Cofactor Requirements of \( \gamma \)-Butyrobetaine Hydroxylase from Rat Liver*

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SUMMARY

The hydroxylation of \( \gamma \)-butyrobetaine (4-trimethylaminobutyrate) to carnitine (3-hydroxy-4-trimethylaminobutyrate) is catalyzed by a soluble enzyme from rat liver which has been partially purified. The enzyme which previously has been shown to require molecular oxygen and ferrous ion has a specific requirement for 2-ketoglutarate. Several reductants stimulate the formation of hydroxylated product; the most active ones are ascorbate and isoascorbate, whereas reduced 2,6-dichlorophenolindophenol and 2-amino-5,6-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine are less active. The previously observed stimulation both by NADPH, isocitrate, isocitrate dehydrogenase (EC 1.1.1.42), and by micromoles has been found to be related to the formation of 2-ketoglutarate.

One mole of 2-ketoglutarate is degraded per mole of carnitine formed. Carbon dioxide and succinate are products of 2-ketoglutarate degradation; no free succinic semialdehyde can be detected.

Several compounds which are chemically or biologically related to \( \gamma \)-butyrobetaine and to 2-ketoglutarate have been tested as inhibitors in the reaction. Only succinic semialdehyde and 3-trimethylaminopropyl-1-sulfonate are effective inhibitors. The sulfhydryl reagents \( \beta \)-chloromercuribenzoate and \( \beta \)-chloromercuriphenylsulfonate in 0.1 mM concentration completely inhibit the formation of carnitine after preliminary incubation with the enzyme for 20 min at 37°. N-Ethylmaleimide, iodosobenzoate, and iodoacetate are less effective inhibitors under these conditions, whereas sodium arsenite, carbarsone, and acetarsone cause appreciable inhibition only in 10 μM concentration. Preliminary incubation of the enzyme with ferrous ion and ascorbate results in significantly lower yield of carnitine than when catalase has been included in the preliminary incubation medium.

It is suggested that \( \gamma \)-butyrobetaine is hydroxylated to carnitine simultaneously with the oxidative decarboxylation of 2-ketoglutarate in a reaction sequence which involves the intermediate formation of a peroxide of the two substrates. Ferrous ion might act as oxygen-activating agent. Free sulfhydryl groups are apparently necessary for enzymic activity and ascorbate and catalase probably act by maintaining these groups as well as ferrous ion in the reduced state.

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\( \gamma \)-Butyrobetaine (4-trimethylaminobutyrate) is hydroxylated to carnitine (3-hydroxy-4-trimethylaminobutyrate) by the rat and the mouse (3–5), and a soluble protein fraction catalyzing this reaction has been obtained from rat liver homogenates (6). The rate-limiting step is probably the dissociation of the carbon-hydrogen bond, as a kinetic hydrogen isotope effect was found with \( \gamma \)-butyrobetaine labeled with tritium in the chain (6). An oxygenase type of reaction is indicated by the requirement for molecular oxygen (6, 7) and evidence has been presented for the requirement for ferrous ion (6, 8). A requirement has been observed for a NADPH-regenerating system, e.g. isocitrate + isocitrate dehydrogenase (6). Ascorbate, catalase, and microsomes from rat liver and kidney stimulated the formation of hydroxylated product (6). Further studies on the cofactor requirement will now be presented.

EXPERIMENTAL PROCEDURES

Materials—Isocitrate dehydrogenase (threo-D, -isocitrate:NADP oxidoreductase (decarboxylating), EC 1.1.1.42), glucose 6-phosphate dehydrogenase (d-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49), crystalline catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.1.1.6), NADH, NADPH, and disodium glucose 6-phosphate were obtained from C. F. Boehringer und Soehne, GmbH, Mannheim, West Germany; glutamate dehydrogenase (L-glutamate : NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) and glutamate-oxaloacetate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England; glucose dehydrogenase \( \beta \)-d-glucose:NAD(P) oxidoreductase (deaminating), EC 1.1.1.47 and p-chloromercuriurepensulfonic acid were obtained from Sigma; 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine, tetrahydrofolic acid, dithiothreitol, and dialuric acid were less effective inhibitors under these conditions, whereas sodium arsenite, carbarsone, and acetarsone cause appreciable inhibition only in 10 mM concentration. Preliminary incubation of the enzyme with ferrous ion and ascorbate results in significantly lower yield of carnitine than when catalase has also been included in the preliminary incubation medium.

It is suggested that \( \gamma \)-butyrobetaine is hydroxylated to carnitine simultaneously with the oxidative decarboxylation of 2-ketoglutarate in a reaction sequence which involves the intermediate formation of a peroxide of the two substrates. Ferrous ion might act as oxygen-activating agent. Free sulfhydryl groups are apparently necessary for enzymic activity and ascorbate and catalase probably act by maintaining these groups as well as ferrous ion in the reduced state.
henzene acid, and \(N\)-ethylmaleimide were obtained from Mann Research Laboratories.

Triose reductone was a gift from Prof. Baganz, Nordmark-Werke GmbH, Uetersen, Holst, West Germany, and carbosarone was a gift from Lilly Research Laboratories, Ltd., Bromborough Port, New Ferry, Wirral, Cheshire, England. Dowex AG50 W-X8 (minus 400 mesh, hydrogen form) and AG 11A8 (50 to 100 mesh, self-adsorbed form) were obtained from BioRad Laboratories.

A solution of 2-ketoglutaric acid was obtained by passing a solution of the zinc salt through a small column of Dowex AG50 W-X8 (hydrogen form) which was then eluted with water. \(\text{Ca(OH)}_2\) Carnitine chloride, 3,trimethylaminopropionate, 4 dimethylaminobutyrate, 4-trimethylamincrotonate, 5-dimethylaminovalerate, 5-trimethylaminovalerate, 6-trimethylaminocaproate, and 4,trimethylaminobutan-1-ol were synthesized as described previously (5, 6). Succinic semialdehyde was synthesized according to the method of Carrière (9) as modified by Albers (cited by Jacoby in Reference 10) and was characterized as the 2,4-dinitrophenylhydrazone and by gas chromatography-mass spectrometry of the trimethylsilyl ether of the oxime.\(^1\) 3-Dimethylaminopropyl-1-sulfonic acid was prepared by treating 3-amino-1-propyl-1-sulfonic acid with formaldehyde and formic acid (6, 11). The product was crystallized twice from hot ethanol, m.p. 224-227° with decomposition.

\[
\text{C}_6\text{H}_{13}\text{NOSO}_2 (167.2)
\]

Calculated: H, 7.84; N, 8.38

Found\(^2\): H, 7.85; N, 8.28

3-Trimethylaminopropyl-1-sulfonic acid was obtained by treating the corresponding dimethylamine in methanol-water (7:3) with a 10-fold excess of methyl iodide. The theoretical amount of 1 M potassium hydroxide was slowly added during 24 hours. The reaction mixture was then taken to dryness, redissolved in water, and passed through a column of AG 11A8, using water for the elution. The fractions containing trimethylaminopropyl-1-sulfonate were free from the dimethylaminic acid, as judged by microanalytical laboratory, Ballerup, Copenhagen, Denmark.

\[
\text{C}_6\text{H}_{13}\text{NOSO}_2\text{Cl} (217.7)
\]

Calculated: H, 7.40; N, 6.65

Found: H, 7.40; N, 6.61

Radioactive Compounds—[Methyl-\(^{14}\)C]\(\beta\)-butyrobetaine (30 mCi per mmole) was prepared as described previously (6). [2,3-\(^{14}\)C]Succinic acid (4 mCi per mmole) was obtained from the Radiochemical Centre, Amersham, England. Sodium [5-\(^{14}\)C]2-ketoglutarate (15 mCi per mmole) was obtained from the Radiochemical Centre, and [1-\(^{14}\)C]2-ketoglutaric acid from Calbiochem (AG, Luzerne, Switzerland). 2-Ketoglutaric acid was purified by silicic acid chromatography (see below and legend to Fig. 3). Some preparations contained 30% or more of impurities which were eluted with higher concentrations of tert-butanol than required for 2-ketoglutaric acid. The fractions containing 2-ketoglutaric acid were lyophilized, redissolved in water, and distributed in several ampoules which were then stored at -15°. Rechromatography regularly disclosed the presence of up to 5% of more polar material which was probably polymerization products. Silicic acid chromatography did not reveal any impurities in the labeled succinic acid.

Chromatography Procedures—Ion exchange chromatography on columns of Dowex AG50 W-X8 (minus 400 mesh, hydrogen form) was carried out as described previously (12). Dialyses were separated from dimethylamino acids by filtration through a column of AG 11A8 (self-adsorbed form) (13). Partition chromatography of carboxylic acids was carried out on columns of silicic acid with 0.25 M sulfuric acid as the stationary phase and benzene with varying concentrations of tert-butanol as the mobile phase according to Prior Ferraz and Relvas (14). For conditions see legend to Fig. 3. Descending paper chromatography of the dinitrophenylhydrazones of 2-ketoglutaric acid and succinic semialdehyde was carried out with 1-butanol-95% aqueous ethanol-0.5 M ammonia in water (13:2:5) as the mobile phase (15). \(R_f\) values were 0.22 for 2-ketoglutaric dinitrophenylhydrazone and 0.56 for succinic semialdehyde dinitrophenylhydrazone. Succinic acid was separated from 2-ketoglutaric acid by descending paper chromatography with iso-amyl alcohol saturated with 4 M formic acid as the mobile phase. \(R_f\) values were 0.44 for 2-ketoglutaric acid and 0.65 for succinic acid. Munkell filter paper No. 312 was used throughout. Thin layer chromatography was carried out on Silica Gel G with methanol-dioxane-25% aqueous ammonia (30:45:25) as the mobile phase (16). The spots were made visible with iodine vapor. Approximate \(R_f\) value for 3-amino-1-propyl-1-sulfonic acid was 0.33, 0.51 for 3-dimethylaminopropyl-1-sulfonic acid and 0.10 for 3-trimethylaminopropyl-1-sulfonic acid.

Fat Liver Homogenates—Male rats of the Sprague-Dawley strain, weighing 200 to 300 g, were used. The animals were killed by a blow on the head and the livers were immediately 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 nicotinamide and recentrifuged at 100,000 x g for 60 min. The procedure was repeated another time. The sediment was then rehomogenized in the sucrose solution and the protein concentration was adjusted to approximately 20 mg per ml. The homogenates were prepared fresh for each series of experiments.

Enzyme Preparation—The procedure used previously (6) was slightly modified. As before, the supernatant fraction of a rat liver homogenate (see above) was fractionated by the addition of a saturated ammonium sulfate solution at 4°. The protein fraction between 40 and 70% saturation was desalted by filtration through a Sephadex G-25 column (coarse) with 20 mM phosphate buffer at pH 6.5 as eluent and then immediately applied to a column of hydroxyapatite in the same buffer (50 g of hydroxyapatite for 2 to 5 g of protein). The column was
eluted with a slightly convex concentration gradient of potassium phosphate buffer at pH 6.5. The bulk of the protein emerged from the column between 50 and 150 nM, after which most of the hydroxylase activity was obtained between approximately 175 and 225 mM phosphate buffer (Fig. 1). The hydroxylapatite chromatography resulted in a purification of about six to eight times with about 70% yield. The partially purified enzyme preparation usually had a specific activity of 3 units per mg. The enzyme was fairly stable, and could be kept frozen for several months with a decrease in specific activity of less than 50%.

**Assay**—The assay procedure which has been described in detail previously (6) was used with some modifications. Phos- phate buffer was usually used instead of Tris-HCl buffer and the NADPH-isocitrate dehydrogenase system was replaced by 2-ketoglu tarate except when otherwise indicated. The incubations were carried out at 37°C for 1 or 2 hours either in 25-ml Erlenmeyer flasks (incubation volume 4.8 to 5.0 ml) or in 5-ml test tubes (incubation volume 0.8 ml). The composition of the incubation mixture before the incubations (“complete system”) was: 0.5 to 3.0 ml of enzyme preparation (0.5 to 5 mg of protein), potassium phosphate buffer, pH 7.0 or 6.5 (20 mM), [methyl-14C]-2-ketoglutarate (0.8 mg per ml), and potassium chloride (20 mM).

In the incubations with NADPH, the concentration of NADPH was 0.2 or 1.0 mM. In the incubations with a NADPH-regenerating system the concentration of NADPH was 0.2 mM. Per ml of incubation mixture there were added also either 0.02 mg of isocitrate dehydrogenase, 4 μmoles of α-ketoglutarate, and 1.3 μmoles of MgCl₂ or 5 mg of glucose dehydrogenase and 100 μmoles of glucose or 0.05 mg of glucose 6-phosphate dehydrogenase, 4 μmoles of glucose 6-phosphate, and 6 μmoles of magnesium chloride. Incubations with a NADPH-regenerating system were carried out both with 20 mM phosphate buffer at pH 7.0 and with 20 mM Tris-HCl buffer, pH 7.8. All these incubations contained 40 mM nicotinamide.

In the preliminary incubation experiments given in Table I, 2 mg of glutamate dehydrogenase, 4 μmoles of NADPH, 10 μmoles of ammonium chloride or 0.5 mg of glutamate-oxalacetate transaminase, and 10 μmoles of L-aspartate were used. The commercial suspensions in ammonium sulfate solutions of glutamate dehydrogenase and glutamate-oxalacetate transaminase had been dialyzed against an excess of 0.1 M phosphate buffer, pH 7.0, at 4°C overnight.

In incubations with microsomes, 2 to 5 mg of microsomal protein were added per ml of incubation mixture.

In the studies of the effect of different pH values on the formation of carnitine, the concentration of the phosphate buffer was 50 mM. The pH values were recorded at room temperature immediately after the incubations. During the incubations the changes in pH value were less than 0.1 unit.

The incubations were terminated by the addition of an equal volume of 10% trichloroacetic acid. After cooling in ice for 1 hour or overnight the protein was spun down, and the super- natant fractionated on columns of Dowex AG50 W-X8 (minus 400 mesh, H⁺ form). The columns were eluted with 0.1 M hydrochloric acid and the amount of radioactivity in the carnitine and γ-butyrobetaine fractions were determined with a methane- flow proportional counter.

In incubations with succinic semialdehyde and [5-14C]2-ketoglu tarate, the dinitrophenylhydrazones were prepared as described previously (17). The dinitrophenylhydrazones were separated by means of paper chromatography (see above) and the amount of radioactivity determined with a paper strip counter. The quantitative determination of succinic semialdehyde was carried out according to the method of Bessman, Rossen, and Layne (18) and Prescott and Waelsch (19). In the incubations with [1-14C]2-ketoglu tarate, the tubes were stoppered, and a 0.5 cm² filter paper was attached to a piece of wire in the stopper; 1 ml solution of Hyamine in methanol (200 μl) was applied to the filter paper. The incubations were terminated by the addition of either trichloroacetic acid or 2 mM sulfuric acid, and the diffusion of labeled carbon dioxide was allowed to proceed for 1 hour at 37°C. The filter papers were then transferred to a scintillation counter vial, containing 10 ml of a mixture of the following composition: 10 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis(2-(4-methyl-5-phenyloxazoly)benzene, 1000 ml of tolu- ene, and 600 ml of methyl cellosolve.

Protein was determined according to the method of Lowry et al. (20) with human serum albumin as standard. The protein concentration after the hydroxylapatite chromatography was followed by measurement of the absorbance at 280 nm.

### RESULTS

**Enzyme Preparation**—Fig. 1 shows the purification of the hydroxylase by hydroxylapatite chromatography. The γ-
butyrobetaine-requiring 2-ketoglutarate-degrading activity (see below) was eluted together with the 2-ketoglutarate-requiring γ-butyrobetaine hydroxylase activity. There was a linear relationship between the γ-butyrobetaine-hydroxylating activity and the concentration of protein in the incubations with the “complete system” of cofactors (see “Assay”).

Requirement for 2-Ketoglutarate as Cofactor—In the previous paper (6) a requirement was reported for a NADPH-regenerating system, viz. NADPH, isocitrate dehydrogenase, and isocitrate. From studies on the specificity of this NADPH-regenerating system it then became apparent that the stimulation was not related to the reduction of NADPH, but to the formation of 2-ketoglutarate (Table I) since (a) other NADPH-regenerating systems were inactive, (b) 2-ketoglutarate could replace the isocitrate dehydrogenase system, and (c) very low hydroxylating activity could be observed after preliminary incubation with two different enzymes which catalyze the conversion of Z-ketoisocitrate to glutamate. The hydroxylating activity was also reduced after preliminary incubation of the soluble fraction of a rat liver homogenate under similar conditions before the incubation with γ-butyrobetaine and the “complete system” of cofactors (minus 2-ketoglutarate). In this case glutamate-oxalacetate transaminase plus aspartate caused almost complete inhibition whereas only about 50% inhibition was noted with glutamate dehydrogenase, ammonium chloride, and NADPH, probably because NADPH was reoxidized during the incubation.

Several compounds were tested in 0.15 M and 1.5 M concentration for their ability to replace 2-ketoglutarate as a cofactor, viz. oxalacetate, 2-ketoacid, 2-ketobutyrate, 2-hydroxyglutarate, and succinyl semialdehyde, glutarate, DL-glutamate, pyruvate, 2-ketobutyrate, 2-ketovalerate, citrate, DL-isocitrate, dl-cis-aconitate, succinate, fumarate, malate. Since none of the tested compounds could replace 2-ketoglutarate there is a highly specific requirement for 2-ketoglutarate as a cofactor in the hydroxylation of γ-butyrobetaine to carnitine. An apparent $K_m$ value for 2-ketoglutarate was calculated to about 0.5 mM (Fig. 2).

Degradation of 2-Ketoglutarate to Succinate—Fig. 3 shows a silicic acid chromatogram of the products obtained after an incubation with [5-$^{14}$C]-2-ketoglutarate. One major metabolite was found, which was eluted together with added unlabeled succinic acid. The radioactive material had the same $R_f$ value as succinic acid on paper chromatography (see “Experimental Procedures”), and three recrystallizations together with unlabeled

| Addition                              | Carnitine μmoles |
|---------------------------------------|------------------|
| Experiment 1                          |                 |
| NADPH                                 | <0.02           |
| NADPH + isocitrate dehydrogenase + isocitrate | 0.06          |
| 2-Ketoglutarate                       | 2.1             |
| NADPH + glucose 6-phosphate dehydrogenase + glucose 6-phosphate | <0.02         |
| NADPH + glucose dehydrogenase + glucose | <0.02         |
| Experiment 2                          |                 |
| No addition                           | <0.005          |
| 2-Ketoglutarate                       | 0.23            |
| 2-Ketoglutarate + glutamate dehydrogenase + HCN | 0.015       |
| 2-Ketoglutarate + glutamate - oxalacetate transaminase + aspartate | 0.008       |

Fig. 2. Lineweaver-Burk plot of the relationship between the rate of hydroxylation of γ-butyrobetaine to carnitine and the initial concentration of 2-ketoglutarate. The incubations were carried out for 15 min as described under “Assay.”

Fig. 3. Partition chromatography on a column (1.2 X 6 cm) of silicic acid (4 g) of the incubation mixture after incubating [5-$^{14}$C]-2-ketoglutarate (3 μCi, 0.2 μmole) for 1 hour at 37° with the purified protein fraction (2 mg), γ-butyrobetaine, ferrous ion, ascorbate, catalase, and phosphate buffer in 0.8 ml as described under “Assay.” Succinic acid (10 mg) was added to a 0.2-ml aliquot of the incubation mixture, which then was acidified with 50 μmoles of sulfuric acid and applied to the column, which was eluted with increasing concentrations of tert-butanol in benzene. The eluate was collected in 5-ml fractions. Aliquots of 0.5 ml were dried on glass planchets and the radioactivity was determined with a methane-flame proportional counter. The remainder of the fractions was titrated with 0.025 M sodium hydroxide. , counts per min; O——O, micromoles of sodium hydroxide.
carnitine from \([\text{methyl}-^{14}\text{C}]\)γ-butyrobetaine and the formation of \(14\text{CO}_2\) from \([1-^{14}\text{C}]\)2-ketoglutarate. The incubations (0.8 ml), which were carried out at 37° for 60 min in stoppered tubes, contained the partially purified protein fraction (8.2 mg), ferrous ion, catalase, phosphate buffer (see "Assay"), different concentrations of \([\text{methyl}-^{14}\text{C}]\)γ-butyrobetaine, and \([1-^{14}\text{C}]\)2-ketoglutarate. The values are from one experiment in which the 2-ketoglutarate concentration was 6.3 mM and the γ-butyrobetaine concentration was 0.38 to 2.5 mM, and another experiment in which the 2-ketoglutarate concentration was 0.63 to 6.3 mM and the γ-butyrobetaine concentration was 2.5 mM. Trichloracetic acid (10%) was added to the stopped tubes after the incubations, and diffusion of \(^{14}\text{CO}_2\) onto pieces of filter paper with Hyamine was allowed to proceed for 1 hour at 37°. The conversion of γ-butyrobetaine to carnitine was then determined by ion exchange chromatography (see "Assay").

succinic acid showed unchanged specific radioactivity of the succinic acid. The other labeled material was eluted at the rate expected for 2-ketoglutarate and its nonenzymic polymerization products (see "Experimental Procedures"). It was ascertained by separate experiments that labeled succinate was not metabolized under the present conditions of incubation, as judged by silicic acid chromatograms. Similar results have previously been obtained with a γ-butyrobetaine hydroxylase from \(Pseudomonas\) sp. AK 1 (21).

**Formation of Carbon Dioxide in 2-Ketoglutarate Degradation**—Fig. 4 shows the relationship between the formation of labeled carbon dioxide and the formation of carnitine in incubations with different initial concentrations of γ-butyrobetaine and of 2-ketoglutarate. The data indicate a stoichiometric relationship between the degradation of 2-ketoglutarate and the hydroxylation of γ-butyrobetaine. No enzymic formation of carbon dioxide was found when γ-butyrobetaine had been omitted from the incubation mixture. In a previous study of the bacterial γ-butyrobetaine hydroxylase (21), a stoichiometric relationship was also found between the hydroxylation of γ-butyrobetaine and the consumption of 2-ketoglutarate.

**Exclusion of Succinic Semialdehyde as Free Intermediate in Formation of Succinate from 2-Ketoglutarate**—There was no appreciable oxidation of succinic semialdehyde in a 1.25 mM solution under the incubating conditions used. No labeled succinic semialdehyde was detected in incubations with \([5-^{14}\text{C}]\)2-ketoglutarate to which had been added unlabeled succinic semialdehyde, i.e., less than 0.3% of the degraded 2-ketoglutarate could be recovered as free succinic semialdehyde. We have previously reported similar results from experiments with the bacterial γ-butyrobetaine hydroxylase (21).

**Inhibition by Compounds Structurally Similar to 2-Ketoglutarate and to γ-Butyrobetaine**—Table II shows that slight inhibition was observed when structural analogues of 2-ketoglutarate were added to the incubations. Succinic semialdehyde and 3-trimethylaminopropionate and 4-dimethylaminobutyrate were effective inhibitors, whereas 3-trimethylaminopropionate and 4-dimethylaminobutyrate were less inhibitory. The nature of the inhibition has not been investigated. No inhibition was observed with 4-trimethylaminobutan-1-ol.

**Ascorbate**—Table III and Fig. 5 show the effect of adding different reductants to incubations with the partially purified protein fraction, 2-ketoglutarate, ferrous ion, and catalase. The formation of carnitine was high with ascorbate and isoascorbate, lower with reduced 2,6-dichlorophenolindophenol and 2-amino-5,6-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine, and still lower with the other reductants which were tested.

**Catalase**—The stimulatory effect of catalase on the rate of carnitine formation was the same with 2-ketoglutarate as cosubstrate as with the NADPH-isocitrate dehydrogenase system (6). An addition of at least 0.3 mg of catalase per ml was required for maximal rate of carnitine formation. A low rate of carnitine formation was observed when the enzyme had been

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**Table II**

| Compound added                        | Carnitine (nmol) |
|---------------------------------------|-----------------|
| None                                  | 27              |
| Hydroxylglutarate                     | 19              |
| Glutamate                             | 27              |
| Glutarate                             | 20              |
| Oxalacetate                           | 15              |
| 2-Ketoadipate                         | 25              |
| Pyruvate                              | 23              |
| Succinate                             | 25              |
| Succinic semialdehyde                 | 4               |
| Acetaldehyde                          | 26              |
| Propionaldehyde                       | 28              |
| Glyoxylate                            | 23              |
| 3-Trimethylaminopropionate            | 12              |
| 4-Dimethylaminobutyrate               | 25              |
| 4-Trimethylaminocrotonate             | 20              |
| 5-Trimethylaminovalerate              | 27              |
| 5 Dimethylaminovalerate               | 27              |
| 6-Trimethylaminocaprate               | 20              |
| 3-Trimethylaminopropyl-1-sulfonate    | 4               |
| 3-Trimethylaminopropyl-1-sulfonate    | 13              |
| 4-Trimethylaminobutan-1-ol            | 27              |
The partially purified protein fraction (0.1 mg) was incubated at 37° for 1 hour with [methyl-14C]γ-butyrobetaine, ferrous ion, catalase, 2-ketoglutarate, potassium phosphate buffer, pH 7.0, and the reductant given in the table. Incubation volume was 0.8 ml. See "Assay" for details.

| Compound                                      | Amount added | Carnitine nmol |
|-----------------------------------------------|--------------|----------------|
| None                                          |              | <0.7           |
| Ascorbate                                     | 10           | 31             |
| Isoascorbate                                  | 10           | 30             |
| Dehydroascorbate                              | 10           | <0.2           |
| 2-Keto-γ-gulonate                             | 10           | <0.5           |
| α-Gulonolactone                               | 10           | <0.5           |
| Trypto reductone                              | 10           | 2.5            |
| 2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine | 10          | 8.7            |
| Tetrahydrofolic acid                          | 10           | 4.8            |
| Glutathione                                   | 10           | 1.0            |
| Glutathione + 2,6-dichlorophenoloidophenol     | 10 + 2       | 11             |
| Glutathione + 2,6-dichlorophenoloidophenol + ascorbate | 10 + 2 + 10 | 30             |
| Dialurate                                     | 10           | <0.5           |
| Hydroquinone                                  | 10           | <0.5           |
| Dihydroxyfumarate                             | 10           | <0.5           |
| Ferrous sulfate                               | 10           | 4.9            |
| NADPH                                         | 2            | <0.2           |
| NADH                                         | 2            | <0.2           |
| Dithiothreitol                                | 1            | 1.0            |
| Dithiothreitol + ascorbate                    | 1 + 10       | 30             |

Effect of catalase on formation of carnitine from γ-butyrobetaine

The partially purified protein fraction (0.5 mg) was incubated in 0.3 ml of 0.15 m potassium phosphate buffer, pH 6.5, for 20 min at 37° with the additions given in the table. The remaining components of the complete system (see "Assay") were then added, and the incubation continued for 1 hour at 37°.

| Preliminary incubation conditions | Carnitine nmol |
|----------------------------------|----------------|
| Enzyme                           | 118            |
| Enzyme + ferrous ion + ascorbate | 38             |
| Enzyme + ferrous ion + ascorbate + catalase | 115 |

Fig. 6. Effect of the addition of microsomes (4 mg of protein) on the formation of [methyl-14C]carntine from [methyl-14C]γ-butyrobetaine in incubations with 100,000 × g supernatant fraction of a rat liver homogenate (20 mg of protein) or with the partially purified protein fraction ("hydroxylapatite fraction," 3 mg of protein). The incubations to which had been added labeled γ-butyrobetaine, ferrous ion, ascorbate, catalase, and phosphate buffer (see "Assay") were carried out either with the isocitrate-dehydrogenase system (NADPH, isocitrate, isocitrate dehydrogenase, and Mg²⁺, see "Assay") and 40 mM nicotinamide or with 3 mM 2-ketoglutarate. The total volume of the incubation mixture was 0.8 ml, and the incubations were carried out at 37° for 2 hours.

The values are given as enzyme activity (i.e. formation of carnitine) in relation to the controls without added microsomes, in which the activity was taken as 1.0.

The apparent Kₐ was about 0.1 mm in 20 mm phosphate buffer at pH 7.0. With 20 mm Tris-HCl buffer at pH 7.8 the apparent Kₐ value was found to be about 0.02 mm (6).

Nicotinamide, Microsomes—A stimulatory effect of nicotinamide was previously observed in incubations with liver homogenates fortified with NADPH, ascorbate, and ferrous sulfate (6). However, the addition of 40 mM nicotinamide had no effect when 2-ketoglutarate was used instead of NADPH. As shown in Fig. 6, the effect of microsomes on the formation of carnitine from γ-butyrobetaine was small when 2-ketoglutarate was used as cofactor instead of the NADPH-isocitrate dehydrogenase system in incubations both with the 100,000 × g supernatant fraction of a rat liver homogenate and with the partially purified protein fraction ("hydroxylapatite fraction").

Inhibition by Sulfhydryl Reagents—In a previous study on the effect of various metal ions on the formation of carnitine in incubations with the 20,000 × g supernatant fraction of rat liver homogenates (8), inhibition was noted by, among others, mercuric ion. Several sulfhydryl reagents were therefore tested as.
Table V

Effect of sulphydryl reagents on formation of carnitine from \(\gamma\)-butyrobetaine

| Compound                        | Amount | Carnitine |
|---------------------------------|--------|-----------|
| None                            |        | 118       |
| p-Chloromercuriphenylsulfonate   | 0.1    | <1        |
| p-Chloromercuriphenylsulfonate   | 0.01   | 80        |
| p-Chloromercuribenzoate         | 0.1    | <1        |
| N-Ethylmaleimide                | 1.0    | 16        |
| N-Ethylmaleimide                | 0.1    | 5         |
| o-Iodosobenzoate                | 1.0    | 40        |
| Iodoacetate                     | 1.0    | 40        |
| Arsenite                        | 10     | 20        |
| Arsenite                        | 1.0    | 102       |
| Carbarsone                      | 10     | 25        |
| Carbarsone                      | 1.0    | 123       |
| Acetarsone                      | 10     | 55        |

Fig. 7. Relation between the enzymic activity (\(\gamma\)-butyrobetaine hydroxylation) and the pH value in the incubations. See “Assay” for details.

In the previous studies of the hydroxylation of \(\gamma\)-butyrobetaine to carnitine in rat liver (6) some results were obtained for which no explanation could be offered, such as a stimulatory effect of microsomes, a dual requirement for a reductant, viz. ascorbate and a NADPH-regenerating system, as well as a stimulating effect of catalase. We had also observed that NADP\(^+\) was as effective as NADPH when crude extracts of acetone-dried rat liver were used as sources of enzyme and ascorbate, ferrous ion, and furmarate were the other cofactors (7). The results now reported indicate a specific requirement for 2-ketoglutarate in \(\gamma\)-butyrobetaine hydroxylation in the rat liver, and that the observed stimulation by NADPH, isocitrate, and isocitrate dehydrogenase is related to the formation of 2-ketoglutarate. As microsomes had negligible effect in the incubations with 2-ketoglutarate, they probably stimulate the formation of carnitine in incubations with the isocitrate dehydrogenase system by oxidizing NADPH thereby causing an increased formation of 2-ketoglutarate. The fact that none of a series of organic acids could replace 2-ketoglutarate and the disappearance of hydroxylating activity in a 100,000 \(\times\) g supernatant fraction of a rat liver homogenate after preliminary treatment with 2-ketoglutarate aminating systems may be taken as evidence that 2-ketoglutarate is the cofactor which participates in \(\text{vivo}\) in the hydroxylation reaction.

2-Ketoglutarate might be bound to the enzyme by way of the carboxyl group and the \(\gamma\)-carboxylate group, as succinic semialdehyde was inhibitory and 2-ketovalerate was not. The other aldehydes which were tested had little or no inhibitory activity. However, kinetic studies with the inhibitors are required to solve this problem.

The stoichiometric relationship between degradation of 2-ketoglutarate and hydroxylation of \(\gamma\)-butyrobetaine suggest an intimate coupling between the oxidative decarboxylation of 2-ketoglutarate and the formation of the hydroxylated product. Similar results were obtained with \(\gamma\)-butyrobetaine hydroxylase from a \textit{Pseudomonas} strain (21), which has the same cofactor requirements as the enzyme from rat liver (22, 23). Probably 2-ketoglutarate acts as the specific reductant of oxygen. The electrons may be transferred either directly to oxygen concomitantly with the decarboxylation or via an intermediate electron carrier. In the latter case the reaction would be similar to that catalyzed by the 2-ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes in the mitochondria in which thiamine pyrophosphate and lipoic acid are cofactors. However, such a mechanism for \(\gamma\)-butyrobetaine hydroxylase appears less probable as no enzymic decarboxylation of 2-ketoglutarate could be demonstrated in the absence of \(\gamma\)-butyrobetaine, whereas in the thiamine pyrophosphate-dependent decarboxylations, degradation of the 2-keto acid may be noted also in the absence of oxidants (24, 25). Recently (17), we proposed a reaction mechanism for 2-ketoglutarate-requiring hydroxylases (Scheme 1) according to which the anion of the substrate to be hydroxylated (I in Scheme 1) is attacked by a positively charged ferrous ion-oxygen complex, after which dissociation of ferrous ion occurs simultaneously with a nucleophilic attack of the hydroperoxide anion (II) on the 2-carbon atom in free or enzyme-bound 2-ketoglutarate. Rearrangement of the resulting peroxide (III) would result in decarboxylation and formation of succinate and the anion of the hydroxylated...
product (IV). Alternatively, the peroxide anion (II), or the hydroperoxide might be formed by a radical mechanism. Support for this view of the oxidative decarboxylation of 2-ketoglutarate is the fact that hydrogen peroxide and hydroperoxides decarboxylate 2-keto acids (for a review see Reference 26) and the recent finding that succinate contains 1 atom of isotopic oxygen after incubations with the bacterial \( \gamma \)-butyrobetaine hydroxylase in an \( ^{18} \text{O} \)-enriched atmosphere (37).

2-Ketoglutarate is required also in the hydroxylation of collagen proline (28–30), collagen lysine (31, 32), and thymine (33). In preliminary experiments we have found a 2-ketoglutarate-requiring oxygenation of hydroxymethyluracil and demonstrated both a thymine-dependent and a hydroxymethyluracil-dependent degradation of 2-ketoglutarate by extracts of a Neurospora strain. The degradation of 2-ketoglutarate in the hydroxylation of proline was recently reported by Rhoads and Udenfriend (34), who obtained results similar to those previously reported for the bacterial \( \gamma \)-butyrobetaine hydroxylase (21).

A reaction mechanism like that in Scheme 1 leaves no role for ascorbate as electron donor in the hydroxylation reaction. The same is true for the oxygenation of \( \beta \)-hydroxyphenylpyruvate to homogentisate and carbon dioxide, in which reaction the 2-keto acid side chain apparently acts as the reductant\(^3\) (35). Ascorbate and other reductants might act by maintaining ferrous ion and sulfhydryl groups in the reduced state as ferrous phosphate buffer. Since mercurials were effective inhibitors, sulfhydryl groups necessary for the enzymic activity are probably present in the hydroxylase, and such groups are readily oxidized by oxygen in the presence of transitional-metal salts (37).

The requirement for ferrous ion appears to be well established, the evidence being (a) inhibition of the enzymic activity in rat liver homogenates by several metal ion complexing agents with different structures (6), (b) reversal of this inhibition only by ferrous ion (6), and (c) a requirement for ferrous ion by the partially purified enzyme. Catalase probably acts by stabilizing the enzyme or protecting it from inactivation (Table IV), possibly from hydrogen peroxide formed in ferrous ion-catalyzed autooxidation of ascorbate (38). The concentrations of ferrous ion, ascorbate, and oxygen used under the present incubation conditions are probably much higher than those present in the tissues, and need for protection from hydrogen peroxide may therefore be lower in vivo. More efficient ways for the reduction of low concentrations of hydrogen peroxide than by the action of catalase exist in animal tissues (30). Also the subcellular localization of catalase speaks against catalase as a natural "cofactor" for \( \gamma \)-butyrobetaine hydroxylase.

A stimulation by catalase as well as by ferrous ion and ascorbate has also been noted for a \( \gamma \)-butyrobetaine hydroxylase from a \( Pseudomonas \) strain (22), for collagen-proline hydroxylase (40) and for thymine 7-hydroxylase and 5-hydroxymethyluracil 5-oxygenase from a \( \text{Neurospora} \) strain. The degradation of 2-ketoglutarate in the hydroxylation of proline was recently reported by Rhoads and Udenfriend (34), who obtained results similar to those previously reported for the bacterial \( \gamma \)-butyrobetaine hydroxylase (21).

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