Hydroxylation of γ-Butyrobetaine to Carnitine in Rat Liver*

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ABSTRACT: γ-Butyrobetaine (4-trimethylaminobutyric acid) was hydroxylated to carnitine (3-hydroxy-4-trimethylaminobutyric acid) by a partially purified soluble protein fraction from rat liver. The reaction, which required molecular oxygen and ferrous ion, was stimulated by a combination of ascorbate and a reduced nicotinamide–adenine dinucleotide phosphate regenerating system, and also by catalase and by liver microsomes. Significant enrichment of tritium in γ-butyrobetaine was obtained when [carboxy-^{14}C-2,3-^{3}H]γ-butyrobetaine had been used as substrate, indicating a kinetic hydrogen isotope effect. Homogenates from rat muscle and kidney did not hydroxylate γ-butyrobetaine. Several amines related to γ-butyrobetaine were tested as substrates. 3-Trimethylaminopropionic acid and 4-dimethylaminobutyric acid were hydroxylated at a lower rate than γ-butyrobetaine, whereas 2-trimethylaminoglutaric acid and quaternary amines without a carboxyl group were not hydroxylated at all or at a very low rate.

The high capacity of the hydroxylating system and the observed substrate specificity may indicate that γ-butyrobetaine is an intermediate in carnitine biosynthesis.

The biosynthesis of carnitine[1] is still largely unknown. The low incorporation of radioactivity from methyl-labeled methionine into carnitine indicates that carnitine is formed in rats at a slow rate (Wolf and Berger, 1961; Bremer, 1961; Strength and Yu, 1962). The formation of carnitine from γ-butyrobetaine[1] in rats and mice has been demonstrated previously (Lindstedt and Lindstedt, 1961, 1965a; Bremer, 1962) and results from preliminary experiments with preparations from rat liver indicated an oxygenase mechanism for this conversion (Lindstedt and Lindstedt, 1962).

The present paper shows that the hydroxylation of γ-butyrobetaine is catalyzed by a soluble protein fraction from rat liver. The reaction requires molecular oxygen and ferrous ion and is stimulated by ascorbate and a NADPH-regenerating system. The high substrate specificity of the enzyme and the capacity for carnitine formation may indicate that γ-butyrobetaine is a physiologic precursor of carnitine.

Experimental Procedure

Materials. 3-Amino-1,2-propandiol was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.; 3-amino-2-hydroxypropionic acid, 4-methylaminobutyric acid, and adenosine 3′,5′-phosphate from Calbiochem, Lucerne, Switzerland; 1-aminobutane from Distillation Products Industries, Eastman Organic Chemicals Department, Rochester, N. Y.; 3-amino-1-propanol, 3-aminopropionic acid, 5-aminovaleric acid, DL-glutamic acid, 3-bromopropionic acid, crotonic acid methyl ester, nicotinamide, sodium DL-isocitrate, methyl iodide, 10% palladium on carbon, and human serum albumin from Fluka AG, Buchs, Switzerland; 4-amino-1-butanol and quinacrine hydrochloride from K & K Laboratories, Inc., Plainview, N. Y.; gold trichloride from Mallinckrodt Chemical Works, St. Louis, Mo.; anhydrous trimethylamine from The Matheson Co., Inc., East Rutherford, N. Y.; silica gel G from E. Merck AG, Darmstadt, West Germany; 2-amino-2-hydroxymethyl-1,3-propanediol (Trizma base) and aminopterin from Sigma Chemical Co., St. Louis, Mo.; sodium ascorbate and benzoyl peroxide from Dr. Theodor Schuchardt GmbH, München, West Germany; argon containing less than 5 ppm of oxygen from AGA AB, Lidingö, Sweden; FAD, FMN, NADPH, isocitrate dehydrogenase ($NADP^+$ specific), and catalase from C. F. Boehringer & Soehne, Mannheim, West Germany; Dowex AG 50 W-X8 (200–400 mesh (dry) and −400 mesh (dry), H^+ form), Retardion AG 11A8 (50–100 mesh), Duolite C-3 (200–400 mesh (wet), H^+ form), Dowex 1-X2 (200–400 mesh (dry), Cl^- form), and hydroxylapatite (HTP) from Bio-Rad Laboratories, Inc., Richmond, Calif.; Sephadex G-25 (coarse) from AB Pharmacia, Uppsala, Sweden; and DEAE-cellulose (0.7 mequiv/g) from Serva Entwicklungslabor, Heidelberg, West Germany.

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[1] Abbreviations and trivial names used: γ-butyrobetaine, 4-trimethylaminobutyric acid; carnitine, 3-hydroxy-4-trimethylaminobutyric acid; SKF 525-A, 2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride; Lilly 18947, 2,4-dichloro-6-phenylphenoxyethyldiethylamine; DPEA, 2,4-dichloro-6-phenylphenoxyethylamine hydrochloride; CIBA, 4885-Su, 1,2-bis(3-pyridyl)-2-methyl-1-propanone; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene; FAD, flavin-adenine dinucleotide; FMN, flavin mononucleotide; $NADP^+$ and NADPH, oxidized and reduced nicotinamide–adenine dinucleotide phosphates.

The following compounds were gifts: 2-trimethylaminoglutaric acid chloride from Dr. Jon Bremer, Rikshospitalet, Oslo, Norway; CIBA 4885-Su (Metopirone)[1] from CIBA Produkter AB, Stockholm, Sweden; DPEA[1] and Lilly 18947[1] from Eli Lilly, S. A., Geneva, Switzerland; and SKF 525-A[1] from Smith Kline and French Laboratories, Philadelphia, Pa.

Previously synthesized compounds are 4-dimethylaminobutyric acid hydrochloride, DL-4-dimethylamino-3-hydroxybutyric acid, 4-trimethylamino-1-butanol chloride, γ-butyrobetaine, carnitine, and 5-trimethylaminovaleric acid chloride (Lindstedt and Lindstedt, 1964, 1965a).

Chromatographic Procedures. Ion-exchange chromatography was performed with Dowex AG 50W-X8 (200–400 mesh (dry) and −400 mesh (dry), H^+ form) with dilute hydrochloric acid as eluent (Lindstedt and Lindstedt, 1965a,b). Carnitine and 2-hydroxy-3-trimethylaminopropionic acid were also chromatographed on Duolite C-3 (200–400 mesh (wet), H^+ form) (Lindstedt and Lindstedt, 1965a). Trimethylamino acids were separated from dimethylamino acids and other ions by chromatography on Retardion AG 11A8 (50–100 mesh (dry), self-adsorbed form) with water as eluent. The radioactive trimethylamines and trimethylamino alcohols were separated from the dimethylamino compounds by ion-exclusion chromatography on Dowex 1-X2 (200–400 mesh (dry), CO_3^{2-} form) with water as eluent. The chlorides of the trimethylammonium ions were obtained by filtering water solutions of the quaternary ammonium salts through columns of Dowex 1-X2 (200–400 mesh (dry), Cl^- form). Thin layer chromatography on silica gel G was carried out as described earlier (Lindstedt and Lindstedt, 1965b; Eneroth and Lindstedt, 1965). For paper chromatography Whatman No. 1 paper was used with ethanol–25% ammonia in water (4:1) as the mobile phase (Lindstedt and Lindstedt, 1965a).

Radioactivity Measurements. In the experiments with doubly labeled γ-butyrobetaine a scintillation counter was used (Tri-Carb Liquid Scintillation Spectrometer, Packard Instrument Co., Inc., La Grange, Ill.). Aliquots of 0.5-ml water solution were added to 16 ml of the following mixture: PPO[1] (10 g), dimethyl-POPOP[1] (0.3 g), toluene (1000 ml), and methyl Cellosolve (600 ml). The efficiency for ^{14}C and ^{3}H was 45 and 15%, respectively. The values obtained were corrected for quenching. In order to determine the amount of radioactive material in the fractions from the ion-exchange columns, 0.5-ml aliquots were evaporated in glass dishes under an infrared lamp. The radioactivity was determined in a methane-flow proportional counter (Frieseke & Hoepfner GmbH, Erlangen-Bruck, West Germany). The efficiency for ^{14}C was about 40%. Paper chromatograms were scanned in a paper strip flow counter with an efficiency for ^{14}C of about 10%.

Chemical Syntheses.[2] DIMETHYLAMINES. 1-Dimethylaminobutane, 3-dimethylamino-1-propanol, 4-dimethylamino-1-butanol, and 5-dimethylaminovaleric acid hydrochloride were prepared in 60–80% yield by heating the formol derivatives of the parent amines in dilute formic acid (Eschweiler-Clarke modification of the Leuckardt reaction (Moore, 1949)). After reflux overnight, an excess of concentrated hydrochloric acid was added to ensure complete removal of remaining formaldehyde and the reaction mixtures were taken to dryness. 5-Dimethylaminovaleric acid hydrochloride was crystallized from ethanol–diethyl ether, mp 163°; picrate,[2] mp 154° dec, lit. (Cornforth and Henry, 1952) mp 153–154°. The free bases of 1-dimethylaminobutane, 3-dimethylamino-1-propanol, and 4-dimethylamino-1-butanol were prepared by adding solid potassium hydroxide to the ice-cold water solutions of the hydrochlorides. The amines were dried with solid potassium hydroxide and distilled; 1-dimethylaminobutane (bp 94° (760 mm), lit. (Clarke *et al.*, 1933) bp 94° (760 mm), n_D^{23} 1.3970), 3-dimethylamino-1-propanol (bp 60–63° (19 mm), n_D^{23} 1.4346, lit. (Kaluszyner and Galun, 1961) n_D^{21} 1.4355), and 4-dimethylamino-1-butanol (bp 88–89° (11 mm), lit. (Ashley and Berg, 1959) bp 90° (12 mm), n_D^{23} 1.4395, lit. (Kaluszyner and Galun, 1961) n_D^{22} 1.4388, (Blicke and Biehl, 1957) n_D^{20} 1.4416). 3-Dimethylaminopropionic acid hydrochloride, DL-3-dimethylamino-2-hydroxypropionic acid hydrochloride, and L-2-dimethylaminoglutaric acid hydrochloride were obtained in about 90% yield by reducing the formol derivatives of the parent amino acids with hydrogen and 10% palladium on carbon (Bowman and Stroud, 1950). Two moles of hydrogen was taken up per mole of amino acid. After removal of the catalyst by filtration an excess of concentrated hydrochloric acid was added to the filtrates which were taken to dryness. The compounds were recrystallized from hot ethanol; 3-dimethylaminopropionic acid hydrochloride (mp 185°, lit. (Cornforth and Henry, 1952) mp 187–188°), DL-3-dimethylamino-2-hydroxypropionic acid hydrochloride (mp 143–144°, lit. (Mannich and Bauroth, 1922) mp 145–146°), and DL-2-dimethylaminoglutaric acid hydrochloride (mp 190°, lit. (Bowman and Stroud, 1950) mp 192°).

TRIMETHYLAMINES. 1-Trimethylaminobutane iodide and 3-trimethylamino-1-propanol iodide were obtained in 90% yield by slowly adding methyl iodide to methanol solutions of the corresponding dimethylamines. After standing overnight at room temperature, the solutions were taken to dryness. The compounds were recrystallized from ethanol–diethyl ether: 1-trimethylaminobutane iodide (mp 229° dec, lit. (Braun, 1911) mp 225–230° dec) and 3-trimethylamino-1-propanol iodide (mp 200°, lit. (Traynelis and Dadura, 1961) mp 201–202°). 3-Trimethylamino-1,2-propanediol chloride was prepared in about 80% yield by methylating the parent amino alcohol with methyl iodide and barium hydroxide

[2] The synthesized compounds were analyzed for purity by thin layer chromatography. The picrates were recrystallized from hot water and the tetrachloroaurates from hot 1 M hydrochloric acid. The melting points were determined on a heating block and are uncorrected. The microanalyses were performed by the Scandinavian Microanalytical Laboratory, Ballerup, Copenhagen, Denmark.

in water–methanol (1:4) at room temperature (Lindstedt and Lindstedt, 1965a). After 2 days the reaction mixture was taken to dryness and then dissolved in boiling water. A slight excess of hot 1 M sulfuric acid was added. The resulting suspension was cooled to 0° and filtered. The filtrate was passed through a column of Dowex 1 in the chloride form. The column was washed with water and the combined eluates were taken to dryness. 3-Trimethylamino-1,2-propandiol chloride was recrystallized from hot ethanol. The chloride was hygroscopic; tetrachloroaurate mp 154°, lit. (Hartmann, 1904) mp 155°. 3-Trimethylaminopropionic acid chloride was obtained in about 70% yield by passing dry trimethylamine into a solution of 3-bromopropionic acid in ice-cooled ethanol. When no more precipitation of 3-trimethylaminopropionate and trimethylammonium bromide was noted, the reaction mixture was allowed to stand at room temperature overnight and then taken to dryness. The residue was redissolved in water and filtered through a column of Retardion AG 11A8 to remove the trimethylammonium bromide. The column was washed with water, and the eluate was collected in fractions. An equivalent amount of hydrochloric acid was added to the fractions which contained trimethylaminopropionate. After evaporation of the solvent 3-trimethylaminopropionic acid chloride was recrystallized from hot ethanol, mp 215° dec, lit. (Willstätter, 1902) mp 195–196°. The tetrachloroaurate slowly decomposed above 180° and melted at 196° dec, lit. (Willstätter, 1902) mp 196–198° dec, "vorher erweichend."

Anal. Calcd for $C_6H_{14}NO_2 \cdot AuCl_4$ (471.0): C, 15.3; H, 3.00; N, 2.97; Au, 41.8. Found: C, 15.2; H, 3.03; N, 2.77; Au, 41.7.

2-Hydroxy-3-trimethylaminopropionic acid chloride was synthesized in about 35% yield by adding methyl iodide to a solution of 3-dimethylamino-2-hydroxypropionic acid hydrochloride in water–methanol (1:4). Barium hydroxide was added in portions during 20 hr to the reaction mixture, which was stirred at room temperature. It was then neutralized with hydrochloric acid and taken to dryness at a temperature below 50°. The residue was dissolved in a small amount of water and passed through a column of Retardion AG 11A8 which was eluted with water as described above. 2-Hydroxy-3-trimethylaminopropionic acid chloride was recrystallized from hot ethanol, mp 191–192° dec.

Anal. Calcd for $C_6H_{14}ClNO_3 \cdot H_2O$ (201.7): C, 35.83; H, 8.04; N, 6.96. Found: C, 35.86; H, 8.06; N, 6.90.

4-Bromocrotonic acid methyl ester was obtained from crotonic acid methyl ester by allylic bromination with *N*-bromosuccinimide and benzoyl peroxide in carbon tetrachloride (Schmid and Karrer, 1946). After removal of the solvent at atmospheric pressure, 4-bromocrotonic acid methyl ester was distilled at reduced pressure, bp 89–90.5° (16 mm), lit. (Owen and Sultanbawa, 1949) bp 78–82° (8 mm), n_D^{23} 1.5007, lit. (Owen and Sultanbawa, 1949) n_D^{20} 1.5021. 4-Trimethylaminocrotonic acid chloride was obtained by passing dry trimethylamine into a solution of 4-bromocrotonic acid methyl ester in ethanol at 0°. When the precipitation of the bromide of 4-trimethylaminocrotonic acid methyl ester had ceased the reaction mixture was taken to dryness. The residue was redissolved in 2 M hydrochloric acid and heated with reflux for 10 hr to hydrolyze the ester. The solvent was evaporated and the residue redissolved in water and filtered through a column of Retardion AG 11A8 as described above. 4-Trimethylaminocrotonic acid chloride was recrystallized from hot ethanol, mp 204–205° dec, lit. (Linneweh, 1928) mp 203–205° dec. The yield from 4-bromocrotonic acid methyl ester was about 40%. The catalytic hydrogenation of 4-trimethylaminocrotonic acid chloride at atmospheric pressure and room temperature was studied under three different conditions (*cf.* Linneweh, 1928).

(a) In 0.1 M hydrochloric acid and with 10% palladium on carbon the uptake of hydrogen was 0.13–0.15 cc/min per mg of catalyst. (b) At pH 7 and with 10% palladium on carbon the uptake was 0.08–0.10 cc of hydrogen/min per mg catalyst. (c) At pH 7 and with 5% palladium on barium sulfate the uptake was 0.008–0.010 cc of hydrogen/min per mg of catalyst. In all cases 1.5–1.8 moles of hydrogen was taken up/mole of 4-trimethylaminocrotonic acid. The reaction mixture after hydrogenation smelled of butyric acid and trimethylamine. No difference in hydrogenolysis of 4-trimethylaminocrotonic acid was found between the three conditions.

Labeled Compounds.[3] 4-Amino[carboxy-^{14}C]butyric acid was obtained from New England Nuclear Corp., Boston, Mass., and [^{14}C]methyl iodide and tritium gas from The Radiochemical Centre, Amersham, England. A reference sample of 2-[methyl-^{14}C]trimethylaminoglutaric acid was a gift from Dr. J. Bremer, Rikshospitalet, Oslo, Norway. 4-Dimethylamino[carboxy-^{14}C]butyric acid was synthesized as described previously (Lindstedt and Lindstedt, 1965a). The synthesis of DL-[methyl-^{14}C]carnitine is described by Lindstedt *et al.* (1967). The labeled compounds were purified by chromatography on columns of Dowex AG 50W-X8 (200–400 mesh (dry), H^+ form, 1.6 × 50 cm). Methyl-labeled quaternary ammonium compounds were first purified by ion-retardation or ion-exclusion chromatography as described under Chromatographic Procedures and then by chromatography on Dowex 50. [Carboxy-^{14}C]γ-butyrobetaine (2.5 mc/mmole) was obtained by stirring 4-amino[carboxy-^{14}C]butyric acid with a threefold excess of methyl iodide and barium hydroxide in methanol–water (4:1) for 2 days. The solvent was then evaporated and the residue was suspended in 0.1 M sodium hydroxide in water for 2 hr at room temperature in order to hydrolyze any methyl ester which might have been formed. The mixture was then acidified with 0.1 M hydrochloric acid and chromatographed on a column of Dowex 50 (Lindstedt and Lindstedt, 1965b). The yield of labeled γ-butyrobetaine was about 95%.

[3] The labeled compounds were purified by ion-exchange chromatography and purity was checked by chromatography on paper and silica gel together with reference compounds.

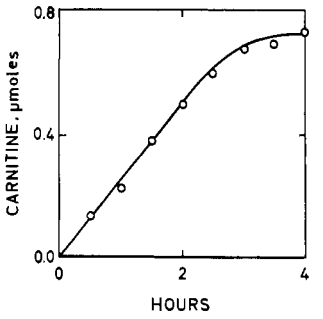

FIGURE 1: Formation of carnitine from γ-butyrobetaine after different times of incubation. The 100,000*g* supernatant fluid (35 mg of protein) was incubated at 37° with liver microsomes (5 mg of protein) and the complete system (see Assay).

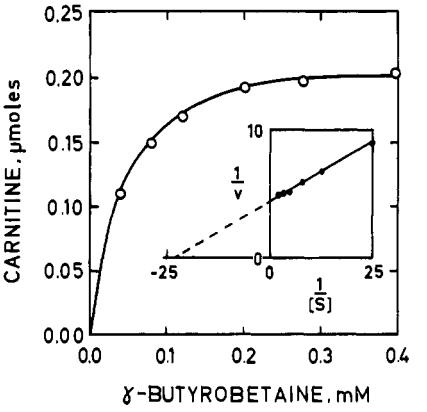

FIGURE 3: Formation of carnitine in incubations with different concentrations of γ-butyrobetaine. The hydroxylapatite fraction (10 mg of protein, see Enzyme Preparation) was incubated for 15 min at 37° with liver microsomes (3 mg of protein) and the complete system (see Assay). In the Lineweaver–Burk plot, *v* is the amount of carnitine formed during the incubation and [S] the initial concentration of γ-butyrobetaine.

4-[Methyl-^{14}C]methylaminobutyric acid (30 mc/mmole) was obtained by adding a tenfold excess of 4-aminobutyric acid and barium hydroxide to a solution of [^{14}C]methyl iodide in methanol–water (4:1) in a glass tube which had been cooled in liquid nitrogen. The tube was sealed in a flame and left for 3 days with occasional shaking. Labeled 4-methylaminobutyric acid was obtained in about 80% yield after ion-exchange chromatography. 3-[Methyl-^{14}C]trimethylaminopropionic acid (30 mc/mmole), 5-[methyl-^{14}C]trimethylaminovaleric acid (12 mc/mmole), 2-[methyl-^{14}C]-trimethylaminoglutaric acid (12 mc/mmole), 3-[methyl-^{14}C]trimethylamino-1-propanol (30 mc/mmole), 1-[methyl-^{14}C]trimethylaminobutane (30 mc/mmole), and 4-[methyl-^{14}C]trimethylamino-1-butanol (30 mc/mmole) were obtained by treating [^{14}C]methyl iodide with a tenfold excess of the parent dimethylamine as described above. Amines and amino alcohols were methylated in ethanol and amino acids in methanol–water (4:1) and barium hydroxide. The water solution of 3-dimethylaminopropionic acid was adjusted to pH 9 before the methylation. The isotopic yields were quantitative except for the labeled 3-trimethylaminopropionic and 2-trimethylaminoglutaric acids which both were obtained in about 50% yield. 4-[Methyl-^{14}C]trimethylaminocrotonic acid (15 mc/mmole) was obtained by dehydrating DL-[methyl-^{14}C]carnitine with hot sulfuric acid (Engeland, 1921) and was purified by Dowex 50 chromatography. About 50% of the isotope was recovered in material which was identical with the reference compound described above, as judged by thin layer and paper chromatography. [2,3-^{3}H]γ-Butyrobetaine (about 200 mc/mmole) was obtained by reducing 4-trimethylaminocrotonic acid with tritium gas in 0.1 M hydrochloric acid with 10% palladium on carbon as catalyst. After treatment overnight with tritium, the mixture was further reduced with hydrogen until no more gas was taken up. The product was purified by chromatography on Retardion AG 11A8 and Dowex 50. The distribution of tritium in the product was not studied. Water solutions of the labeled compounds were kept frozen at −20°.

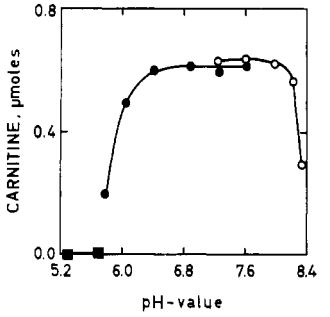

FIGURE 2: Formation of carnitine from γ-butyrobetaine at different pH values. The ammonium sulfate fraction (20 mg of protein, see Enzyme Preparation) was incubated for 2 hr at 37° with liver microsomes (5 mg of protein) and the complete system (see Assay). (■——■) Sodium acetate buffer. (●——●) Potassium phosphate buffer. (○——○) Tris-HCl buffer. The pH values were measured after 1 and 2 hr of incubation and had changed less than 0.1 unit between the measurements.

Tissue Homogenates. Rats of the Sprague-Dawley strain and of the Danish State Serum Institute Strain V.S. weighing 200–300 g were used. The animal was killed by a blow on the head and the respective tissue was excised and cooled in ice-cold 0.25 M sucrose. Homogenates (33%, wet weight per volume) were prepared in cold 0.25 M sucrose with 0.03 M nicotinamide in a Potter-Elvehjem homogenizer with a Teflon pestle. A tightly fitting pestle was used for liver homogenates. The tissue was cut in approximately 5-mm pieces and homogenized in the cold at 1400 rpm for

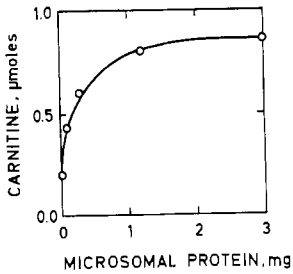

FIGURE 4: Effect of liver microsomes on the formation of carnitine from γ-butyrobetaine. The hydroxylapatite fraction (6 mg of protein, see Enzyme Preparation) was incubated for 2 hr at 37° with the complete system (see Assay) and different amounts of microsomes.

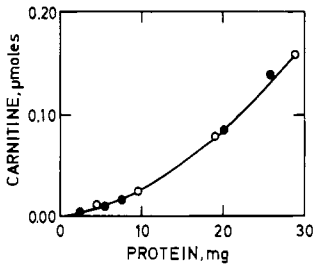

FIGURE 5: Formation of carnitine from γ-butyrobetaine in incubations with the 100,000*g* supernatant fraction of a rat liver homogenate (33% in 0.25 M sucrose with 0.03 M nicotinamide). (●——●) Protein fraction obtained after filtration through a column of Sephadex G-25 in 100 mM KCl and 10 mM Tris-HCl buffer at pH 7.8. (○——○) Untreated supernatant fluid. Liver microsomes (4 mg of protein) and the complete system *minus* catalase (see Assay) were added to the incubations, which were carried out for 2 hr at 37°.

about 45 sec. Heavy particles were spun down at 700*g* for 10 min, mitochondria at 15,000*g* for 20 min, and microsomes at 100,000*g* for 60 min. The microsomes were washed three times, resuspended in 0.25 M sucrose with 0.03 M nicotinamide, and kept frozen at −20°. For the enzyme purification, the supernatant fraction after centrifugation at 70,000*g* for 60 min was used.

Enzyme Preparation. The purification of the soluble protein fraction was carried out at 4° as follows. A saturated ammonium sulfate solution, which had been brought to pH 7 with aqueous ammonia, was added during 10 min with stirring to the 70,000*g* supernatant fraction of homogenates from the livers of 25 rats. The suspension was stirred for another 30 min. The first fraction was collected at 40% saturation and the second at 70% saturation. The pH value was usually 6.7–6.8 in the homogenate, 6.4–6.5 in the first ammonium sulfate supernatant, and 6.6 in the second supernatant. The sediment at 70% saturation was dissolved in about 60 ml of 0.1 M Tris-HCl buffer (pH 7.8) containing 0.2 M potassium chloride and was then desalted by filtration through a 500-ml column of Sephadex G-25 (coarse) which was eluted with 10 mM Tris-HCl buffer (pH 7.8). The protein fraction from this column will be called the ammonium sulfate fraction. The protein solution was put onto a 50-g column of DEAE-cellulose (130–170 ml) which had been equilibrated with 10 mM Tris-HCl buffer (pH 7.8). The column was eluted stepwise with potassium chloride solutions containing 10 mM Tris-HCl buffer (pH 7.8). Three bed volumes of each solution were used and the concentrations of potassium chloride were 0, 20, 50, 75, and 200 mM. The last fraction was then put onto a 25-g column of hydroxylapatite (about 60 ml), which had been equilibrated with 10 mM Tris-HCl buffer (pH 7.8). The column was eluted first with three bed volumes of 10 mM phosphate buffer (pH 7.8) and then with three bed volumes of 50 mM phosphate buffer (pH 7.8). The last fraction was dialyzed against a saturated ammonium sulfate solution at pH 7.0 overnight. The precipitate was collected by centrifugation, dissolved in 5–10 ml of 10 mM Tris-HCl buffer (pH 7.8), and then desalted by filtration through a 100-ml column of Sephadex G-25 (coarse). The protein fraction thus obtained will be referred to as the hydroxylapatite fraction.

Ordinary distilled water which had been further purified by passage through a mixed-bed ion-exchange resin followed by glass distillation was used throughout. Occasionally, tests for copper and nickel ions were performed with diethyl dithiocarbamate and dimethylglyoxime, respectively. The detection limit was less than 0.1 μM of both ions. The tests gave consistently negative results.

Assay. The hydroxylating activity was assayed by incubating radioactive substrate with enzyme and cofactors. The deproteinized incubation mixtures were then fractionated on Dowex 50 (see below) and the amount of radioactive material in substrate and product was determined. The composition of the incubation mixture was as follows (this will be referred to as the complete system). The soluble protein fraction (2 ml) (5–80 mg of protein), 1 ml of 0.10 M Tris-HCl buffer (pH 7.8) (except when otherwise indicated), 100 μmoles of potassium chloride, 230 μmoles of nicotinamide, 6.5 μmoles of magnesium chloride, 20 μmoles of sodium DL-isocitrate, 0.1 mg of isocitrate dehydrogenase, 4 mg of catalase, substrate (about 1 μc; 4 μmoles of γ-butyrobetaine), 1 μmole of NADPH, 50 μmoles of sodium ascorbate, and 1 or 4 μmoles of ferrous sulfate depending on if Tris-HCl or potassium phosphate buffer was used. If not indicated otherwise, 1 ml of 0.25 M sucrose with 0.03 M nicotinamide was also added. In incubations with microsomes, 1 ml of microsomal suspension (2–5 mg of protein) was added instead of this. The final volume was 4.8–5.0 ml. All solutions were cooled in ice, and the solutions of NADPH, sodium ascorbate, and ferrous sulfate were made immediately before incubation. Microsome

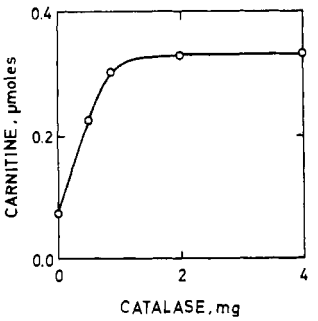

FIGURE 6: Effect of catalase on the formation of carnitine from γ-butyrobetaine. The hydroxylapatite fraction (2.2 mg of protein, see Enzyme Preparation) was incubated for 2 hr at 37° with liver microsomes (3 mg of protein), the complete system (see Assay), and different amounts of catalase.

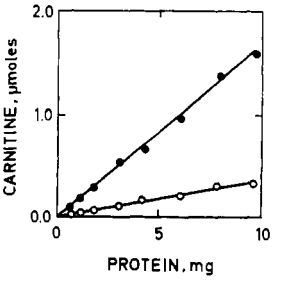

FIGURE 7: Formation of carnitine from γ-butyrobetaine in incubations with different amounts of the hydroxylapatite fraction (see Enzyme Preparation) with (●——●) and without (○——○) liver microsomes (3 mg of protein). The complete system, including catalase (see Assay), was added to the incubations, which were carried out for 2 hr at 37°.

suspensions could be kept frozen for 2 weeks without loss in activity. All incubations were carried out in duplicate in stoppered 25-ml erlenmeyer flasks by shaking in a water bath at 37° for 2 hr and were terminated by adding an equal volume of 10% trichloroacetic acid in water. The mixtures were kept at 4° overnight, and the precipitated proteins were then spun down. The supernatant fractions were fractionated on columns of Dowex AG 50W-X8 (−400 mesh, 1.2 × 12 cm) which were eluted with 1.0 M hydrochloric acid. The fraction volumes were 40, 40, 10, 10, 10, and 125 ml. Carnitine was recovered in fraction 2 or fractions 2 and 3. γ-Butyrobetaine was usually recovered in fraction 6. The amount of radioactive material in the fractions was determined with a flow counter. In the experiments with doubly labeled γ-butyrobetaine fractions 6 were taken to dryness and redissolved in 5 ml of water, and 0.1-ml aliquots were then counted in a liquid scintillation counter. Incubations with other amines were fractionated on columns of Dowex AG 50W-X8 (200–400 mesh, 1.6 × 50 cm) which were eluted with 1.0 to 6 M hydrochloric acid. The reference compounds which had been added before chromatography were located by thin layer chromatography and the amount of radioactive material was determined.

Incubations with argon as gas phase were carried out in the following way. The complete system except for γ-butyrobetaine was added to the ice-cold flasks which then were evacuated to 10 torr and refilled with argon five times. The evacuation and refilling with argon was repeated another five times after the addition of γ-butyrobetaine. The incubations were carried out for 45 min with a slow stream of moistened argon through the flasks. The volumes did not change during the incubations. Control incubations were evacuated in the same way, but air was introduced before placing the flasks in the water bath.

Protein was determined as described by Lowry *et al.* (1951) with human serum albumin as standard. Glucose 6-phosphatase was used as an index of microsomes and was determined according to Harper (1965).

Results

Identification of Reaction Product. A Dowex 50 chromatogram from an incubation with [methyl-^{14}C]-γ-butyrobetaine, the 15,000*g* supernatant fraction of a rat liver homogenate, and the complete system (see Assay) showed two peaks with radioactive material which were eluted together with nonradioactive carnitine and γ-butyrobetaine which had been added after the incubation. The radioactive material in the first peak was also eluted, together with carnitine when chromatographed on a Duolite C-3 column. After oxidation with chromic acid (Wolf and Berger, 1961) the radioactive material migrated together with trimethylaminoacetone on Dowex 50 and on paper. Crystallizations together with the gold salt of trimethylaminoacetone chloride resulted in crystals with unchanged specific radioactivity. No degradation of methyl-labeled DL-carnitine was observed under the same conditions of incubation.

Effect of Incubation Time, pH, and Substrate Concentration. Figure 1 shows the formation of carnitine after different times of incubation. The rate of carnitine formation was the same during the first 3 hr of incubation and then declined. An incubation time of 2 hr was generally used in following experiments. The hydroxylating activity was nearly the same at pH values between 6.4 and 8.0 (Figure 2). Figure 3 shows the formation of carnitine at different concentrations of γ-butyrobetaine. In these experiments, the incubations were carried out for 15 min. A Lineweaver–Burk plot (Figure 3) gives a K_m value of about 5×10^{-5} M. No substrate inhibition was noted in 2 mM γ-butyrobetaine. DL-Carnitine in 2 mM concentration did not inhibit the hydroxylation of γ-butyrobetaine.

Subcellular Distribution of Hydroxylating Activity. When the fractions from differential centrifugations of rat liver homogenates were incubated with the complete system (see Assay) the enzymic activity was found in the 100,000*g* supernatant fraction. Particles alone were inactive. With 2-ml aliquots of the super-

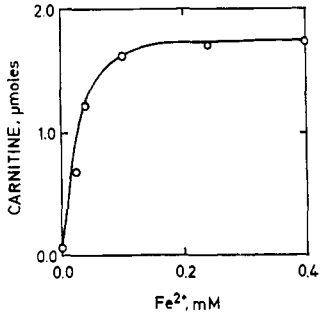

FIGURE 8: Effect of ferrous ion on the formation of carnitine from γ-butyrobetaine. The hydroxylapatite fraction (10 mg of protein, see Enzyme Preparation) was incubated for 2 hr at 37° with liver microsomes (3 mg of protein), the complete system (see Assay), and different amounts of ferrous sulfate.

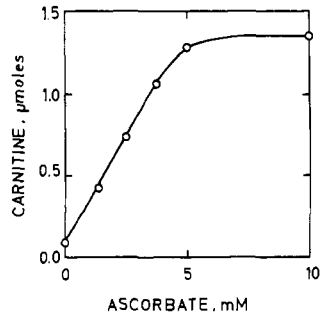

FIGURE 9: Effect of sodium ascorbate on the formation of carnitine from γ-butyrobetaine in incubations with the ammonium sulfate fraction (40 mg of protein, see Enzyme Preparation), liver microsomes (5 mg of protein), the complete system (see Assay), and different amounts of sodium ascorbate. The incubations were carried out for 2 hr at 37°.

natant fluid the addition of suspensions of mitochondria or microsomes did not affect carnitine formation. However, the addition of small amounts of microsomes to the hydroxylapatite fraction (see Enzyme Preparation) resulted in approximately fourfold stimulation (Figure 4). Determinations of glucose 6-phosphatase activity showed that 5–10% of the microsomes present in the 15,000*g* supernatant fraction of the crude homogenate were recovered in the 100,000*g* supernatant fluid. No glucose 6-phosphatase activity could be found in the hydroxylapatite fraction.

Figure 5 shows the formation of carnitine in incubations with different amounts of the 100,000*g* supernatant fraction. The incubations were carried out both directly with the supernatant fluid and with the protein fraction which was obtained after gel filtration through Sephadex G-25. In both cases, a nonlinear relationship was found between the hydroxylating activity and the concentration of the soluble protein fraction from the homogenate.

Effect of Catalase. Figure 6 shows that catalase stimulated the formation of carnitine. When an excess of catalase was added to the incubations a linear relationship was obtained between the hydroxylating activity and the concentration of the purified protein fraction (Figure 7). This result was obtained both in the absence of microsomes and when microsomes had been added.

Purification of Soluble Protein. Table I shows the results of the purification of the soluble protein fraction. When the hydroxylapatite fraction had been heated at 60° for 5 min with argon as gas phase only about 6% of the hydroxylating activity was recovered. Heating for 15 min resulted in complete inactivation. γ-Butyrobetaine (1 mM) did not significantly protect the enzymic activity from heat inactivation. Microsomes no longer stimulated carnitine formation when they had been heated at 100° for 10 min before the incubations.

Cofactors. Figures 8 and 9 show that very low hydroxylating activity was obtained when ferrous ion or ascorbate had been omitted from the complete system. Previously (Lindstedt, 1967) evidence was obtained for the formation of an inhibitor when ferrous ion was present in higher concentrations than 0.04 mM. However, as shown by Figure 8, no inhibition by ferrous ion was observed when an excess of catalase had been added to the incubations. The stimulation by ascorbate was the same at pH 6.4 in potassium phosphate buffer as at pH 7.8 in Tris-HCl buffer. Figure 10 shows that the NADPH-regenerating system stimulated carnitine formation about sixfold, but an absolute requirement for NADPH was not demonstrated.

In preliminary experiments with the 20,000*g* supernatant fraction of rat liver homogenates nicotinamide

TABLE I: Purification of Soluble Protein Fraction.[a]

Protein Fraction	Protein (mg)	Sp Act. (mU[b]/mg)	Total Act. (U)	Recov (%)
70,000*g* supernatant fluid	6100	0.12	0.71	
Ammonium sulfate fraction (40–70%)	2400	0.28	0.66	93
DEAE-fraction	810	0.80	0.65	92
Hydroxylapatite fraction	220	1.4	0.32	44

[a] See Enzyme Preparation for details. The hydroxylating activity was assayed in Tris-HCl buffer (pH 7.8) with microsomes (3–5 mg of protein) and the complete system (see Assay). The incubations were carried out for 2 hr at 37°. [b] One unit (U) = 1 μmole of carnitine formed/min of incubation.

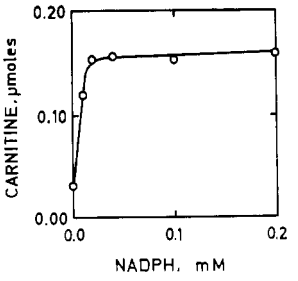

FIGURE 10: Effect of NADPH on the formation of carnitine from γ-butyrobetaine in incubations with the hydroxylapatite fraction (4.2 mg of protein, see Enzyme Preparation), the complete system (see Assay), and different amounts of NADPH. No microsomes were added. The incubations were carried out for 2 hr at 37°.

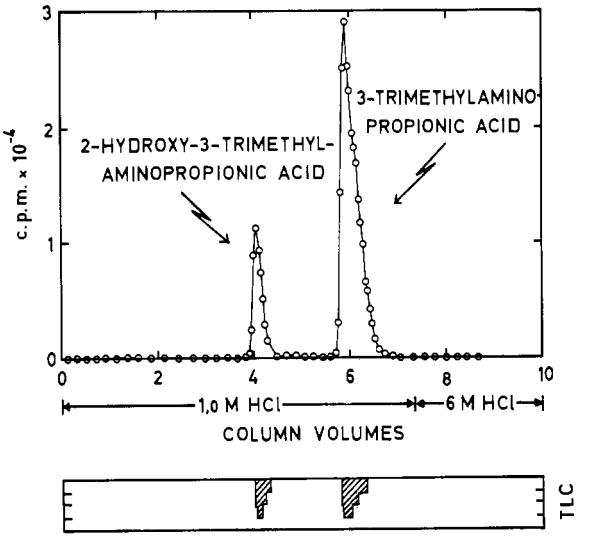

FIGURE 11: Hydroxylation of 3-[methyl-^{14}C]trimethylaminopropionic acid (6 μc, 0.4 μmole) in an incubation for 2 hr at 37° with the ammonium sulfate fraction (50 mg of protein, see Enzyme Preparation), liver microsomes (5 mg of protein) and the complete system (see Assay) with Tris-HCl buffer (pH 7.8). After deproteinization with trichloroacetic acid, 15 mg of 2-hydroxy-3-trimethylaminopropionic acid chloride and 15 mg of 3-trimethylaminopropionic acid chloride were added to the incubation mixtures, which were fractionated on a column of Dowex 50 (diameter 1.6 cm; height 50 cm). The *upper* graph shows radioactivity and the *lower* graph shows estimates of relative concentrations of betaines in the effluent from the ion-exchange column. Silica gel G was used for thin layer chromatography (tlc), with HCl–acetone–methanol (10:90:10, v/v) as mobile phase.

stimulated the formation of carnitine. An amount of 230 μmoles of nicotinamide was, therefore, routinely added to the incubations. Adenosine 3′,5′-phosphate, which stimulates the steroid C-11β and C-18 hydroxylases (Creange and Roberts, 1965), did not affect the hydroxylation of γ-butyrobetaine in 1 mM concentration.

Oxygen Requirement. When the incubations were carried out with argon as gas phase the amount of carnitine formed was less than 1% of that in the controls incubated in air (Table II).

Isotope Effect in the Hydroxylation of γ-Butyrobetaine. Table III shows that there was a significant isotope effect when [carboxy-^{14}C]γ-butyrobetaine was incubated together with γ-butyrobetaine prepared by reducing 4-trimethylaminocrotonic acid with tritium gas. In contrast, no isotope effect was noted when the substrate was [carboxy-^{14}C-methyl-^{3}H]γ-butyrobetaine.

Inhibitors. SKF 525 A,[1] DPEA,[1] and Lilly 18947,[1] which inhibit microsomal drug hydroxylations (for a review, see Gillette, 1963), and CIBA 4885-Su,[1] which inhibits some steroid hydroxylations (Liddle *et al.*, 1958), did not affect the hydroxylation of γ-butyrobetaine (Table IV). Aminopterin, which inhibits the hydroxylation of phenylalanine (Kaufman, 1963), had no effect on carnitine formation. Quinacrine and FMN, in high concentrations, inhibited the formation of carnitine. The amount of carnitine formed was only 50% of that in the control when 15 μmoles of hydrogen peroxide had been added every tenth min during incubation with the hydroxylapatite fraction. No inhibition was noted when the enzyme had been treated with 200 μmoles of hydrogen peroxide at 4° for 10 min before the incubation.

TABLE II: Requirement for Molecular Oxygen in the Hydroxylation of γ-Butyrobetaine.[a]

Conditions	Carnitine (μmole)
Incubated in air	0.58
Flushed with argon, incubated in air	0.56
Flushed with argon, incubated in argon	0.004

[a] The ammonium sulfate fraction (50 mg of protein, see Enzyme Preparation) was incubated in Tris-HCl buffer (pH 7.8) for 45 min at 37° with liver microsomes (5 mg of protein) and the complete system. The procedure is described under Assay.

Inability of Muscle and Kidney to Form Carnitine from γ-Butyrobetaine. The 15,000*g* supernatant fractions of muscle and kidney homogenates were incubated with and without liver microsomes. No carnitine formation was observed under conditions where 2 μmoles of carnitine was formed in liver homogenates. Microsomes from kidney and muscle did not stimulate the formation of carnitine in incubations with the hydroxylapatite fraction from liver, whereas the same amount

TABLE III: Hydrogen Isotope Effect in the Hydroxylation of γ-Butyrobetaine to Carnitine.[a]

Substrate	^3H:^{14}C Ratio Before Incubn	^3H:^{14}C Ratio After Incubn	Conversion of ^{14}C Labeled Substrate (%)
[Carboxy-^{14}C-methyl-^{3}H]γ-butyrobetaine	1.5	1.5	90
[Carboxy-^{14}C-2,3-^{3}H]-γ-butyrobetaine	1.0	6.7	92

[a] γ-Butyrobetaine (0.4 μmole, 1 μc of ^{14}C) was incubated for 0 or 6 hr at 37° with the ammonium sulfate fraction (60 mg of protein, see Enzyme Preparation), microsomes (5 mg of protein), and the complete system (see Assay) in Tris-HCl buffer (pH 7.8). γ-Butyrobetaine was isolated from the incubation mixtures by chromatography on Dowex 50.

of microsomal protein from liver doubled carnitine formation.

Substrate Specificity. Table V shows the results of experiments in which various amines related to γ-butyrobetaine were incubated under conditions which were optimal for carnitine formation. Amines without a carboxyl group were not hydroxylated to any appreciable extent. The introduction of a carboxyl group on the γ-carbon atom in γ-butyrobetaine resulted in a substrate which was not attacked by the hydroxylase. 4-Dimethylamino-3-hydroxybutyric acid was identified as a metabolite of 4-dimethylaminobutyric acid by ion-exchange chromatography on Dowex 50 and Duolite C-3 and by paper chromatography. Figure 11 shows a Dowex 50 chromatogram from an incubation with methyl-labeled 3-trimethylaminopropionic acid. The first peak of radioactive material migrated together with DL-2-hydroxy-3-trimethylaminopropionic acid, and similar results were obtained by chromatography on Duolite C-3 and on paper.

Discussion

The requirement for molecular oxygen and reducing agents classifies the hydroxylation of γ-butyrobetaine in the rat as a monooxygenase reaction (Hayaishi, 1964) or a mixed-function oxidation (Mason, 1957), but experiments with isotopic oxygen are required before this can be definitely established. The rate-limiting reaction is the dissociation of carbon and hydrogen, as indicated by the kinetic hydrogen isotope effect (*cf.* Samuelsson, 1960; Wood and Ingraham, 1962; Prockop *et al.*, 1964; Pomerantz, 1966). The physiologic electron donor is unknown at present time, but reduced pyridine nucleotides (for reviews, see Hayaishi, 1962; Mason, 1965), flavin nucleotides (Sutton, 1955; Conrad *et al.*, 1961; Katagiri *et al.* 1962; Yano *et al.*, 1966), pteridines (Kaufman, 1958), and porphyrins (Mason *et al.*, 1965; Estabrook *et al.*, 1963; Omura *et al.*, 1965), as well as enediols (Levin *et al.*, 1960; Peterkofsky and Udenfriend, 1965; Prockop and Juva, 1965) may be considered. High hydroxylating activity (see below) was obtained with a combination of ascorbate and the NADPH-regenerating

TABLE IV: Inhibitors of γ-Butyrobetaine Hydroxylation.

Enzyme	Compound(s)	Amt (μmoles)	Inhibn (%)
15,000*g* supernatant[a]	Aminopterin	1, 10	0, 0
Hydroxylapatite fraction[a]	Aminopterin	1, 10	0, 0
	FAD	0.1, 10	0, 0
	FMN	0.1, 10	0, 50
Hydroxylapatite fraction[a]	Quinacrine	2, 20	70, 95
	Quinacrine + EDTA	20 2	95
	SKF 525-A	10	0
15,000*g* supernatant[b]	DPEA	10	0
	Lilly 18947	10	0
	CIBA 4885-Su	10	0
Ammonium sulfate fraction	Fumarate	20	30

[a] The inhibitors were preincubated with the enzyme in the cold for 10 min. The incubations were then carried out for 2 hr at 37° as described under Assay. [b] In these experiments ferrous sulfate, sodium ascorbate, NADPH, nicotinamide, potassium chloride, and [methyl-^{14}C]γ-butyrobetaine were added after preincubating the enzyme with the inhibitors for 10 min in the cold.

TABLE V: Substrate Specificity of the Hydroxylating System.[a]

Substrate	Amt, μmoles (μc)	Conversion (%)
[Carboxy-^{14}C]γ-butyrobetaine	4.0 (0.5)	46
4-[Methyl-^{14}C]trimethylaminocrotonic acid	0.5 (7.5)	<0.1
4-Dimethylamino[carboxy-^{14}C]butyric acid	4.0 (1.5)	1.0
4-[Methyl-^{14}C]methylaminobutyric acid	0.2 (6.0)	<0.1
4-Amino[carboxy-^{14}C]butyric acid	1 (3)	0.1
DL-2-[Methyl-^{14}C]trimethylaminoglutaric acid	0.3 (3.6)	<0.1
4-[Methyl-^{14}C]trimethylamino-1-butanol	0.1 (3)	0.1
1-[Methyl-^{14}C]trimethylaminobutane	0.2 (6)	<0.1
3-[Methyl-^{14}C]trimethylaminopropionic acid	4 (6)	10.5
3-[Methyl-^{14}C]trimethylamino-1-propanol	0.2 (6)	<0.1
5-[Methyl-^{14}C]trimethylaminovaleric acid	0.5 (6)	<0.1

[a] Incubations with the ammonium sulfate fraction (60 mg of protein, see Enzyme Preparation), microsomes (5 mg of protein), the complete system (see Assay) in Tris-HCl buffer (pH 7.8), and the respective substrate were carried out for 2 hr at 37°. The deproteinized incubation mixtures were analyzed by chromatography on Dowex 50 (*cf.* Figure 11).

system, but their specificity as electron donors has not been studied so far. A requirement for a pteridine appears less probable as high concentrations of aminopterin did not affect the hydroxylation of γ-butyrobetaine (*cf.* Kaufman, 1963). High concentrations of FMN and quinacrine were inhibitory, but the mechanism for this inhibition is unknown at present (*cf.* Webb, 1966a).

Metal ions, such as iron and copper ions, have been implicated as catalysts in the activation of oxygen in oxygenase reactions (for reviews, see Mason, 1957; Hayaishi, 1962, 1964; Ingraham, 1966). A number of dioxygenases have been shown to contain ferrous ion or require ferrous ion for activity (for reviews, see Mehler, 1962; Hayaishi, 1964; Crandall, 1965). The presence of copper ion in some monooxygenases has been demonstrated (Friedman and Kaufman, 1965; Blumberg *et al.*, 1965; Keilin and Mann, 1938; Kubowitz, 1938), and ferrous ion stimulates several hydroxylase reactions (Conrad *et al.*, 1961; Gholson *et al.*, 1963; Kusunose *et al.*, 1964; Nagatsu *et al.*, 1964; Guroff and Ito, 1965; Lovenberg *et al.*, 1965; Peterkofsky and Udenfriend, 1965; Prockop and Juva, 1965). The hydroxylation of γ-butyrobetaine in rat liver homogenates is inhibited by several metal-complexing agents (Lindstedt, 1967). The results from these studies as well as the observed stimulation by ferrous ion in the present study indicate a requirement for ferrous ion in γ-butyrobetaine hydroxylation. Interestingly, several ions of elements in the first transition series were effective inhibitors (Lindstedt, 1967). This might indicate that the enzyme will bind several transition-metal ions, but that only ferrous ion is catalytically active. Studies on the kinetics of the inhibition by metal ions, however, are necessary to solve this problem.

Most hydroxylases in animal tissue are particle bound. The hydroxylation of γ-butyrobetaine is catalyzed by a soluble enzyme, which can be purified by ammonium sulfate precipitation and reversible adsorption to DEAE-cellulose and hydroxylapatite. However, the mechanism for the stimulation by microsomes is obscure at present. Tryptophan pyrrolase has recently been shown to be stimulated by particles by an unknown mechanism (Greengard *et al.*, 1966).

The relation between the hydroxylating activity and the protein concentration in the incubations with the 1000,000*g* supernatant fluid (Figure 5) suggested the requirement for more than one high-particle-weight component in the soluble fraction of the homogenate. One component might be catalase or some other hydrogen peroxide destroying system (Mills, 1960), as a linear relationship was noted when large amounts of catalase had been added to the incubations. Catalase activity has been found both in the supernatant and particulate fractions of liver homogenates (Greenfield and Price, 1956; Radhakrishnan and Sarma, 1964; Higashi and Shibata, 1965), but it is apparently not yet established if the soluble catalase is released from the microbodies when the tissue is homogenized (De Duve and Baudhuin, 1966). Previously, evidence was obtained for the formation of an inhibitor at high concentrations of ferrous ion and ascorbate (Lindstedt, 1967). Hydrogen peroxide is formed in the iron-catalyzed autoxidation of ascorbate (Euler *et al.*, 1933; Tanner, 1958; Flesch, 1960) and may inactivate several biologically active proteins (Webb, 1966b; Dedman *et al.*, 1961; Neumann *et al.*, 1962; Weinryb, 1966). In our system, only about 50% inhibition was obtained when large amounts of hydrogen peroxide were added to the incubations. Furthermore, as shown in Figure 6, high concentrations of catalase were needed for optimal effect. These findings may indicate a role of catalase other than that of protecting the enzyme from hydrogen peroxide. However, to our knowledge, catalase has never been found to participate in monooxygenase reactions (*cf.* Staudinger and Ullrich, 1964). Dopamine β-oxidase, a copper-protein-requiring ascorbate, is protected from inactivation by catalase (Levin and Kaufman, 1961). Possibly, hydrogen peroxide formed in ascorbate autoxidation close to enzyme-bound iron is a more potent inhibitor than exogenous hydrogen peroxide.

The enzyme had a high degree of specificity. The lower homolog of γ-butyrobetaine was hydroxylated at about one-fifth the rate of γ-butyrobetaine, whereas no hydroxylation of the higher homolog or of amines without a carboxyl group could be demonstrated. Small amounts of 4-dimethylamino-3-hydroxybutyric acid were formed from 4-dimethylaminobutyric acid as has previously been shown in *in vivo* experiments (Bremer, 1962; Lindstedt and Lindstedt, 1965a). The specificity of the enzymic reaction may indicate that γ-butyrobetaine is a natural compound, although attempts to demonstrate its biosynthesis have met with limited success (Bremer, 1962; Lindstedt and Lindstedt, 1965a; *cf.* Hosein *et al.*, 1962). However, small amounts of γ-butyrobetaine have been found in the urine of dog and man (Broekhuysen and Deltour, 1961; *cf.* Takeda, 1910; Reinwein and Thielmann, 1924). Carnitine has been reported to occur in several microorganisms (Fraenkel and Friedman, 1957; Strack *et al.*, 1960) and γ-butyrobetaine is hydroxylated in a newly isolated *Pseudomonas* strain, apparently by a similar mechanism as in rat liver (Lindstedt *et al.*, 1967).

In mammals carnitine is found mainly in muscle. As homogenates of rat muscle and kidney did not hydroxylate γ-butyrobetaine, carnitine is probably formed in the liver and then transported to the muscle (*cf.* Strength *et al.*, 1965). About 1 μmole of carnitine was formed/g of rat liver per hr under optimal conditions for γ-butyrobetaine hydroxylation *in vitro*. From data given by Khairallah and Mehlman (1965), the daily formation of carnitine would be of the order of 30 μmoles in a 200-g rat. Thus, the capacity of the liver γ-butyrobetaine hydroxylating system is sufficient for the estimated rate of carnitine synthesis.

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