β-CH), 3.15 (2 H, t, γ-CH), 3.81 (1 H, t, α-CH). *Anal.* Calcd for $C_5H_8N_2O_2S$: C, 37.5; H, 5.03; N, 17.5; S, 20.0. Found: C, 37.3; H, 4.98; N, 17.3; S, 20.0.

Materials. $K^{14}CN$ (8.8 Ci/mol) was purchased from New England Nuclear and crystalline bovine serum albumin from Pentex. Protamine sulfate, L- and DL-homocystine, DL-penicillamine, D-cycloserine, and molecular weight marker proteins were from Schwarz/Mann. DL-Homocysteine (Hcy) was obtained from Nutritional Biochemicals and 3,3′-dithiodipropionic acid from Aldrich. L-Hcy (du Vigneaud and Brown, 1957), *O*-acetyl-L-homoserine (Wiebers and Garner, 1967), L-γ-CNabu (Brysk and Ressler, 1970; Ressler and Ratzkin, 1961), and L-cystathionine (Snow *et al.*, 1967) were synthetic materials. The mixed disulfides of L- and DL-homocysteine and 2-mercaptoethanol, of L-cysteine and 2-mercaptoethanol, and of L-homocysteine and 2-mercaptoacetic acid were synthesized by procedures described separately (Abe *et al.*, 1974). Cm-Hcy (Kodama *et al.*, 1969) was a gift of Dr. S. Mizuhara. Nutrient Broth was Difco's and the beef liver rhodanese (6.7 units/mg) was from Sigma; Sepraphore III strips were from Gelman, QAE-Sephadex A-50 and Sephadex G-25 and G-200 from Pharmacia, and AG1-X4 and Dowex 50-X4 resins from Bio-Rad Labs.

Growth of Cells. C. violaceum strain D 341 was cultivated in a glutamate–salt medium (Brysk and Ressler, 1970; Michaels and Corpe, 1965) and later in Nutrient Broth. Four-liter batches in ten-liter carboys were inoculated from fresh slants and aerated at 30° until an optical density of 0.7–0.75 at 600 mμ was reached. The cells were harvested by centrifugation at 13,000*g* for 15 min, washed with 0.1 M potassium phosphate buffer (pH 7.0), and stored at −20°; 25 l. yielded 70 g of wet cells.

To test the utilization of γ-SCNabu and other substances cells were grown for the indicated periods in the glutamate salt medium, then were harvested and incubated for 24 hr with NaCN and the substance in 50 ml of salt medium. γ-CNabu levels in the incubation fluid and cell protein were determined.

Enzyme Assays: γ-CNabu Synthase. Enzyme activity was measured at 30° by following the formation of γ-CNabu or thiocyanate. Reaction mixtures contained in 1 ml 20 mM DL-homocystine (a suspension), 50 mM potassium cyanide (fresh solution), 50 mM Tris-HCl (pH 9.1), 140 mM potassium chloride, and 10–40 munits of the enzyme. The reaction was initiated by the addition of KCN, incubated for 20 min, and terminated with 0.3 ml of 1 N HCl. The mixture (pH 1–2) was placed on the amino acid analyzer or was placed on a 0.9 × 10 cm column of Dowex 50-X4 (H^+) resin equilibrated with 0.5 M pyridine acetate buffer (pH 3.35). The column was developed with the same buffer at a flow of 0.5 ml/min. The eluate (20–45 ml) was collected in 5-ml fractions which were adjusted to pH 5 with 0.5 ml of 3.6 N NaOH. Samples (1 ml) were analyzed with ninhydrin (Moore and Stein, 1954) with synthetic L-γ-CNabu as standard (Figure 1).

In an alternative chromatographic radioassay about 1.5 μCi (300 nmol) of $K^{14}CN$ was substituted for the KCN in the above incubation mixture. Samples (1 ml) of the Dowex 50 fractions were counted (Figure 1).

For the colorimetric estimation of thiocyanate, 0.5 ml each of 5 N HNO_3 and 0.3 M $FeCl_3$ were added to 1 ml of the acidified reaction mixture. After 5 min, absorbance at 470 mμ was determined (Sorbo, 1955). In both assays reaction rates were linear with respect to time for 20 min. For linearity of the rate in the thiocyanate assay, less than 30 munits, or one-fifth the amount of enzyme allowable for the γ-CNabu assay could be used. This observation remains unexplained at present, since a direct effect of the enzyme on thiocyanate was not detected.

One unit of enzyme is defined as that quantity catalyzing the formation of 1 μmol of γ-CNabu or of thiocyanate per min from DL-homocystine and cyanide under these conditions of assay. Specific activity is expressed in units per milligram of protein.

Cystathionase was assayed in reaction mixtures containing 6 mM L-cystathionine, 3.5 mM ME, 0.4 mM pyridoxal-P, and 0.1 M Tris-HCl buffer (pH 8.6) that were incubated for 30 min at 30°. Keto acid was determined colorimetrically as described by Greenberg (1962) with the exception that it was read at 435 mμ against sodium pyruvate as standard. For rhodanese the method of Sorbo (1955) was used except that thiocyanate was determined as described for γ-CNabu-synthase. For homocysteine desulfhydrase the method of Kallio (1951) was used except that the buffer contained 0.6 mM pyridoxal-P and no glutathione; α-ketobutyrate was determined. *O*-Acetylhomoserine sulfhydrylase was measured by the procedure of Kerr (1971) with Na_2S except that pH was 7.2 (Wiebers and Garner, 1967) and the formation of Hcy and homocystine was determined by analysis on the amino acid analyzer.

Identification of Enzymatic and Nonenzymatic Products. γ-CNabu, γ-SCNabu, and Hcy were identified by chromatography and cochromatography with synthetic materials on the amino acid analyzer (Table III). Hcy was confirmed by carrying out the reactions in the presence of iodoacetate; the formed Cm-Hcy was chromatographed and cochromatographed on the analyzer with authentic material. Elution volumes (milliliter) for the system for physiological fluids (Spackman *et al.*, 1958) are: γ-CNabu (190), γ-SCNabu (409), Hcy (495), Cm-Hcy (330), and Cm-HcySH (6 ml before Hcy and 9 ml before Met). Hcy was also determined colorimetrically by a modified diazo procedure for thiols (Liddell and Sayville, 1959; Becker *et al.*, 1969). γ-CNabu was confirmed by cochromatography on the analyzer the products of a radioassay containing 11.5 nmol of [γ-^{14}C]Nabu and 0.46 mg of added γ-CNabu. Good coincidence of ninhydrin and radioactivity (2.8×10^4 cpm/μmol) resulted at the position of γ-CNabu. In addition, the product of a radioassay with 2.5 μCi of K^{14}CN was similarly diluted with γ-CNabu and subjected to electrophoresis on paper at pH 9.3. Eluted γ-CNabu was diluted with 15.1 mg of γ-CNabu and crystallized from water–dioxane to constant specific activity in three crystallizations: 2587, 2027, and 2089 cpm per mg. Thiocyanate was identified colorimetrically as described in the assay for γ-CNabu-synthase.

Other Methods. Protein was determined by Lowry's method (Lowry *et al.*, 1951) with bovine serum albumin as standard, or by ultraviolet (uv) absorbance. Gel electrophoresis was carried out by the method of Ornstein and Davis (Davis, 1964). Stained gels were scanned on a Joyce Loebl and Co. recording microdensitometer. Unstained gels were sliced into 2-mm sections, placed directly into the γ-CNabu-synthase assay mixture, and assayed for thiocyanate formation. For preparative purposes the resolving gel was 2.8 × 14 cm and was developed for 10 hr at 4°. The gel was cut into 2.8-mm sections, and the enzyme was removed electrophoretically and collected in a dialysis bag.

The isoelectric point of the enzyme was determined by electrophoresis on Sepraphore III cellulose polyacetate strips. Migration was effected at 100 V for 60 min in potassium phosphate or sodium acetate buffer, pH 7.4–4.6, ionic strength 0.1. The strip was then cut lengthwise; half was stained as in disc gel electrophoresis; the other half was cut into sections and assayed for activity. The isoelectric point was obtained by extrapolation of a plot of electrophoretic mobility against pH to zero mobility.

Molecular weight was determined by gel filtration on a 2.4 × 120 cm column of Sephadex G-200 (Leach and O'Shea, 1965) equilibrated with 0.1 M potassium phosphate buffer, pH 8.0 (Gomori, 1955), containing 0.01 mM pyridoxal-P (buffer A). γ-CNabu-synthase was located by enzyme assay; other proteins by absorbance (Andrews, 1965). Molecular weight was obtained by interpolation of a plot of V_e/V_0 values against the logarithm of the molecular weights of known proteins (Leach and O'Shea, 1965; Andrews, 1965) (Figure 2).

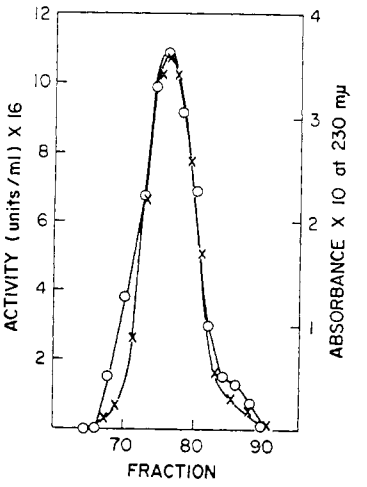

FIGURE 2: Second chromatographic passage of γ-CNabu-synthase on Sephadex G-200 (see text, step 6): (○) protein absorbance; (×) enzymatic thiocyanate formation.

Enzyme stability was examined in solutions (0.2 ml) containing 50 munits, specific activity 0.3, and 10 μmol of sodium acetate, citrate, carbonate, potassium phosphate, or Tris-HCl (pH 4.3–10.1). Apoenzyme was prepared by an adaptation of the method for resolving rabbit muscle phosphorylase *b* (Shaltiel *et al.*, 1966). K_m and V_{max} values were calculated from Lineweaver–Burk plots. The catalytic constant was calculated from V_{max}. pH optima were measured at various pH values in 0.1 M carbonate buffer containing 0.6 mM pyridoxal-P and 50 mM KCN at 30°. For the homocystine–cyanide reaction, the data for a given pH run were treated according to the first-order rate law, since the reaction went to completion (97.5% after 4 hr at pH 9.6) and was carried out at a 30-fold ratio of total cyanide to homocystine. The pseudo-first-order rate constant was obtained from the slope of the usual log plot (Figure 7) as in the cystine–cyanide reaction (Gawron *et al.*, 1964).

Infrared spectra, optical rotations, amino acid, and elemental analyses were obtained, and paper electrophoresis was carried out as described elsewhere (Brysk and Ressler, 1970). Tlc was on strips of Eastman Chromagram 6064 cellulose in 1-butanol–acetic acid–pyridine–water (4:1:1:2). Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. Nmr was examined on a Varian EM-360 proton magnetic resonance spectrometer with trimethylsilane as an external standard. Radioactivity was determined in a Nuclear-Chicago 722 liquid scintillation system. Samples were counted in Bray (1960) solution, or the effluent from the amino acid analyzer was counted in a 2-ml flow cell with a Nuclear-Chicago 6770 scintillation flow adapter.

Results

Purification of γ-CNabu-synthase. All steps were carried out at 0–4° unless indicated otherwise. Table I summarizes the results.

Steps 1–3. Extraction and Protamine and Heat Treatments. Cells, 25 g wet wt, suspended in 90 ml of buffer A, were dis-

TABLE I: Purification Procedure Summary for γ-Cyanoaminobutyric Acid Synthase Extracted from *C. violaceum*.[a]

Fraction	Vol (ml)	Total Protein (mg)	Total Act. (IU)	Sp Act. (IU/mg)	Recov (%)	Purifcn (-fold)
1. Cell extract	97	3230	113	0.035		
2. Protamine sulfate	115	1850	113	0.061	100	1.7
3. Heat treatment	110	681	90	0.132	80	3.8
4. QAE-Sephadex A-50	73	70.7	70.5	0.997	62	29
5. Sephadex G-200	46.5	11.2	41.0	3.69	36	105
6. Sephadex G-200	39.2	5.2	35.4	6.80	31	194
7. Sephadex G-200	36	2.2	19.0	8.50	17	243

[a] From 25 g wet cells.

rupted with a Bronwill Biosonik sonifier for 10 min while cooled in an ice–water bath. The sonicate was centrifuged at 27,000*g* for 20 min. To the supernatant (fraction 1) adjusted to 23 mg/ml of protein, 1% aqueous protamine sulfate was added dropwise with stirring to a final concentration of 0.15 mg/mg of protein. After 30 min the precipitate was centrifuged off at 1000*g* for 15 min. The supernatant (fraction 2) in 20-ml portions in 30-ml centrifuge tubes was inserted for 3 min in a 60° water bath with manual shaking. The solutions were immediately cooled to 2° in an ice–water bath, again with shaking, and centrifuged at 27,000*g* for 5 min. The supernatant was retained (fraction 3). At this point the enzyme activity was stable at −20° for at least 6 months.

Step 4. Chromatography on QAE-Sephadex A-50. Fraction 3 (110 ml) was applied to a 2.4 × 40 cm column of QAE-Sephadex A-50 containing 196 ml of gel previously equilibrated with buffer A. Protein was eluted with 700 ml of a linear gradient (0.05–0.5 M) of KCl in buffer A. Fractions of 4 ml were collected at a flow of 30 ml/hr, and the samples were assayed for enzyme activity. Fractions 96–104 were combined, lyophilized to dryness, dissolved in a small volume of water, and dialyzed overnight at 4° against 1 l. of buffer A (fraction 4).

Steps 5–7. Gel Filtration. Fraction 4 (*ca.* 10 ml) was concentrated fivefold by ultrafiltration (Schleicher and Schull collodion bags 10) and then was placed onto a 24 × 104 cm column of Sephadex G-200 containing 493 ml of gel previously equilibrated with buffer A. The same buffer was used for filtration. Fractions of 4 ml were collected at a flow rate of 8 ml/hr and assayed. Fractions 76–88 were combined, lyophilized to dryness, and dialyzed as in step 4. The dialysate (fraction 5), 7 ml, was concentrated by ultrafiltration to 2 ml and rechromatographed on a Sephadex G-200 column under the same conditions, except the flow was 10 ml/hr (Figure 2). Fractions 70–79 were combined, concentrated by ultrafiltration, and rechromatographed. The specific activity of the peak material before concentration was 9.4; after a week's storage at −20°, 4.6; and after 2 weeks, 2.8. When subjected to preparative gel electrophoresis, this stored material yielded a band with a specific activity of 7.4 that was estimated to be 87% pure by disc gel electrophoresis.

Properties of the Enzyme. Partially purified γ-CNabu-synthase (stage 3) was stable for 3 hr at 30° at pH 5.2–10.1, but it was inactivated at pH 4.3. γ-CNabu-synthase has a molecular weight near 130,000 (Figure 3) and an isoelectric point near 4.2–4.4 at $\mu = 0.1$. The rates of formation of γ-CNabu and thiocyanate from 10 mM homocystine were op-

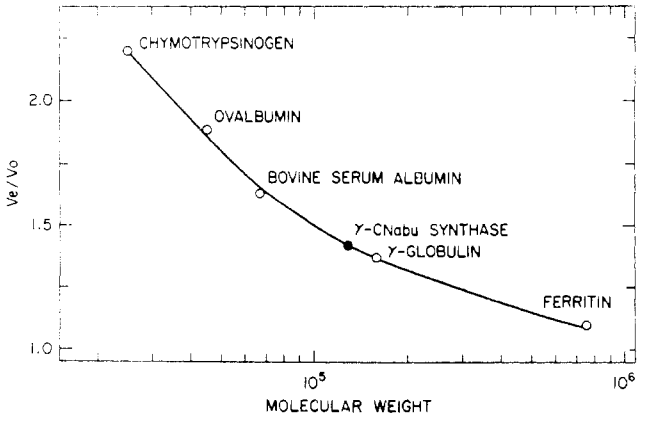

FIGURE 3: Estimation of the molecular weight of γ-CNabu-synthase on a Sephadex G-200 column; variation of elution volume with molecular weight for (●) γ-CNabu-synthase and (○) reference proteins. V_e/V_0 is the ratio of protein elution volume to column void volume determined with blue dextran.

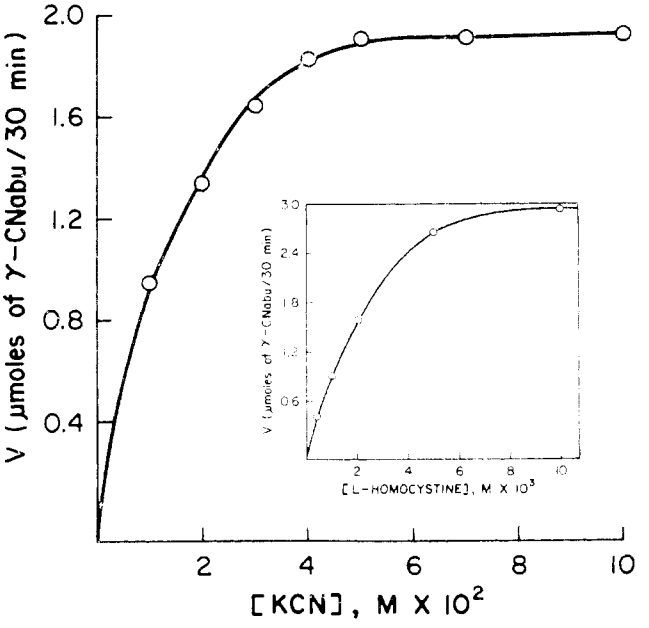

FIGURE 4: Kinetics of γ-cyano-α-aminobutyric acid synthesis as a function of KCN concentration, with 20 mM DL-homocystine. Reaction mixtures (pH 9.1) also contained per ml 64 munits of the enzyme (0.28 mg) and 0.6 mM pyridoxal-P and were incubated for 30 min at 30°. The chromatographic ninhydrin assay was used. Insert: kinetics of γ-cyano-α-aminobutyric acid synthesis as a function of the concentration of L-homocystine (in suspension at 5 mM and above), with 50 mM KCN. Reaction mixtures (pH 9.1) also contained enzyme and pyridoxal-P and were incubated and assayed as for the other curve.

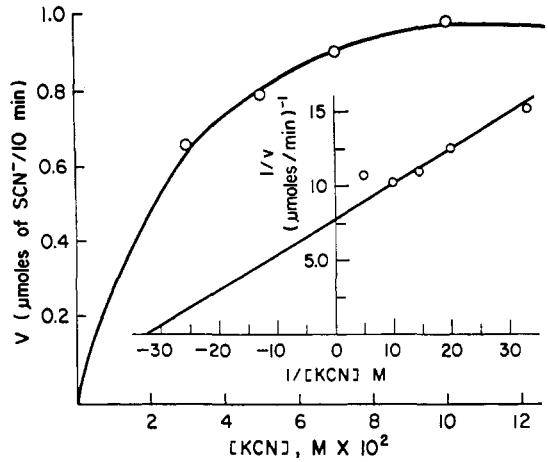

FIGURE 5: Kinetics of γ-cyano-α-aminobutyric acid synthesis as a function of KCN concentration, with 0.1 M γ-thiocyano-α-L-aminobutyric acid. Mixtures also contained per ml 7.9 munits of the enzyme (11.1 μg); other components and conditions as for Figure 6.

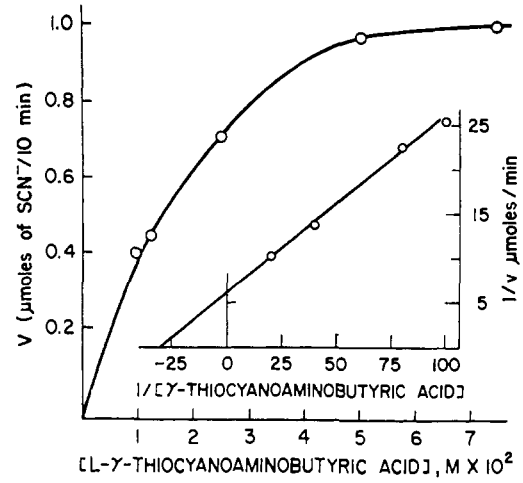

FIGURE 6: Kinetics of γ-cyano-α-aminobutyric acid synthesis as a function of γ-L-thiocyanoaminobutyric acid concentration, with 50 mM KCN. Reaction mixtures also contained per ml 10 munits of the enzyme (9.5 μg), 0.6 mM pyridoxal-P, and 40 μmol of potassium carbonate buffer (pH 10) and were incubated for 10 min at 30°. The thiocyanate assay was used.

timal in the pH range 8.3–9.6. Figure 4 shows the dependence of the reaction rate on KCN concentration, with 20 mM DL-homocystine. The insert of Figure 4 shows the dependence of the rate on L-homocystine concentration, with 50 mM KCN.

The optimal pH range for thiocyanate formation from γ-SCNabu was near 8.5–9.7 with 1.6 and 5 mM substrate and 9.3–10.5 with 50 mM substrate.[2] Figure 5 shows the dependence of the reaction rate on KCN concentration, with 0.1 M L-γ-SCNabu; Figure 6, the dependence of the rate on γ-SCNabu concentration, with 50 mM KCN. For KCN, K_m = 30 mM and V = 16.2 μmol/min per unit of synthase. For L-γ-SCNabu, K_m = 29 mM, V = 15.4 (inserts of Figures 5 and 6). The catalytic constant = 1.9×10^4 min^{-1}.

There was no evidence for the reverse synthesis of γ-SCNabu and cyanide from γ-CNabu (50 mM) and thiocyanate (0.13 M) under conditions (20 min and 92 munits of enzyme) that would have allowed detection of at least 0.25% reversibility. With both γ-SCNabu and homocystine as substrates, the enzyme was inhibited by the end-product thiocyanate by about 40% at 10 mM and 90% at 0.1 M but was unaffected by 0.1 M γ-CNabu.

Preparation and Activation of the Apoenzyme; Effect of Hydroxylamine. L-Cysteine (50 μmol) was added to a solution of the enzyme (500 munits, specific activity 0.6) in 2 ml of potassium phosphate buffer (50 μmol, pH 8.1), and the mixture was stirred magnetically for 2 hr at 4°. It was then clarified by centrifugation and passed over a 0.9 × 53 cm column of Sephadex G-25 previously equilibrated with the same buffer. Fractions of 2 ml were collected and assayed for activity. The essentially inactive apoenzyme was present in fraction 8. In the presence of 0.6 mM pyridoxal-P the specific activity was the same as for the starting material; the recovery of activity was 82–92% (Table II). When not treated with cysteine before gel filtration the enzyme retained 80% of its activity without requiring addition of pyridoxal-P.

After gel filtration the enzyme (stage 3) was preincubated for 5 min before assay with 0.1 mM hydroxylamine which resulted in 99% inactivation. When this was followed by preincubation for 5 min with 0.6 mM pyridoxal-P, activity was 88% restored.

[2] Such variation of pH optimum with substrate concentration is a known phenomenon (Reiner, 1969).

Nonenzymatic Cyanolysis of Homocystine in Relation to the Enzymatic Utilization of Homocystine. The formation of γ-SCNabu from homocystine and cyanide had a maximal rate near pH 9.6 with a pseudo-first-order rate constant for homocystine of 0.037 min^{-1} (Figure 7). In short reaction periods γ-SCNabu and Hcy (as Cm-Hcy in the presence of

TABLE II: Requirements for the Enzymatic Synthesis of Thiocyanate from KCN and Homocystine or γ-Thiocyanoaminobutyric Acid.[a]

Compound Omitted	Sp Act.[b] A	Sp Act.[b] B
None	0.55[c]	1.40
KCN	0	0.11
Homocystine	0	
γ-Thiocyanoaminobutyric acid		0.07
Pyridoxal-P	0	0.07
Enzyme: heated holoenzyme was substituted[d]	0.08	0.11

[a] The reaction mixtures in 1 ml contained for A: apoenzyme corresponding to 14 munits (see Preparation and Activation of Apoenzyme), 0.6 mM pyridoxal-P, 20 mM DL-homocystine, 50 mM KCN, and 50 mM Tris-HCl buffer (pH 9.1) containing 140 mM KCl; for B: apoenzyme corresponding to 7.8 munits, 0.6 mM pyridoxal-P, 20 mM L-γ-SCNabu, 30 mM KCN, and 80 mM potassium carbonate buffer (pH 10). The mixtures were preincubated for 10 min before reaction was initiated by the addition of KCN. [b] A and B should not be compared directly, since the specific activity of the starting enzyme for A was 2.45 times that for B, and only in A were the substrate concentrations saturating. [c] Pyridoxal, pyridoxamine, pyridoxine, deoxypyridoxine (at 0.1 mM) or pyridoxamine-5′-P (0.3 mM) were unable to replace pyridoxal-P effectively. [d] The holoenzyme in 0.1 M phosphate buffer (pH 8) was placed in a water bath at 100° for 5 min before addition of the other components.

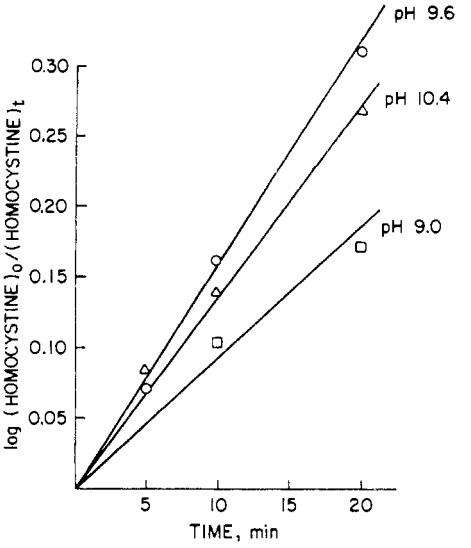

FIGURE 7: First-order plots for the cyanolysis of DL-homocystine at different pH values at 30° (see text): total homocystine concentration, 1.6 mM; total cyanide concentration, 50 mM; γ-SCNabu was determined.

iodoacetate, Table III, mixture 5) formed in stoichiometrically equivalent amounts.

When γ-CNabu-synthase was present the enzyme product γ-CNabu formed at the apparent expense of the nonenzymatically formed γ-SCNabu (*cf.* mixtures 3 and 5; *cf.* mixtures 1 and 2). In the presence of iodoacetate the enzymatic synthesis of γ-CNabu from homocystine (mixture 3) was no faster than the nonenzymatic cyanolysis of homocystine (as judged by Cm-Hcy in mixtures 3 or 5, or by γ-SCNabu in mixture 5). With very large amounts of enzyme when virtually no γ-SCNabu remained, the rates of these two reactions were almost the same. In the absence of iodoacetate the rate of the enzymatic synthesis of γ-CNabu from homocystine (mixture 1) varied over the range of 0.4–1.9 times that of the nonenzymatic cyanolysis (as judged by γ-SCNabu in mixture 2), depending on the amount of enzyme.

γ-Thiocyanoaminobutyric Acid as a Precursor of γ-Cyanoaminobutyric Acid for C. violaceum in Vivo. With 1 mM NaCN and 20 mM L-γ-SCNabu, an excess, the specific activity of 24-hr cultures was 18.5 μmol of γ-CNabu/mg of protein per 24 hr. Doubling the concentration of NaCN increased the production of γ-CNabu to 21.5 μmol. In a direct comparison at 5 mM with 18-hr cultures, γ-SCNabu yielded, per mg of protein/24 hr, 20 μmol of γ-CNabu; L-aspartic acid, 0.3 μmol of γ-CNabu; and DL-homocystine, none.

Discussion

Of various four- and three-carbon compounds previously tested as precursors of γ-CNabu with intact *C. violaceum* cells, only L-aspartic acid was effective (Brysk and Ressler, 1970). Isotopic experiments in those studies suggested that a substance derivable from aspartic acid participated without degradation of its carbon chain in a condensation reaction with cyanide. When the cell-free extract was prepared, homoserine, homocysteine, cystathionine, and methionine, as well as aspartic acid, were retested as cosubstrates of cyanide. Of these only homocysteine led to activity. Generally associated with γ-CNabu-synthase was some cystathionase activity. Since the latter was known to utilize cystine as a substrate, homocystine was also tested, and it proved to be far more effective than homocysteine as a cosubstrate of cyanide for γ-CNabu-synthase. That activity with homocysteine (6% of the disulfide) could be due to the presence of the disulfide in the assay has not been excluded; the reducing agent mercaptoethanol suppressed almost all activity with homocysteine but only part of that with homocystine. That γ-CNabu and thiocyanate are coproducts found support in the close agreement that resulted when both these compounds were determined when the pH dependency of the reaction rate and of the enzyme's stability were examined. Moreover, γ-CNabu and thiocyanate formed in close to stoichiometrically equivalent amounts.

A requirement of pyridoxal-P as a cofactor of γ-CNabu-synthase was suggested by the inactivation of the holoenzyme by hydroxylamine followed by its reactivation by pyridoxal-P and was supported by a full reactivation of the inactive apoenzyme by pyridoxal-P. To obtain the apoenzyme it was necessary to pretreat γ-CNabu-synthase with cysteine before passage over Sephadex G-25 as with several known pyridoxal-P enzymes (Shaltiel *et al.*, 1966). A number of monovalent metal chlorides enhanced the activity of γ-CNabu-synthase, an effect that may correspond to the activation by cations observed with some other pyridoxal-P enzymes (Newton and Snell, 1964).

γ-CNabu-synthase was inhibited by the vicinal thioamines L-cysteine and DL-penicillamine: presumably these bound the pyridoxal-P cofactor as thiazolidines (du Vigneaud *et al.*, 1957; Mardashev, 1963). Simpler thiols, including mercaptoethanol, glutathione, and dithioerythritol, however, tended to activate the enzyme. Hcy also activated, although at 25 mM in the absence of substrate and with gel filtration, it inactivated 90% and was effective in removing the cofactor presumably by forming a thiazane (Mardashev, 1963). Three of the four disulfides tested tended to inhibit γ-CNabu-syn-

TABLE III: Stoichiometry and Product Identification for the Reaction of Homocystine with Cyanide in the Presence and Absence of γ-Cyanoaminobutyric Acid Synthase.[a]

Reaction Mixture[b]	Product Formation (μmol/20 min) γ-CNabu	Cm-Hcy	γ-SC-Nabu	Hcy
1. Complete mixture[c]	5.0		1.4	3.4
2. − Enzyme			3.7	1.7
3. + Iodoacetate[c]	3.7	4.8	1.6	0.1
4. + Iodoacetate + 2.4 μmol of Cm-Hcy[d]	3.7	7.2	1.5	0.1
5. + Iodoacetate − Enzyme		5.0	5.7	0.1

[a] The complete reaction mixture contained 10 mM L-homocystine, 50 mM KCN, 0.6 mM pyridoxal-P, 160 munits (350 μg) of enzyme, 100 mM Tris-HCl + 280 mM KCl buffer (pH 9.1) in 2 ml and was incubated for 20 min at 30°. Where indicated, 11 mM iodoacetate was included. Products were determined on the amino acid analyzer. [b] Reactions with 40 and 300 munits of enzyme and with 20 mM DL- and 1.4 mM L-homocystine gave compatible results. [c] Thiocyanate was present in a ratio to γ-CNabu of approximately 0.9. [d] Cm-Hcy was added as a marker to mixture 3 before amino acid analysis.

thase (Table IV). Generally, the effects of simple thiols and of thiol alkylating agents were understandably obscured with homocystine as the substrate instead of γ-SCNabu. It remains to be established whether the observed effects of thiols and disulfides reflect the presence of essential sulfhydryl group(s) on the enzyme, or suggest a reaction mechanism involving such a grouping, or whether some other phenomenon may be taking place.

Since some enzymes have multiple activities, *e.g.*, the pyridoxal enzymes tyrosinase, tryptophan synthetase, and cystathionine γ-synthetase that catalyze both elimination and replacement reactions (Davis and Metzler, 1972), some attempt was made to determine whether γ-CNabu-synthase represents a secondary activity of a known enzyme. Purified γ-CNabu-synthase had 8% cystathionase activity. It catalyzed a cystathionase type of γ elimination (Matsuo and Greenberg, 1958) of thiocyanate from γ-SCNabu to only 0.4% the extent of its γ replacement with cyanide. Homocysteine desulfhydrase activity (Kallio, 1951) likewise was negligible. In converting cyanide into thiocyanate γ-CNabu-synthase resembles rhodanese (Sorbo, 1955) and β-mercaptopyruvate transsulfurase (Fiedler and Wood, 1956), and is further similar in utilizing a sulfur-containing substrate, but it is readily distinguished from these enzymes by molecular weight (Bowen *et al.*, 1965) and cofactor requirement. Moreover, beef liver rhodanese was unable to catalyze the synthesis of γ-CNabu from homocystine and KCN. Partially purified γ-CNabu-synthase (specific activity 0.5) had 5% rhodanese activity which may represent an impurity, since the crude extracts of *C. violaceum* D 341 cells had approximately equal rhodanese and γ-CNabu-synthase activities. Under specified conditions this organism also synthesizes β-cyanoalanine (Brysk and Ressler, 1970), thus suggesting at least three routes for it to detoxify cyanide.

γ-CNabu-synthase had negligible activity for *O*-acetylhomoserine sulfhydrylation (Kerr, 1971), the reaction that would be homologous or similar to that utilized by the β-cyanoalanine synthases of *Bacillus megaterium*, *Escherichia coli* and blue lupin seedlings (Castric and Conn, 1971; Dunnill and Fowden, 1965; Hendrickson and Conn, 1969). Moreover, *O*-acetylhomoserine was only a poor cosubstrate with cyanide for the synthesis of γ-CNabu with γ-CNabu-synthase. Although the synthesis of γ-CNabu from homocystine and cyanide appears to differ in type from that reported for β-CNala, it should be noted that in contrast to γ-CNabu-synthase the β-CNala-synthases were from noncyanide-forming species. To metabolize cyanide in quantity, the *C. violaceum* strain may have developed an additional enzyme.

One of the mechanisms considered for the enzymatic formation of γ-CNabu from homocystine and cyanide envisioned the nonenzymatic cyanolysis of homocystine (**1**) to give homocysteine (**2**) and γ-thiocyanoaminobutyric acid (**3**) (reaction

$$\underset{\mathbf{1}}{(SCH_2CH_2CHNH_2COOH)_2} \xrightarrow{CN^-}$$

$$\underset{\mathbf{2}}{HOOCCHNH_2CH_2CH_2SH} + \underset{\mathbf{3}}{HOOCCHNH_2CH_2CH_2SCN} \quad (2)$$

$$SCN^- + \underset{\mathbf{4}}{HOOCCHNH_2CH_2CH_2C{\equiv}N} \xleftarrow{CN^-,\ \text{enzyme}} \quad (3)$$

2) followed by the enzymatic displacement of thiocyanate ion from **3** by cyanide ion to give γ-CNabu (**4**) (reaction 3). Reaction 2 represents the generally accepted nucleophilic attack of cyanide ion on a polarized disulfide bond to give as products a mercaptide and an alkyl thiocyanate. Reaction 3 depicts an enzymatic γ-replacement reaction (see Davis and Metzler, 1972).[3]

TABLE IV: Effect of Various Additives on Enzyme Activity.[a]

Additive	Substrate: L-γ-SCNabu[b] % Act.[d]	Substrate: DL-Homocystine[c] % Act.[d]
$CdCl_2$[e]	19	47
$CuSO_4$[e]	2	12
$HgSO_4$[e]	11	28
p-Chloromercuribenzoate	53	103
Iodoacetate	80	104
L-Cysteine	75	21
DL-Homocysteine	129	44
DL-Penicillamine	21	
L-Cystine	41	51
3,3′-Dithiodipropionic acid	28	
5,5′-Dithiobis-2-nitrobenzoic acid	55	
GSH	197	64
2-Mercaptoethanol	184	43
1,4′-Dithioerythritol	135	18

[a] Reaction mixtures in 1 ml contained either 8.4 munits of the enzyme, 29 mM γ-SCNabu, and 40 mM potassium carbonate buffer (pH 10) (in experiments with metals, 250 mM sodium glycinate buffer was substituted), or 31 munits, 20 mM DL-homocystine, and 100 mM Tris-HCl buffer containing 280 mM KCl (pH 9.1). All experiments also contained 0.6 mM pyridoxal-P and 10 mM additive. Enzyme specific activity was 3.4. After 10-min incubation at 30°, the reactions were started with 30 and 50 mM KCN, and after 10 and 30 min, respectively, they were assayed for thiocyanate. [b] D-Cycloserine, *N*-ethylmaleimide, EDTA, and GSSG had little effect. [c] $MnSO_4$, $FeCl_3$, $MgSO_4$, and $CaCl_2$ inhibited by 15%. [d] Compared to mixture lacking additive. [e] At 1 mM these inhibited by 20–40%.

The cyanolysis of homocystine had been studied by Schöberl and Kawohl (1957) who identified Hcy and 2-aminopentthiazoline-4-carboxylic acid as the reaction products. γ-SCNabu was presumed to be the primary unstable product but was not detected. The synthesis of this previously unknown sulfur amino acid was therefore undertaken and accomplished by a separate route involving alkylation of homocysteine with cyanogen bromide. Chromatographic comparison with the synthetic amino acid allowed the unknown material observed in the homocystine–cyanolysis mixture that decreased in the presence of enzyme to be identified as γ-SCNabu (Table III). This amino acid was then detected when the reaction mixture of Schöberl and Kawohl was reexamined on the analyzer (11% **3** from homocystine, cyanide, and 7 equiv of benzyl chloride after 2 hr at pH 10.1). The small amount of γ-SCNabu apparently had been missed in their isolation of the main product as a copper salt. In alkaline solution

[3] An alternate mechanism considers an enzymatic cleavage of homocystine to thiohomocysteine ($COOHCHNH_2CH_2CH_2SSH$, HcySH) analogous to the cystathionase-catalyzed cleavage of cystine (Cavallini *et al.*, 1962; Flavin, 1962). The HcySH could then undergo nonenzymatic cyanolysis to thiocyanate and Hcy (Hylin and Wood, 1959). This seemed unlikely since thiocyanate and no Cm-HcySH (homocysteine–mercaptoacetic acid mixed disulfide) formed when the γ-CNabu-synthase reaction with homocystine and cyanide was carried out in the presence of iodoacetate (Table III, *cf.* Flavin, 1962).

TABLE V: Substrate Specificity of γ-Cyanoaminobutyric Acid Synthase.[a]

Compound[b]	Act. Ratio
L-Homocystine	100
DL-Homocystine	56
DL-Homocysteine[c]	3
DL-Homocysteine–2-mercaptoethanol mixed disulfide	48
L-Homocysteine–2-mercaptoethanol mixed disulfide	87
L-Homocysteine–2-mercaptoacetic acid mixed disulfide	50
L-Cystine	3
L-Cysteine–2-mercaptoethanol mixed disulfide	3
L-γ-Thiocyanoaminobutyric acid	359
O-Acetyl-L-homoserine	5

[a] Reaction mixtures in 1 ml included 50 mM KCN, 6–38 munits of the enzyme (specific activity 1.1 or 0.55), 50 mM of Tris-HCl containing 140 mM KCl (pH 9.1), and 20 mM compound except for L-cystathionine, 0.66 mM. The thiocyanate assay was used except for *O*-acetylhomoserine when amino acid analysis was used. [b] Homoserine, cystathionine, methionine, aspartic acid, and threonine (all L) with 55 munits and the chromatographic radioassay led to no detectable activity. [c] Assayed in the presence of N_2; without this precaution, the activity ratio was 7.

synthetic γ-SCNabu was in fact labile, with a half-life of 1 hr in the enzyme assay buffer.

The synthetic γ-SCNabu proved to be an excellent cosubstrate of cyanide for γ-CNabu-synthase providing 3.5 times the reaction rate as did L-homocystine when both were at 20 mM, and about seven times at saturating concentrations. The mixed disulfides of homocysteine with 2-mercaptoethanol and 2-mercaptoacetic acid were the only other good substrates observed (Table V). Like homocystine their activities possibly depend upon their degree of cyanolysis to γ-SCNabu. The presence of γ-SCNabu in the incubation mixtures of the mixed disulfides was in favor of this. The efficient utilization of γ-SCNabu by intact *C. violaceum* cells for the synthesis of γ-CNabu backed its action as a good substrate for γ-CNabu-synthase. Moreover, γ-SCNabu was far more effective and reproducible in this *in vivo* process than aspartate. The synthesis of γ-CNabu from aspartate presumably depends upon elaboration of a series of intermediates, a route expected to be less efficient and more subject to variation than the apparently more direct conversion of γ-SCNabu to γ-CNabu.

In the presence of iodoacetate the rate of the overall enzymatic utilization of homocystine (reaction 1) was in general compatible with a scheme that proceeds through the nonenzymatic cyanolysis step (reaction 2) since the rate of the overall reaction did not exceed the rate of the latter step. With large amounts of enzyme in the absence of iodoacetate, however, the rate of overall reaction 1 seemed to be somewhat too fast to be mediated only by reaction 2. If the utilization of homocystine does proceed through the nonenzymatic formation and enzymatic utilization of γ-SCNabu, perhaps the homocysteine, also formed nonenzymatically, participates somehow to accelerate the overall reaction. The γ-SCNabu route as the pathway for the enzymatic utilization of homocystine thus warrants further examination. The detailed mechanism for the enzymatic displacement of the thiocyano group from γ-SCNabu by cyanide ion and the biological properties of γ-SCNabu should also be of interest to study.

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Spectra of 3-Hydroxypyridines. Band-Shape Analysis and Evaluation of Tautomeric Equilibria†

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ABSTRACT: Absorption spectra of individual ionic and nonionic forms of pyridine, pyrazine, phenol, and 19 3-hydroxypyridines have been measured and have been described as sums of log normal curves. The log normal parameters are tabulated and are discussed. In favorable cases band positions are located to $\pm 10\ cm^{-1}$ (± 0.1 nm at 316 nm) and band widths are measured to $\pm 0.5\%$. It is concluded that band width is a highly reproducible quantity that can be used as an index of homogeneity and as an indicator of alterations in chemical environment. The data obtained provide the basis for precise resolution of overlapping spectral bands. Limits of error are considered as are the effects of minor "buried" absorption bands. The latter have been identified in spectra of highly purified 3-hydroxypyridines in uncharged and anionic forms. They are absent in cationic and dipolar ionic forms, however. Their origin is uncertain. A new method for evaluating tautomeric equilibria depends upon the observation that for many pure substances, areas of absorption bands are constant with changes in solvent composition or temperature. Precise resolution of overlapping absorption bands at two or more temperatures or in two or more solvent mixtures permits evaluation of the tautomerization constant. The latter, together with stepwise pK_a values, allows evaluation of microscopic dissociation constants. The analysis has been extended to a system of three tautomers with closely overlapping bands (5-deoxypyridoxamine). Band-shape analysis often reveals hidden vibronic fine structure in absorption bands. We show that distinct changes in the fine structure of the pyridoxamine phosphate spectrum take place upon binding to the apoenzyme of aspartate aminotransferase.

The use of log normal distribution curves, fitted by a computer-assisted iterative process to experimental data (plotted against wave number), permits a precise mathematical description of electronic absorption spectra. This *band-shape analysis* has been applied to spectra of 3-hydroxypyridines (Siano and Metzler, 1969; Johnson and Metzler, 1970), proteins (Metzler *et al.*, 1972), potassium iodide (Siano and Metzler, 1972), purines, pyrimidines, and other substances (Metzler *et al.*, 1973). The method is in general superior to the use of gaussian curves to resolve spectra plotted against wavelength. It provides information about band shape as well as intensity and position. It also provides a convenient way to visualize fine structure in absorption bands, *e.g.*, those of proteins (Metzler *et al.*, 1972).

The present paper documents the utility of the log normal distribution curve in the quantitative description of spectra of substituted pyridines and of free and enzyme-bound derivatives of vitamin B_6. A new method of evaluating tautomeric equilibria is described.

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Experimental Procedures

Sources of compounds (for numbering see tables) studied are as follows: compounds **2** and **4–8** were purchased from Aldrich Chemical Co; **9**, **11**, **13**, **15**; **19**, and **22** were from Sigma Chemical Co.; **1** was from Baker; and **3** was from Mallinckrodt. The following were gifts: 6-methylpyridoxine, from M. Karpeisky; isopyridoxamine phosphate from A. Pocker; 3-hydroxy-4-pyridinecarboxaldehyde from M. O'Leary. Other compounds were prepared in this laboratory using published procedures (see Korytnyk and Ikawa, 1970). Samples of 3-methoxypyridine (compound **6**) were prepared from 3-hydroxypyridine by treatment with diazomethane in anhydrous methanol by T. Fisher and by T. D. Bolden in this laboratory. The solution was acidified with aqueous HCl and extracted with ether; then the aqueous phase was made basic. The product was extracted with ether and crystallized from methanol and ether. Deoxypyridoxamine was synthesized by T. Fisher by reduction of the oxime of 5-deoxypyridoxal (Fisher, 1971; Testa and Fava, 1957). In every case we have attempted to obtain highly pure samples and have recrystallized several compounds to constant spectrum. Usually little change was observed except at the high-energy end in which improvement was often obtained by recrystallization from "Spectroquality" methanol and water. For some compounds