γ-Butyrobetaine Hydroxylase from *Pseudomonas* sp AK 1*

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ABSTRACT: A soluble γ-butyrobetaine hydroxylase has been partially purified from cells of a *Pseudomonas* strain (*Pseudomonas* sp AK 1) by chromatography on DEAE-cellulose and on hydroxylapatite. Carnitine is the only product of γ-butyrobetaine; trimethylamine or trimethylaminoacetone is not formed. The enzyme has a low isoelectric point (around pH 4.5). The $K_{M,app}$ value for γ-butyrobetaine is 2.4 mM. There is an absolute and specific requirement for 2-ketoglutarate and for ferrous ion. The $K_{M,app}$ values are 0.5 and 0.05 mM, respectively. Ascorbate and several other reducing agents stimulate the reaction and catalase increases the rate of carnitine production about threefold. Preincubation of the enzyme with ascorbate and ferrous ion results in appreciable loss of enzymic activity, which does not occur if catalase is present in the preincubation mixture. Sulfhydryl reagents and several metal ions are potent inhibitors of the enzyme.

In the previous studies of carnitine metabolism, evidence was obtained for a low turnover rate of carnitine in the rat (Lindstedt and Lindstedt, 1961a). Of various hypothetical precursors, only γ-butyrobetaine (4-trimethylaminobutyrate) was converted efficiently into carnitine (Lindstedt and Lindstedt, 1961b, 1965a). Results from studies with preparations from rat liver indicated an oxygenase type of mechanism for this reaction (Lindstedt and Lindstedt, 1962; Lindstedt, 1967a). The pathway for carnitine degradation in the rat is unknown. A β-oxidation type of reaction for the degradation of carnitine in *Pseudomonas aeruginosa* NCTC A 7244 was suggested from the finding of glycine betaine as a metabolite of carnitine (Lindstedt and Lindstedt, 1962). The possibility that this type of mechanism occurred in the formation of carnitine from γ-butyrobetaine in bacteria was therefore considered. A *Pseudomonas* strain (*Pseudomonas* sp AK 1) was obtained by enrichment culture on γ-butyrobetaine media (Lindstedt *et al.*, 1967b). Carnitine was formed from γ-butyrobetaine in incubations with acetone-dried cells under conditions which suggested a monooxygenase reaction. The hydroxylase from *Pseudomonas* sp AK 1 has been used for studies of the reaction mechanism (Lindblad *et al.*, 1969) as high specific activity is more easily obtained with the bacterial preparations than with those from rat liver. The partial purification of the enzyme and studies of cofactor requirements are now presented. Some of the results have been reported in preliminary form (Lindstedt *et al.*, 1967a,c).

Material and Methods

Chemicals. Chemicals were obtained from the following sources: γ-butyrobetaine chloride, ferrous sulfate, and 1,10-phenanthroline from E. Merck, AG, Darmstadt, West Germany; oxalacetic acid, mercaptoethanol, L-glutamic acid, sodium DL-isocitrate, zinc 2-hydroxyglutarate, sodium pyruvate, succinic acid, isoascorbate, dichlorophenolindophenol, and sodium *p*-mercuribenzoate from Fluka AG, Buchs, Switzerland; 2-ketopimelic acid, 2-ketoadipic acid, 2-ketovaleric acid, 2-ketobutyric acid, and *cis*-aconitic acid from K & K Laboratories, Inc., Plainwood, N. Y.; NADH, NADPH, sodium 2-ketoglutarate, and catalase from C. F. Boehringer und Soehne GmbH, Mannheim, West Germany; sodium ascorbate from Dr. Th. Schuchardt GmbH, Munich, West Germany; streptomycin sulfate from Glaxo Laboratories Ltd., Greenford, England; reduced glutathione and sodium metaarsenite from British Drug Houses, Ltd., Pool, England; coenzyme A, tetrahydrofolate, sodium *p*-mercuriphenylsulfonate, tris(hydroxymethyl)aminomethane (Trizma Base), and glutaric acid from Sigma Chemical Co., St. Louis, Mo.; dithiothreitol, 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine from Calbiochem AG, Luzerne, Switzerland; silicic acid from Mallinckrodt Chemical Works, St. Louis, Mo.; hydroxylapatite and Dowex AG 50W-X8 (−400 mesh, hydrogen form), Dowex 2-X8 (200–400 mesh, chloride form), and Retardion AG 11A8 (50–100 mesh, self-absorbed form) from Bio-Rad Laboratories, Inc., Richmond, Calif.; diethylaminoethylcellulose from Serva Entwicklungslabor, Heidelberg, West Germany; Sephadex G-25 (coarse) from AB Pharmacia, Uppsala, Sweden; and Hyamine from Packard Instrument Co., Downers Grove, Ill.; *N*-ethylmaleimide, iodoacetate, iodosobenzoate, and acetarsone from Mann Research Laboratories, Inc., New York 6, N. Y.; citric acid from Riedel de Haen AG, Seelze, Hannover, West Germany; malic acid from Pfahnstiehl Laboratory, Inc., Wanteegan, Ill.; fumaric acid from Eastman Organic Chemicals, Rochester, N. Y.; Ampholine from LKB-Produkter AB, Stockholm, Sweden.

The chloride of 3-trimethylaminopropionic acid was obtained by passing dry trimethylamine into a solution of 3-bromopropionic acid in ice-cooled ethanol until no more precipitation of 3-trimethylaminopropionate and trimethylammonium bromide was noted. The reaction mixture was kept overnight at room temperature and taken to dryness. The residue was dissolved in water and filtered through a column of Retardion AG 11A8 which was eluted with water. Hydrochloric acid was added to the fractions containing trimethylaminopropionate and the solvent was evaporated. Trimethylaminopropionic acid chloride was recrystallized from hot ethanol (Lindstedt, 1967a: Lindstedt and Tofft, 1969). 4-Tri-

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methylaminocrotonic acid chloride was obtained by passing dry trimethylamine into a solution of 4-bromocrotonic acid methyl ester under the same conditions. When no more precipitation of the bromide of the methyl ester of 4-trimethylaminocrotonic acid was noticed the reaction mixture was taken to dryness, dissolved in 2 M hydrochloric acid, and allowed to stand for 24 hr at room temperature to hydrolyze the ester. The solution was then taken to dryness, redissolved in water, and filtered through a column of Retardion AG 11 A8. Trimethylaminocrotonic acid chloride was recrystallized from hot ethanol (Lindstedt, 1967a). Succinic semialdehyde was prepared according to Carrière (1922) as modified by Albers (Jacoby, 1962).

Labeled Compounds. [*methyl*-$^{14}C_3$]-γ-Butyrobetaine (15 mCi/mmole) was prepared as described previously (Lindstedt and Lindstedt, 1965a,b: Lindstedt and Tofft, 1969). [*1*-$^{14}C_1$]-2-Ketoglutaric acid (11.0 mCi/mmole) was obtained from Calbiochem AG, Luzerne, Switzerland. Aliquots were analyzed by partition chromatography on silicic acid (see Figure 1). Preparations, which contained more than 90% 2-ketoglutaric acid, such as the one in Figure 1, were used without further purification. Less pure preparations were chromatographed on silicic acid. Lyophilization of the material in the 2-ketoglutaric acid peak resulted in material which, on rechromatography, was found to contain less than 5% of more polar material. It is important that the purity of the labeled 2-ketoglutarate is checked before use. One commercial sample of [*1,2*-$^{14}C_2$]-2-ketoglutarate was found to contain less than 5% of 2-ketoglutarate. The remainder of the radioactivity was eluted more slowly, and probably represented polymerization products. Eight different samples of [*1*-$^{14}C_1$]-2-ketoglutarate and [*5*-$^{14}C_1$]-2-ketoglutarate have been analyzed so far. Purity has ranged between 35 and 95%.

Analytical Procedures. The separation of γ-butyrobetaine from carnitine and trimethylaminoacetone was carried out on 1.6 × 7.5 cm columns of Dowex AG 50W-X8 (minus 400 mesh, hydrogen form) (Lindstedt and Lindstedt, 1965b; Lindstedt *et al.*, 1967b). The separation of γ-butyrobetaine from trimethylamine was carried out as follows. The lyophilized fractions from the ion-exchange column were dissolved in 0.2 ml of water, nonradioactive reference compounds (10 mg of each) were added, and the solution was applied to a column of aluminum oxide (Woelm, basic, grade III, 5 g, 5 ml). The column was eluted with acetone (five bed volumes) and methanol–acetone (1:1, v/v) (ten bed volumes). Partition chromatography of carboxylic acids on columns of silicic acid (8 g) was performed as described by Prior Ferraz and Relvâs (1965), with 0.25 M sulfuric acid as stationary phase and varying concentrations of *tert*-butyl alcohol in benzene as mobile phase. The acid concentration of the eluate was determined by titration with 0.02 M sodium hydroxide from a microburet. Small amounts of the stationary phase leaked out when the concentration of *tert*-butyl alcohol in benzene was 60%. Diethylaminoethylcellulose (DEAE-cellulose) was washed as described by Peterson and Sober (1962), brought to pH 6.5 with dilute hydrochloric acid, suspended in 10 mM Tris-HCl solution (pH 6.5), and poured into columns. Columns of hydroxylapatite were prepared from suspensions of hydroxylapatite in 2 mM potassium phosphate buffer (pH 6.5).

Isoelectric focusing was carried out according to Vesterberg and Svensson (1966) and Vesterberg *et al.* (1967) with an apparatus from LKB-Produkter, Stockholm, Sweden. Samples

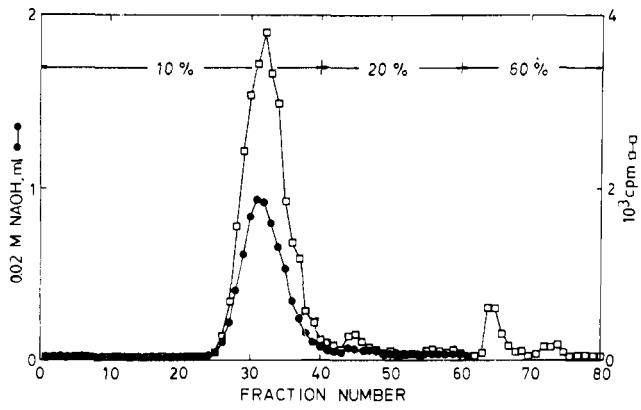

FIGURE 1: Chromatography on a column of silicic acid (8 g, 21 ml) of a sample of commercial [1-$^{14}C_1$]-2-ketoglutarate The column was eluted with increasing concentrations of *tert*-butyl alcohol in benzene in 4-ml fractions. Nonlabeled 2-ketoglutarate (10 mg) had been added before chromatography. The isotope content of the fractions was determined by scintillation counting and the fractions were then titrated with 0.02 M sodium hydroxide.

were dialyzed against 1% (w/v) aqueous glycine solution overnight before electrophoresis.

The protein concentration in the effluent from the chromatographic columns was estimated by measuring the absorbance at 280 nm; otherwise the protein was measured according to Lowry *et al.* (1951).

Enzyme Assay. The γ-butyrobetaine-hydroxylating activity was assayed by incubating enzyme, 2-ketoglutarate, and cofactors with labeled γ-butyrobetaine. Labeled carnitine was separated from γ-butyrobetaine by ion-exchange chromatography. The composition of the incubating mixture was ("complete system") enzyme (10–500 μg of protein), [*methyl*-$^{14}C_3$]-γ-butyrobetaine (2.9 mM, 1 μCi/ml), 2-ketoglutarate (2.9 mM), ferrous sulfate (0.6 mM), sodium ascorbate (14 mM), catalase (1.4 mg/ml), and potassium phosphate buffer at pH 7.0 (15 mM) in a total volume of 0.7 ml. The incubations were carried out in air at 37° for 10–45 min and were terminated by the addition of 0.5 ml of 10% aqueous trichloroacetic acid. After cooling in ice for 1 hr the suspension was centrifuged and the supernatant fraction was put onto a column of Dowex AG 50W-X8, which was eluted with 1 M HCl. The eluate was collected in 10-ml fractions. Aliquots were dried in glass planchets under an infrared lamp, and the radioactivity was then determined in a methane-flow proportional counter.

The 2-ketoglutarate-degrading activity was assayed by incubating enzyme, γ-butyrobetaine, and cofactors with [*1*-$^{14}C_1$]-2-ketoglutarate and trapping the labeled carbon dioxide, which had been formed in the reaction. The composition of the incubating mixture was enzyme (10–500 μg of protein), γ-butyrobetaine (29 mM), [*1*-$^{14}C_1$]-2-ketoglutarate (2.9 mM, 0.1 μCi/ml), ferrous sulfate, ascorbate, catalase, and phosphate buffer in the same concentrations as above. The total volume was 0.7 ml and the incubations were carried out in air for 10–45 min in 10-ml stoppered centrifuge tubes. The incubations were terminated by the addition to the stoppered tubes of either 1 M sulfuric acid (1 ml) or 10% trichloroacetic acid (1 ml). Labeled carbon dioxide was trapped on 1-cm² pieces of filter paper with 20 μl of Hyamine (1 M in methanol). The filter paper had been attached to a wire in the rubber stopper before the

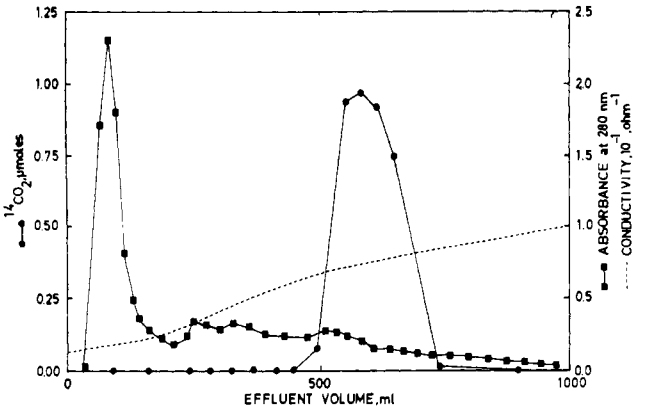

FIGURE 2: Enzyme purification by DEAE-cellulose chromatography (see text). The column (45 ml) was eluted with increasing concentrations of potassium chloride in 10 mM Tris-HCl (pH 6.5). The enzyme activity was determined by incubating 300 μl of the fractions with γ-butyrobetaine, [*1*-$^{14}C_1$]-2-ketoglutarate, ascorbate, ferrous ion, and catalase as described under Assay.

incubations. After diffusion of labeled carbon dioxide for 60 min the filter papers were transferred to a scintillation counter vial, containing 10 ml of scintillation mixture (2,5-diphenyloxazole, 10 g, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, 0.3 g, toluene, 1000 ml, and ethylene glycol monomethyl ether, 600 ml).

Enzyme activity was expressed in units (U) equal to 1 μmole of carnitine formed per min (γ-butyrobetaine-hydroxylating activity) or 1 μmole of CO_2 formed per min (2-ketoglutarate-degrading activity). Both assays were used with enzyme activities in the range 1–40 mU.

Bacteria. Pseudomonas sp AK 1 was grown with aeration at 37° for 24–48 hr in a salt medium, containing 20 mM γ-butyrobetaine as sole source of carbon and nitrogen (Lindstedt *et al.*, 1967b). At the end of the exponential growth phase, the cells were collected by centrifugation and washed three times with 0.9% aqueous sodium chloride. The bacterial paste was kept frozen at −15°.

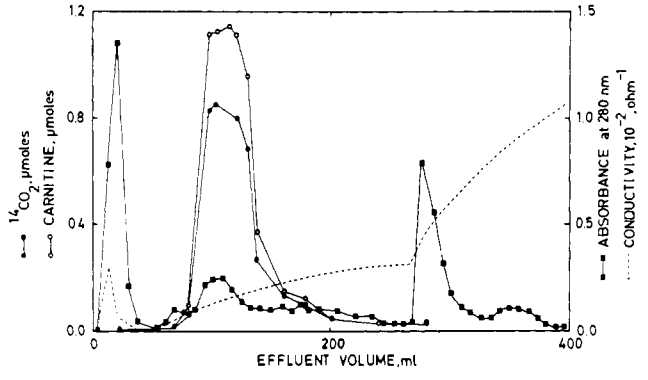

FIGURE 3: Enzyme purification by hydroxylapatite chromatography (see text). The column (5 ml) was eluted with increasing concentrations of potassium phosphate buffer at pH 6.5. Incubations were carried out with 300 μl of the fractions γ-butyrobetaine, [*1*-$^{14}C_1$]-2-ketoglutarate, ferrous ion, ascorbate, and catalase as described under Assay.

TABLE I: Enzyme Purification (γ-Butyrobetaine Hydroxylase Assay; See Methods).

Purification Step	Protein (mg)	Sp Act. (U/g)[a]	Total Act. (U)
1. Cell extract	470	12.4	5.85
2. Streptomycin treatment	426	12.4	5.30
3. DEAE chromatography	35	24	0.80
4. Hydroxylapatite chromatography	1.6	220	0.35

[a] For definition of the enzyme unit, see text.

Results

Enzyme Purification. STEP 1. SONICATION OF BACTERIA. About 20 g of bacterial paste was suspended in 200 ml of ice-cold 50 mM potassium chloride in 10 mM Tris-HCl (pH 6.5) and sonicated for 15 min in a "Rosette" cell (Branson Instrument Co.) with a Branson type S 75 sonifier operating at 75 W and 20 KHz. The mixture was then centrifuged for 60 min at 75,000*g*, and the sediment resuspended in about 50 ml of the same buffer, sonicated under the same conditions, and centrifuged. The supernatant fractions were combined and aliquots were kept frozen for periods up to 2 months. Usually, about 0.5 g of protein was used as starting material for the purification.

STEP 2. STREPTOMYCIN TREATMENT. A 20% streptomycin solution in water (*a* ml) was added during stirring in the cold for 15 min to the combined supernatant fractions from step 1 (*b* ml) ($a = b \times 0.75 \times$ extract A_{260nm}). The precipitate was spun down by centrifugation at 4° at 75,000*g* for 45 min. The pH changed from 6.5 to 6.2 on treatment with streptomycin.

STEP 3. DEAE-CELLULOSE CHROMATOGRAPHY. The protein fraction from step 2 was added to a column of DEAE-cellulose (2.2 × 11.0 cm) which was eluted, first with 10 mM Tris-HCl at pH 6.5, then with a convex gradient between 50 and 300 mM potassium chloride in 10 mM Tris-HCl (pH 6.5). The enzymic activity was eluted between about 100 and 200 mM potassium chloride (Figure 2).

STEP 4. HYDROXYLAPATITE CHROMATOGRAPHY. The fractions from the DEAE-cellulose chromatography were applied to a column (1.1 × 5.2 cm) of hydroxylapatite. The column was eluted, first with a 2 mM potassium phosphate buffer (pH 6.5), then with a convex gradient between 2 and 75 mM potassium phosphate buffer, and finally with a gradient between 75 and 400 mM potassium phosphate buffer (pH 6.5). The γ-butyrobetaine-hydroxylating activity was eluted together with 2-ketoglutarate-degrading activity between approximately 20 and 40 mM potassium phosphate buffer (Figure 3). The chromatographies were performed at 4°. The results from the purification of the enzyme are given in Table I.

Isoelectric Focusing. After isoelectric focusing electrophoresis the enzymic activity was located in the pH region around 4.5 (Figure 4). The procedure, however, resulted in appreciable losses of enzymic activity and could not be used for purification of the enzyme. Inactivation occurred also when the material from step 3 was brought to pH 4.5 with dilute acetic acid and centrifuged, and the sediment dissolved in 10 mM po-

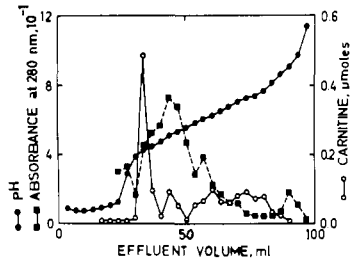

FIGURE 4: Isoelectric focusing electrophoresis of protein fraction from step 2 of the purification procedure (see Table I). A pH gradient between pH 3 and 10 was established with 1% solution of the ampholyte. A potential of about 400 V was applied for 48 hr and contents of the column were then collected. γ-Butyrobetaine hydroxylase activity was determined (see Assay).

tassium phosphate buffer (pH 6.5). The supernatant from this experiment was without enzymic activity.

Formation of Carnitine from γ-Butyrobetaine. Chromatography on Dowex AG 50W-X8 and on aluminium oxide revealed only carnitine as a metabolite of labeled γ-butyrobetaine in incubations with 2-ketoglutarate, ferrous sulfate, ascorbate, and catalase. Neither trimethylaminoacetone nor trimethylammonium ion could be detected (detection limit about 0.5% of incubated γ-butyrobetaine).

Effect of Enzyme Concentration, Time, and pH. The enzyme activity was proportional to the concentration of protein in the range 0–75 mg/l. A linear relation was obtained between enzyme activity and incubation time from 0 to 1 hr. An incubation time of 45 min was chosen for the following experiments, except in the K_M determinations, when the incubation time was 10 min.

Figure 5 shows the effect of pH value in the incubation on the formation of carnitine from γ-butyrobetaine. Optimal enzyme activity was obtained between pH 7.0 and 7.2. During the incubations the pH value was found to change less than 0.1 pH unit.

γ-Butyrobetaine and 2-Ketoglutarate. A series of incubations with different initial concentrations of γ-butyrobetaine were performed to determine the $K_{M,app}$ value for this substrate; from a Lineweaver–Burk plot it was calculated to be 2.4 mM. In separate experiments it was ascertained that the product, carnitine, was not inhibitory in 3 mM concentration. There was an absolute requirement for γ-butyrobetaine for formation of labeled carbon dioxide from [*1*-$^{14}C_1$]2-ketoglutarate. The following analogs of γ-butyrobetaine were tested as substrate: 3-trimethylaminopropionate, 5-trimethylaminovalerate, 6-trimethylaminocaproate, 4-dimethylaminobutyrate, and 4-trimethylaminocrotonate; in no case was 2-ketoglutarate-degrading activity observed. As seen from Table II, they were however moderately effective as inhibitors. There was an absolute requirement for 2-ketoglutarate for hydroxylation of γ-butyrobetaine to carnitine; the $K_{M,app}$ value was 0.45 mM. None of the following compounds could replace 2-ketoglutarate as cofactor: succinic semialdehyde, pyruvate, 2-ketobutyrate, 2-ketovalerate, *p*-hydroxyphenylpyruvate, oxalacetate, 2-ketoadipate, 2-ketopimelate, glutarate, 2-hydroxyglutarate, glutamate, citrate, isocitrate, *cis*-aconitate, succinate, fumarate, or malate. These acids were also tested as

TABLE II: Inhibition of γ-Butyrobetaine Hydroxylation and 2-Ketoglutarate Decarboxylation by Substrate Analogs.[a]

Labeled Substrate	Inhibitor	Amt (μmoles)	Inhibn (%)
γ-Butyrobetaine	3-Trimethylamino-propionate	8	18
	5-Trimethylamino-valerate	8	30
	6-Trimethylamino-caproate	8	23
	4-Dimethylamino-butyrate	8	41
	4-Trimethylamino-crotonate	8	50
2-Ketoglutarate	Oxalacetate	5	40
	2-Ketoadipate	5	20
	2-Ketopimelate	5	80
	Citrate	5	50

[a] The partially purified enzyme (50 μg of protein, 200 U/g) was incubated with either [*methyl*-$^{14}C_3$]-γ-butyrobetaine or [*1*-$^{14}C_1$]-2-ketoglutarate as the labeled substrate (see Assay for details), and with the inhibitor given in the table. The incubations (0.7 ml) were carried out for 45 min at 37°.

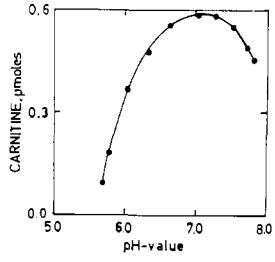

FIGURE 5: Formation of carnitine from γ-butyrobetaine in potassium phosphate buffer at different pH values. The enzyme (66 μg of protein, 195 U/g) was incubated at 37° for 45 min with [*methyl*-$^{14}C_3$]-γ-butyrobetaine as described under Assay.

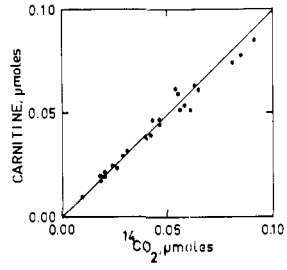

FIGURE 6: Relationship between the formation of carnitine from γ-butyrobetaine and the production of CO_2 from 2-ketoglutarate. The formation of carnitine is plotted against the formation of [^{14}C]-CO_2 during incubation with different concentrations of [*methyl*-$^{14}C_3$]-γ-butyrobetaine (0.1–2.0 μmoles) and [1-$^{14}C_1$]-2-ketoglutarate (0.1–2.0 μmoles). The incubations were carried out during 10 min at 37° with 66 μg of protein (260 U/g).

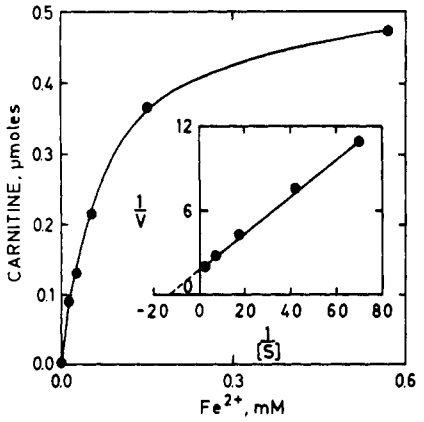

FIGURE 7: Effect of ferrous ion on the formation of carnitine from γ-butyrobetaine. The enzyme (55 μg of protein, 190 U/g) was incubated at 37° for 45 min with [*methyl*-$^{14}C_3$]-γ-butyrobetaine and cofactors as described under Assay with different amounts of ferrous ion. In the Lineweaver–Burk plot, v is the amount of carnitine formed during the incubation and [S] the initial concentration of ferrous ion.

TABLE III: Inhibition of Enzymic 2-Ketoglutarate Decarboxylation by Metal Ions.[a]

Inhibitor	Inhibn (%)
$MgCl_2$	5
$CaCl_2$	12
$CrCl_3$	18
$MnCl_2$	35
$K_4Fe(CN)_6$	20
$CoCl_2$	93
$NiCl_2$	92
$CuCl_2$	80
$ZnCl_2$	93
$CdCl_2$	86
$HgCl_2$	80

[a] The partially purified enzyme preparation (20 μg of protein, 195 U/g) was incubated with the complete system and with 0.4 μmole of inhibitor. The incubations (0.7 ml) were carried out for 45 min at 37°.

inhibitors; only those given in Table II were active. Succinic semialdehyde was also slightly inhibitory when tested in amounts of 1–7 μmoles/incubation. It has been shown previously (Lindstedt *et al.*, 1968) that 2-ketoglutarate is consumed during the hydroxylation of γ-butyrobetaine. As shown in Figure 6 a stoichiometric relationship could be demonstrated between the formation of [^{14}C]CO_2 from [1-$^{14}C_1$]-2-ketoglutarate and the formation of carnitine from γ-butyrobetaine.

Effect of Ferrous Ion, Ascorbate, and Catalase. Figure 7 shows the formation of carnitine from γ-butyrobetaine in incubations with various concentrations of ferrous sulfate. The $K_{M,app}$ value for ferrous ion was about 0.06 mM in 15 mM phosphate buffer. As shown in Figure 8 the formation of carnitine was lower when the concentration of phosphate buffer was increased. The inhibition by phosphate could not be overcome by increasing the concentration of ferrous ion in the incubations. In extracts from *Pseudomonas* cells and with some partially purified enzyme preparations appreciable hydroxylase activity was observed also in the absence of added ferrous sulfate. Addition of 1,10-phenanthroline (10 μmoles) resulted in complete inhibition, which, however, was relieved by addition of ferrous sulfate (4 μmoles). None of the other salts in Table III could relieve the inhibition caused by 1,10-phenanthroline. Several metal ions were potent inhibitors; the most active ones were Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+} (Table III).

Figure 9 shows the formation of carnitine from γ-butyrobetaine with various concentrations of ascorbate in the incubation mixture. As shown in Table IV the reaction was stimulated also by other reductants, *i.e.*, by isoascorbate, tetrahydrofolate, 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine, and reduced 2,6-dichlorophenolindophenol. Figure 10 shows the stimulation of carnitine formation when catalase had been added to the incubations.

The yield of carnitine was low when the enzyme had been preincubated with ferrous ion and ascorbate (Table V). Preincubation with catalase, ferrous ion, and ascorbate resulted in only moderate inhibition.

Preincubation with sulfhydryl reagents resulted in appreciable inhibition of enzyme activity (Table VI). Most effective inhibitors were *p*-mercuriphenylsulfonate and *p*-mercuriben-

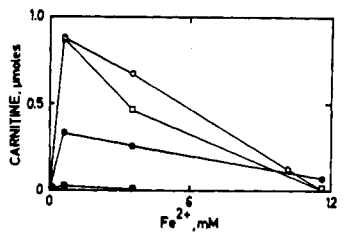

FIGURE 8: Formation of carnitine from γ-butyrobetaine with various concentrations of ferrous ion and phosphate buffer (pH 7.0). The enzyme (530 μg of protein, 188 U/g) was incubated with [*methyl*-$^{14}C_3$]-γ-butyrobetaine and cofactors as described under Assay for 90 min at 37°. Concentration of phosphate buffer: (○—○), 15 mM; (□—□), 20 mM; (●—●) 125 mM; (■—■), 320 mM.

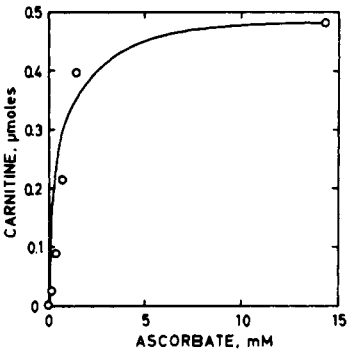

FIGURE 9: Effect of sodium ascorbate on the formation of carnitine from γ-butyrobetaine. The enzyme (55 μg of protein, 195 U/g) was incubated at 37° for 45 min with [*methyl*-$^{14}C_3$]-γ-butyrobetaine as described under Assay.

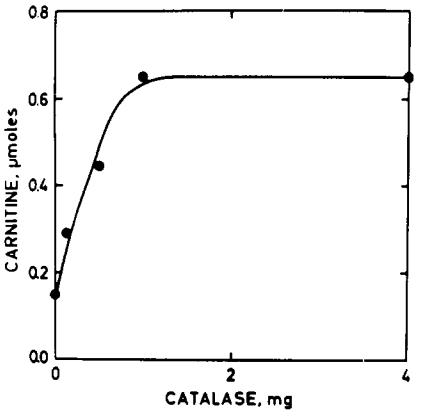

FIGURE 10: Effect of catalase on the formation of carnitine from γ-butyrobetaine. The enzyme (55 μg of protein, 263 U/g) was incubated at 37° for 45 min with [*methyl*-$^{14}C_3$]-γ-butyrobetaine as described under Assay.

zoate. There was no stimulation of the enzyme activity when 1–15 mM mercaptoethanol, dithiothreitol, or reduced glutathione had been added to the incubation mixture.

Discussion

Hydroxylases catalyze the insertion of one half of an oxygen molecule into the substrate. The other half is reduced, apparently in most cases to water. Cofactors are a reductant and an oxygen-activating agent, such as a metal ion, a cytochrome, a flavin nucleotide, or, possibly, a pteridine. The γ-butyrobetaine hydroxylase from *Pseudomonas* has not been purified to homogeneity. However, the present degree of purification has been sufficient to establish certain characteristic properties of the enzyme. Thus, ferrous ion is a cofactor as judged from the following findings: (a) inhibition of the activity in

TABLE IV: Effect on the Formation of Labeled Carbon Dioxide of Adding Various Reductants to Incubations with [*1*-$^{14}C_1$]-2-Ketoglutarate, the Enzyme Preparation (80 μg of Protein, 167 U/g), γ-Butyrobetaine, Ferrous Ion, and Catalase.[a]

Compound	Amt Added (μmoles)	[^{14}C]CO_2 (μmole)
None		0.03
Ascorbate	1	0.40
Ascorbate	10	0.60
Isoascorbate	10	0.58
Tetrahydrofolate	1	0.14
2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine	1	0.13
2,6-Dichlorophenolindophenol + glutathione	1 + 10	0.18
Glutathione	10	0.03
Mercaptoethanol	10	0.02
Dithiothreitol	10	0.03
Coenzyme A	5	0.05
NADPH	1	0.04
NADH	1	0.04

[a] The incubations (0.7 ml) were carried out for 45 min at 37°.

TABLE V: Effect of Preincubating the Enzyme with Ferrous Ion and Ascorbate with and without Catalase.[a]

Preincubation Conditions	[^{14}C]CO_2 (μmole)
Enzyme	0.40
Enzyme + ferrous ion + ascorbate	0.043
Enzyme + ferrous ion + ascorbate + catalase	0.35

[a] After preincubation of the partially purified enzyme preparation (80 μg of protein, 185 U/g) for 20 min at 37° in 15 mM potassium phosphate buffer (pH 7.0), the remaining components of the assay system were added (2-ketoglutarate-decarboxylase assay; see Methods). The incubation was then continued for 45 min.

TABLE VI: Inhibition of γ-Butyrobetaine Hydroxylation by Sulfhydryl Reagents.[a]

Compound	Amt (μmoles)	Inhibn (%)
p-Mercuriphenylsulfonate	0.001	0
p-Mercuriphenylsulfonate	0.01	100
p-Mercuribenzoate	0.001	33
p-Mercuribenzoate	0.01	100
Iodosobenzoate	0.001	0
Iodosobenzoate	0.01	51
Iodosobenzoate	0.1	99
Iodosobenzoate	1.0	100
Iodoacetate	0.1	44
Iodoacetate	1.0	50
Iodoacetate	10	100
N-Ethylmaleimide	0.1	27
N-Ethylmaleimide	1	47
N-Ethylmaleimide	10	100
Arsenite	0.1	24
Arsenite	1.0	29
Arsenite	10	68
Carbarsone	1	0
Carbarsone	10	60
Acetarsone	1	0
Acetarsone	10	73

[a] A partially purified enzyme preparation (44 μg of protein, 200 U/g) was preincubated in 0.3 ml of potassium phosphate buffer (pH 7.0) for 15 min at 37° with the respective compound. The remaining components of the Assay system (including [*methyl*-$^{14}C_3$]-γ-butyrobetaine) were then added and the incubation was carried out for 45 min at 37°.

crude extracts by 1,10-phenanthroline, and the reversal of this inhibition by ferrous ion, and (b) absence of enzyme activity of the partially purified enzyme in the absence of metal salts and the specific stimulation by ferrous ion with Michaelis–Menten kinetics. Present data also indicate a highly specific requirement for γ-butyrobetaine and 2-ketoglutarate whereas several reductants may replace ascorbate. The previously reported data which were obtained with the present enzyme preparation may be summarized as follows. (a) 2-Ketoglutarate is degraded to succinate in amounts stoichiometric with the formation of carnitine from γ-butyrobetaine; no degradation of 2-ketoglutarate can be observed in the absence of γ-butyrobetaine (Lindstedt *et al.*, 1968). (b) Succinic semialdehyde is apparently not metabolized under these conditions; it is not a free intermediate during 2-ketoglutarate decarboxylation nor can 2-ketoglutarate be replaced by succinic semialdehyde (Holme *et al.*, 1968). (c) After incubation with $[^{18}O]O_2$ as gas phase about 0.8 mole of oxygen atom is recovered per mole of succinate (Lindblad *et al.*, 1969). These findings indicate that 2-ketoglutarate acts as the reducing cofactor for the enzyme. Ascorbate and other reductants may possibly serve to keep ferrous ion and/or essential sulfhydryl groups in the reduced state. The results from preincubation experiments with and without catalase indicate that the latter protects the hydroxylase from inactivation.

The bacterial γ-butyrobetaine hydroxylase has similar cofactor requirements as some other hydroxylases. The specific requirement for ferrous ion and 2-ketoglutarate is found also for rat liver γ-butyrobetaine hydroxylase (Lindstedt, 1967a–c), collagen proline hydroxylase (Hutton *et al.*, 1966, 1967; Kivirikko and Prockop, 1967a,b), collagen lysine hydroxylase (Hausmann, 1967), and for thymine 7-hydroxylase and 5-hydroxymethyluracil 7-oxygenase from *Neurospora* (Abbott *et al.*, 1967, 1968). A stoichiometry between 2-ketoglutarate degradation and hydroxylation occurs also for the hydroxylation of collagen proline (Rhoads and Udenfriend, 1968) and for the hydroxylation of thymine to 5-hydroxymethyluracil (Holme *et al.*, 1970).

We recently suggested a reaction mechanism for 2-ketoglutarate-requiring hydroxylases with the formation of a peroxide between the substrate to be hydroxylated and 2-ketoglutarate. Decarboxylation would lead to the formation of hydroxylated product, succinate, and carbon dioxide (Holme *et al.*, 1968; Lindblad *et al.*, 1969). The initial step would be loss of a proton; in the case of γ-butyrobetaine the carbanion might be degraded by Hofmann elimination to trimethylamine and an unsaturated acid. No trimethylamine could be found, however, under the present conditions of incubation. The carbanion may either be stabilized by the enzyme or be only a short-lived one. In the case of γ-butyrobetaine hydroxylase from rat liver, the rate-limiting step appears to be the dissociation of the carbon–hydrogen bond (Lindstedt, 1967a). Attack on the carbanion by a positively charged ferrous ion–oxygen complex would lead to the formation of a hydroperoxide with subsequent attack on the carbonyl group of 2-ketoglutarate. The hydroperoxide might dehydrate to a ketone or to an epoxide after elimination of a proton from the hydroperoxide carbon atom or from the α-carbon atom, respectively. However, neither trimethylaminoacetone, the product of nonenzymic decarboxylation of 2-keto-4-trimethylaminobutyrate, nor any dihydroxybetaine from an epoxide could be detected.

References

Abbott, M. T., Dragila, T. A., and McCroskey, R. P. (1968), *Biochim. Biophys. Acta 169*, 1.

Abbott, M. T., Schandl, E. K., Lee, R. F., Parker, T. S., and Midgett, R. J. (1967), *Biochim. Biophys. Acta 132*, 525.

Carrière, E. (1922), *Ann. Chim. (Paris) 17*, 38.

Hausmann, E. (1967), *Biochim. Biophys. Acta 113*, 591.

Holme, E., Lindstedt, G., Lindstedt, S., and Tofft, M. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 29.

Holme, E., Lindstedt, G., Lindstedt, S., and Tofft, M. (1970), *Biochim. Biophys. Acta 212*, 50.

Hutton, J. J., Jr., Tappel, A. L., and Udenfriend, S. (1966), *Biochem. Biophys. Res. Commun. 24*, 179.

Hutton, J. J., Jr., Tappel, A. L., and Udenfriend, S. (1967), *Arch. Biochem. Biophys. 118*, 231.

Jacoby, W. B. (1962), *Methods Enzymol. 5*, 774.

Kivirikko, K. I., and Prockop, D. J. (1967a), *Arch. Biochem. Biophys. 118*, 611.

Kivirikko, K. I., and Prockop, D. J. (1967b), *Proc. Nat. Acad. Sci. U. S. 57*, 782.

Lindblad, B., Lindstedt, G., Lindstedt, S., and Tofft, M. (1969), *J. Amer. Chem. Soc. 91*, 4604.

Lindstedt, G. (1967a), *Biochemistry 6*, 1271.

Lindstedt, G. (1967b), *Biochim. Biophys. Acta 141*, 492.

Lindstedt, G. (1967c), Ph.D. Dissertation, Karolinska Institutet, Stockholm.

Lindstedt, G., and Lindstedt, S. (1961b), *Biochem. Biophys. Res. Commun 6*, 319.

Lindstedt, G., and Lindstedt, S. (1962), *Biochem. Biophys. Res. Commun. 7*, 394.

Lindstedt, G., and Lindstedt, S. (1965a), *J. Biol. Chem. 240*, 316.

Lindstedt, G., and Lindstedt, S. (1965b), *in* Recent Research on Carnitine, Wolf, G., Ed., Cambridge, Mass., Massachusetts Institute of Technology, p 11.

Lindstedt, G., Lindstedt, S., Midtvedt, T., and Tofft, M. (1967a), *Biochem. J. 103*, 19P.

Lindstedt, G., Lindstedt, S., Midtvedt, T., and Tofft, M. (1967b), *Biochemistry 6*, 1262.

Lindstedt, G., Lindstedt, S., Midtvedt, T., and Tofft, M. (1967c), Procedings of 4th Meeting of the Federation of European Biochemical Societies, Abstract No. 190, Oslo.

Lindstedt, G., Lindstedt, S., Olander, B., and Tofft, M. (1968), *Biochim. Biophys. Acta 158*, 503.

Lindstedt, G., and Tofft, M. (1969), *J. Chromatog. 39*, 78.

Lindstedt, S., and Lindstedt, G., (1961a), *Acta Chem. Scand. 15*, 701.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol. 5*, 3.

Prior Ferraz, F. G., and Relvâs, M. E. (1965), *Clin. Chim. Acta 11*, 234.

Rhoads, R. E., and Udenfriend, S. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 1473.

Vesterberg, O., and Svensson, H. (1966), *Acta Chem. Scand. 20*, 820.

Vesterberg, O., Wadström, T., Vesterberg, K., Svensson, H., and Malmgren, B. (1967), *Biochim. Biophys. Acta 133*, 435.