Registry No. Ammonium, 14798-03-9; ammonia, 7664-41-7; hydroxylamine, 7803-49-8; asparagine, 70-47-3; asparagine synthetase, 9023-69-2.

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γ-Butyrobetaine Hydroxylase: Stereochemical Course of the Hydroxylation Reaction†

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ABSTRACT: The stereochemical course of the aliphatic hydroxylation of γ-butyrobetaine by calf liver and by *Pseudomonas* sp AK1 γ-butyrobetaine hydroxylases has been determined. With [3(*RS*)-3-^{3}H]-γ-butyrobetaine or [3(*R*)-3-^{3}H]-γ-butyrobetaine as substrate, a rapid and significant loss of tritium to the medium occurred. On the other hand, with [3(*S*)-3-^{3}H]-γ-butyrobetaine, only a negligible release of tritium to the aqueous medium was observed. Indeed, on hydroxylation of [3(*S*)-3-^{2}H]-γ-butyrobetaine by either the calf liver or bacterial hydroxylase, the isolated product L-carnitine was found to have retained all of the deuterium initially present in the 3(*S*) position. Since the absolute configuration of the product L-carnitine has been determined to be *R*, such results are only compatible with a hydroxylation reaction that proceeded with retention of configuration. With [*methyl*-^{14}C,3(*R*)-3-^{3}H]-γ-butyrobetaine as substrate for the calf liver hydroxylase, the percentage of tritium retained in the [*methyl*-^{14}C]-L-carnitine product was determined as a function of percent reaction. The results of these studies indicated that *pro-R* hydrogen atom abstraction exceeded 99.9%. Experiments using racemic [*methyl*-^{14}C,3(*RS*)-3-^{3}H]-γ-butyrobetaine as substrate yielded similar results and additionally allowed us to estimate α-secondary tritium kinetic isotope effects of 1.10 and 1.31 for the bacterial and calf liver enzymes, respectively. These results are discussed within the context of the radical mechanism for γ-butyrobetaine hydroxylase previously proposed [Blanchard, J. S., & Englard, S. (1983) *Biochemistry 22*, 5922], and the required topographical arrangement of enzymic oxidant and substrate is illustrated.

The enzyme γ-butyrobetaine hydroxylase [4-trimethylaminobutyrate,2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1] catalyzes the hydroxylation of γ-butyrobetaine to form L-carnitine in accordance with the reaction

γ-butyrobetaine + O_2 + α-ketoglutarate → L-carnitine + CO_2 + succinate

The enzyme has been obtained in highly purified form from bacteria (Lindstedt et al., 1977), calf liver (Kondo et al., 1981), and human kidney (Lindstedt et al., 1982) and is representative of that group of non-heme iron dioxygenases in which hydroxylation of the substrate is coupled to the oxidative decarboxylation of α-ketoglutarate [for reviews, see Abbott & Udenfriend (1974) and Hayaishi et al. (1975)]. α-Ketoglutarate is the only α-keto acid known to support hydroxylation, and its oxidative decarboxylation supplies two of the four electrons for reduction of molecular oxygen.

We recently reported on the primary and secondary tritium kinetic isotope effects exhibited by the bacterial and calf liver γ-butyrobetaine hydroxylases and proposed that the hydroxylation reaction proceeds via a homolytic carbon–hydrogen bond cleavage to yield a carbon radical (Blanchard & Englard, 1983). We also previously reported preliminary results suggesting that hydroxylation proceeds with retention of configuration (Englard & Midelfort, 1978), but those data were unable to distinguish unequivocally between a stereoselective process and a stereospecific hydrogen atom abstraction. Such a stereoselective process operates during the P-450$_{CAM}$-catalyzed aliphatic hydroxylation of camphor (Gelb et al., 1982).

We report here an analysis of the stereochemical course of aliphatic hydroxylation catalyzed by the calf liver and bacterial γ-butyrobetaine hydroxylases. We show that the hydroxylation reaction proceeds with retention of configuration and occurs with greater than 99.9% stereospecificity. The pertinence of these results to the mechanism of action of the two enzymes and the relative topography of the substrate and enzymic oxidant are discussed.

MATERIALS AND METHODS

γ-Butyrobetaine hydroxylase, isolated and purified from *Pseudomonas* sp AK1 (Linstedt et al., 1970, 1977), was generously provided by Dr. Göran Lindstedt (University of

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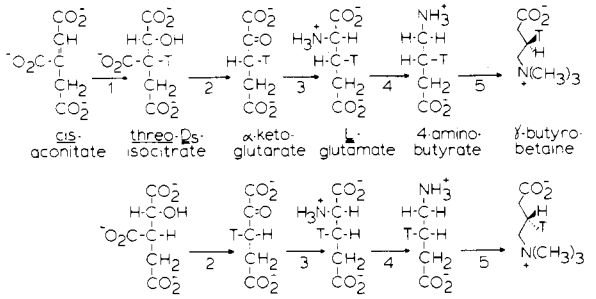
Scheme I: Enzymatic and Chemical Syntheses of [3(*R*)-3-^{3}H]-γ-Butyrobetaine and [3(*S*)-3-^{3}H]-γ-Butyrobetaine Using the Procedures Described under Materials and Methods[a]



[a] Reagents: (1) aconitase, ^{3}HOH, pH 7.4; (2) isocitrate dehydrogenase, $NADP^+$, H_2O or ^3H_2O, pH 7.4; (3) glutamate dehydrogenase, NADPH, NH_4^+, pH 7.4; (4) glutamate decarboxylase, dithiothreitol, pyridoxal phosphate, pH 5.0; (5) CH_3I, base.

Gothenburg, Gothenburg, Sweden). The enzyme was homogeneous as determined by SDS[1]–polyacrylamide gel electrophoresis and had a specific activity of 21 units/mg. Unless otherwise noted, calf liver γ-butyrobetaine hydroxylase was purified through the ion exchange and gel permeation chromatography steps previously described (Kondo et al., 1981). The activity of these enzyme preparations ranged between 0.02 and 0.04 unit/mg; by comparison, the specific activity of the homogeneous calf liver enzyme was 0.053 unit/mg as previously reported (Kondo et al., 1981).

Glutamate decarboxylase, yeast alcohol dehydrogenase, isocitrate dehydrogenase, and glutamate dehydrogenase were purchased from Sigma Chemical Co. Highly purified aconitase was the generous gift of Dr. Jenny Glusker of the Institute for Cancer Research (Philadelphia, PA). NADP and NADPH were obtained from Boehringer-Mannheim. [^{3}H]-Methyl iodide (82 mCi/mmol) was purchased from Amersham International Ltd. ^3H_2O (1 Ci/mL) and [3,3-$^3H_2$]-L-glutamate ([3(*RS*)-3-^{3}H]-L-glutamate, 22.7 Ci/mmol) were products from New England Nuclear. Methyl iodide (purissimum) and the proton sponge 1,8-bis(dimethylamino)-naphthalene were purchased from Tridom-Fluka and Aldrich Chemical Co., respectively. D_2O was obtained from Bio-Rad Laboratories. All other chemicals and reagents were of the highest purity commercially available. The preparation of stereospecifically labeled deuterated and tritiated isomers of γ-butyrobetaine as described below followed the sequence of enzymatic and chemical steps depicted in Scheme I.

Syntheses. [*methyl*-^{3}H,3(*S*)-3-^{2}H]-γ-Butyrobetaine was prepared by the exhaustive N-methylation of [3(*S*)-3-^{2}H]-4-aminobutyric acid. The latter compound was obtained by enzymatic decarboxylation of [3(*S*)-3-^{2}H]-L-glutamate in the presence of 20 units of glutamate decarboxylase, 2 mM dithiothreitol, 50 μM pyridoxal phosphate, and 2 mg/mL bovine serum albumin. The progress of the glutamate decarboxylase reaction was monitored with ninhydrin spray reagent after chromatographic separation of small aliquots of the reaction mixture on silica gel G in a developing system composed of 95% ethanol and H_2O (70:30). Following completion of L-glutamate decarboxylation, the reaction mixture was acidified to pH 2 with perchloric acid and filtered, and the clear filtrate was applied to a 1 × 20 cm column of Dowex 50W-X8 (H^+). The column was eluted with a linear gradient of HCl from 0 to 3 N, and the fractions containing the stereospecifically labeled [3(*S*)-3-^{2}H]-4-aminobutyrate were pooled and concentrated by rotary evaporation. That material was then subjected to exhaustive methylation in the presence of C^3H_3I as described previously (Englard et al., 1978), and the quarternized product was purified chromatographically on Dowex 50 (H^+). Elution of the column was carried out with a linear gradient of HCl from 0 to 5.8 N, peak radioactive fractions emerging at the position of elution of γ-butyrobetaine were pooled, and HCl was removed by repeated rotary evaporation. [*methyl*-^{3}H,3(*S*)-3-^{2}H]-γ-Butyrobetaine (0.37 μCi/μmol) migrated on thin-layer chromatography (silica gel G) with an R_f value identical with that of authentic γ-butyrobetaine in three solvent systems [systems I, III, and IV of Eneroth & Lindstedt (1965)], and its identity and radiochemical purity were further established by analytical ion exchange chromatography on Technicon type C resin (LaBadie et al., 1976).

[3(*S*)-3-^{2}H]-L-Glutamate was prepared from D$_s$-*threo*-isocitrate by the coupled action of isocitrate dehydrogenase and glutamate dehydrogenase. The reaction mixture contained in 50 mL of 99% D_2O 50 mM PIPES (free acid) titrated to pH 6.9 with NH_4OH, 1 mM NADP, 100 μM NADPH, 1 mM $MnSO_4$, 12 mM D$_s$-*threo*-isocitrate, 6 units of isocitrate dehydrogenase, and 400 units of glutamate dehydrogenase. Both enzymes were dialyzed against D_2O prior to addition to the reaction mixture. The course of the reaction was monitored with ninhydrin spray reagent as described above. After completion of the reaction, the incubation mixture was acidified to pH 2 with perchloric acid, and the [3(*S*)-3-^{2}H]-L-glutamate was purified on Dowex 50WX8 (H^+) as described above. Fractions containing ninhydrin-positive material were pooled and concentrated by rotary evaporation. The [3(*S*)-3-^{2}H]-L-glutamate migrated on thin-layer chromatography with authentic L-glutamate. The ^{1}H NMR spectrum of this material was as expected for [3(*S*)-3-^{2}H]-L-glutamate: δ 2.46 (dt, 1 H, J_{H-H} = 6.5 and 7.3 Hz), 2.93 (d, 2 H, J_{H-H} = 7.3 Hz), 4.48 (d, 1 H, J_{H-H} = 6.5 Hz).

[3,3-$^2H_2$]-L-Glutamate was prepared by reduction of [3,3-$^2H_2$]-α-ketoglutarate with glutamate dehydrogenase. The reaction mixture contained in 20 mL 30 mM PIPES titrated to pH 6.9 with NH_4OH, 100 μM NAD, 200 mM ethanol, 200 units of yeast alcohol dehydrogenase, and 100 units of glutamate dehydrogenase. [3,3-$^2H_2$]-α-Ketoglutarate (free acid in D_2O) was added slowly dropwise, and the pH was maintained at 6.9 ± 0.1 by addition of 0.2 N NaOH. [3,3-$^2H_2$]-α-Ketoglutarate was prepared by refluxing 1.0 g of the free acid in 10 mL of >99% D_2O followed by flash evaporation to dryness; the procedure of evaporation from D_2O was then repeated 2 times. The solid [3,3-$^2H_2$]-α-ketoglutaric acid dissolved in D_2O then was reduced to L-glutamate as described above. The ^{1}H NMR spectrum of this material was as expected for [3,3-$^2H_2$]-L-glutamate [δ 2.93 (s, 2 H), 4.48 (s, 1 H)].

[3(*R*)-3-^{3}H]-γ-Butyrobetaine was synthesized from [3-(*R*)-3-^{3}H]-4-aminobutyrate by exhaustive methylation as described above. [3(*R*)-3-^{3}H]-4-Aminobutyrate was obtained from [3(*R*)-3-^{3}H]-L-glutamate by the action of glutamate decarboxylase also as described above. [3(*R*)-3-^{3}H]-L-Glutamate was synthesized from [3(*R*)-3-^{3}H]-D$_s$-*threo*-isocitrate as follows. To 6.2 mL of a solution containing 16 mM Tris-HCl, pH 7.4, 2.9 mM NADP, 76 mM [3(*R*)-3-^{3}H]-D$_s$-*threo*-isocitrate, 80 mM NH_4Cl, and 0.4 mM $MnCl_2$ was added 11.2 units of isocitrate dehydrogenase and 120 units of glutamate dehydrogenase. After a 5-h incubation period at room temperature, no detectable isocitrate remained, and the

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

reaction mixture was quenched with trichloroacetic acid (to 5%). [3(*R*)-3-^{3}H]-L-Glutamate was purified by chromatography on Dowex 50W-X8 (H^+) as described above.

[3(*R*)-3-^{3}H]-D_s-*threo*-Isocitrate was synthesized from aconitate and ^3H_2O as follows. A solution (1.2 mL) containing 40 mM Tris-HCl, pH 7.45, and 380 mM *cis*-aconitate was lyophilized in one arm of a Rittenberg flask. The residue was dissolved in 2.4 mL of ^3H_2O (~0.8 Ci/mL) and reacted at room temperature for 5 h with 38 units of aconitase that had been activated for 80 min at 0 °C in the presence of 7.5 mM L-cysteine, 3.8 mM $Fe(NH_4)_2(SO_4)_2$, and 225 mM sodium ascorbate, pH 7.6. The reaction was terminated by freezing the mixture, and the ^3H_2O was removed by sublimation into the other arm of the flask. The residue was taken up in 5% $HClO_4$, and the precipitated protein was removed by centrifugation. The supernatant was adjusted to pH 7.7 with KOH and the $KClO_4$ removed by filtration. The filtrate was reduced in volume by rotary evaporation, and [3(*R*)-3-^{3}H]-D_s-*threo*-isocitrate was purified chromatographically on a column of silicic acid that was developed sequentially with 25, 40, and 50% butanol in benzene mixtures (Varner, 1957). [3(*R*)-3-^{3}H]-D_s-*threo*-Isocitrate (59 μmol) with a specific activity of 1.87×10^7 dpm/μmol was recovered.

[3(*S*)-3-^{3}H]-γ-Butyrobetaine was prepared by the procedure described for the synthesis of [3(*S*)-3-^{2}H]-γ-butyrobetaine, and [3(*RS*)-3-^{3}H]-γ-butyrobetaine was synthesized from [3,3-$^3H_2$]-L-glutamate as described above for the preparation of specifically labeled γ-butyrobetaine from the various labeled glutamate precursors.

Enzymatic Hydroxylation of Tritiated γ-Butyrobetaines. The stereochemical course of hydroxylation of γ-butyrobetaine was investigated by determining the relative rates of ^{3}H release and L-carnitine formation for each of the tritiated isomers of γ-butyrobetaine. Reaction mixtures contained, in a final volume of 7 mL, 50 mM Tris-HCl, pH 7.7, 15 mM sodium ascorbate, 3 mg/mL catalase (65 000 units/mg), 2 mM $Fe(NH_4)_2(SO_4)_2$, 20 mM KCl, 1.01 mM [3(*RS*)-3-^{3}H]- or [3-(*S*)-3-^{3}H]-γ-butyrobetaine or 0.85 mM [3(*R*)-3-^{3}H]-γ-butyrobetaine, and 4.62 mg of a partially purified preparation of calf liver γ-butyrobetaine hydroxylase. The enzyme preparation was purified through the hydroxylapatite chromatography step of Lindstedt & Linstedt (1970). Reaction mixtures were preincubated at 37 °C for 5 min prior to initiation of the reaction by addition of the labeled substrate. At the indicated time intervals, 600-μL aliquots were removed and added to 120 μL of 0.5 mM 2,2′-bipyridine to terminate the hydroxylation reaction. After all samples were collected, suitable aliquots were removed for the enzymatic radioisotopic determination of carnitine (McGarry & Foster, 1976). ^3H_2O was collected from the remainder of each sample by sublimation as previously described (Englard et al., 1978). Aliquots of the sublimate (400 μL) were counted in Hydromix (Yorktown) scintillation cocktail in a Searle Model 6880 Mark III liquid scintillation counter. As noted previously (Englard et al., 1978), the calculation of carnitine formed from the measured quantity of ^{3}H released into the medium (Figure 1) is based on the known specific activity of a single hydrogen at C_3 that must be displaced as the result of hydroxylation.[2]

For experiments using [*methyl*-^{14}C,3(*R*)-3-^{3}H]-γ-butyrobetaine or [*methyl*-^{14}C,3(*RS*)-3-^{3}H]-γ-butyrobetaine, the experimental protocol was identical with that previously reported (Blanchard & Englard, 1983).

[2] These calculations are not corrected for the isotope effect and assume a stereospecific hydrogen atom abstraction.

Enzymatic Hydroxylation of [3(*S*)-3-^{2}*H*]-γ-*Butyrobetaine.* A 50-mL flask was prepared containing the following in 10 mL: 100 mM HEPES, pH 7.8, 15 mM sodium ascorbate, 2 mg/mL catalase (65 000 units/mg), 1.2 mM $Fe(NH_4)_2(SO_4)_2$, 10 mM α-ketoglutarate, 5 mM K_2HPO_4, and 184 μM [*methyl*-^{3}H,3(*S*)-3-^{2}H]-γ-butyrobetaine. The flask was vigorously shaken in an incubator maintained at 30 °C to ensure oxygen saturation, and the reaction was initiated by addition of either 16 μg of *Pseudomonas* sp AK1 γ-butyrobetaine hydroxylase or 1.2 mg of calf liver hydroxylase. The reaction was terminated 8 h later by addition of TCA to 5%, and the precipitated protein was removed by centrifugation (20 min at 24000*g*). The supernatant was applied to a 1.2 × 12 cm column of Dowex 50W-X8 (H^+) that was then sequentially eluted with 100 mL of water and 100 mL of 4 N HCl. The peak HCl fractions containing radioactivity were evaporated repeatedly to remove HCl, and the residue was taken up in 1.0 mL of 0.25 N sodium citrate, pH 3.4, and applied to a column containing Technicon type C resin. L-Carnitine and γ-butyrobetaine were separated as previously described (La-Badie et al., 1976), and the carnitine peak was isolated, concentrated, and freed of citrate by absorption on Dowex 50W-X8 (H^+) and elution with 4 N HCl. HCl was removed by repeated rotary evaporation, and the amount of carnitine obtained was determined enzymatically (Pearson et al., 1969). The NMR samples were prepared by repeated evaporation of carnitine from 99% D_2O.

^{1}H NMR Experiments. ^{1}H NMR spectra were obtained at 200 MHz on a Varian XL-200 spectrometer, operating in the pulsed Fourier-transform mode and using an internal deuterium lock. For obtaining spectra of the relatively low concentrations of enzymatically generated L-carnitines, the residual HOD signal was suppressed by presaturation (Gupta et al., 1976). A pulse (90°) of 13 μs was used, and 4K data points were obtained with a repetition time of 8 s.

Data Analysis. Primary kinetic isotope effects measured by ^3H_2O release were fit to eq 1 where f is the fractional

$$^T(V/K) = \log (1.0 - f)/\log (1.0 - DPM_f/DPM_0) \quad (1)$$

percent conversion of γ-butyrobetaine to carnitine and DPM_f and DPM_0 are the dpm of ^3H_2O at f and 100% reaction, respectively. Primary kinetic isotope effects measured by analyzing the $^3H/^{14}C$ ratio of reisolated substrate were fit to eq 2 (Melander, 1960) where R_f and R_0 are the $^3H/^{14}C$ ratio

$$^T(V/K) = 1.0/[1.0 + \log (R_f/R_0)/\log (1.0 - f)] \quad (2)$$

of γ-butyrobetaine at fractional percentages of reaction and at 0% reaction, respectively.

Secondary isotope effects, measured by analyzing the $^3H/^{14}C$ ratio of isolated L-carnitine, were calculated from eq 3 where R_f and R_0 are the $^3H/^{14}C$ ratio of carnitine at frac-

$$^T(V/K) = \log (1.0 - f)/\log (1.0 - fR_f/R_0) \quad (3)$$

tional percentages of reaction and at 100% reaction, respectively.

The observed ^{3}H retention in product was calculated by correcting for 13% spillover of ^{14}C counts into the ^{3}H channel and dividing net ^{3}H counts by expected ^{3}H counts at the various percentages of reaction. The predicted percent of ^{3}H retained was calculated by assuming a primary tritium kinetic isotope effect of 15 and 0.033% *pro-S* hydrogen abstraction. The integrated substrate R_f was calculated by averaging the calculated R_f (eq 2) values between 0 and f for each 1% reaction between 0–99% reaction and for each 0.1% reaction between 99.0–99.8% reaction.

Table I: ^3H Retention in L-Carnitine during Hydroxylation of [*methyl*-^{14}C,3(*R*)-3-^3H]-γ-Butyrobetaine by Calf Liver Hydroxylase

		isolated L-carnitine					% ^3H retained in carnitine	
f^a	^3H_2O	^{14}C channel	^3H channel	channel ratio	^3H counts (net)^b	integrated substrate $^3H/^{14}C$	obsd^d	predicted^e
0.00	40	85 368^c	97 756^c	1.145	86 664	1.145		
0.16	638	8956	1356	0.153	203	1.25	1.46	0.62
0.29	1897	19 913	3038	0.153	449	1.34	1.78	0.66
0.49	4285	36 418	4910	0.135	175	1.55	0.41	0.77
0.67	8701	52 756	7653	0.145	794	1.84	1.37	0.91
0.95	31 910	72 165	11 444	0.158	2062	3.37	2.50	1.67
0.97	53 790	76 172	11 162	0.146	1260	3.85	1.49	1.90
0.993	69 890	78 443	12 707	0.162	2509	5.37	2.92	2.66
0.998	86 664	85 368	13 843	0.162	2745	6.46	3.16	3.20

^{a} Calculated from ratio of ^{14}C in carnitine to total ^{14}C. ^{b} Calculated using channel ratio of 0.13 for ^{14}C spillover into ^3H channel. ^{c} Substrate was [*methyl*-^{14}C,3(*R*)-3-^3H]-γ-butyrobetaine. ^{d} Net ^3H counts − (f × 86 664), assuming constant substrate specific radioactivity. ^{e} Predicted tritium retained for $^T(V/K)$ = 15 and 0.03% 3(*S*) hydrogen atom abstraction.

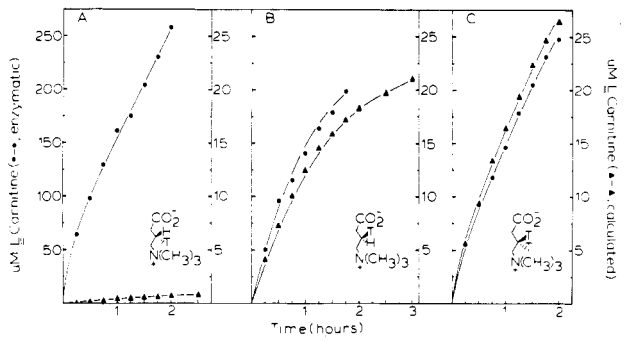


FIGURE 1: Relationship between ^3HOH release from C_3 enantiomers and racemic γ-butyrobetaine. Experimental conditions are as described under Materials and Methods: (A) [3(*S*)-3-^3H]-γ-butyrobetaine, sp act. 7.25 × 10^6 dpm/μmol; (B) [3(*R*)-3-^3H]-γ-butyrobetaine, sp act. 1.47 × 10^7 dpm/μmol; (C) [3-(*RS*)-3-^3H]-γ-butyrobetaine, sp act. 1.44 × 10^7 dpm/μmol.

RESULTS

A shown in Figure 1A, formation of carnitine from [3-(*S*)-3-^3H]-γ-butyrobetaine by the partially purified preparation of calf liver γ-butyrobetaine hydroxylase proceeded with only negligible release of ^3H into the medium water. Under similar conditions of incubation, [3(*R*)-3-^3H]-γ-butyrobetaine showed considerable loss of ^3H to the aqueous medium (Figure 1B). The quantitative relationship between ^3H release from [3-(*R*)-3-^3H]-γ-butyrobetaine and the amount of carnitine actually formed was nearly equivalent to that observed for the detritiation of [3(*RS*)-3-^3H]-γ-butyrobetaine (Figure 1C) and of [2,3(*N*)-^3H]-γ-butyrobetaine, as previously reported (Englard et al., 1978). In view of the known absolute configuration of the stereospecifically labeled [3-^3H]-γ-butyrobetaine substrates and the determined *R* configuration of naturally occurring carnitine (Kaneko & Yoshida, 1962), the results of these studies are compatible with the conclusion that calf liver γ-butyrobetaine hydroxylase catalyzes the hydroxylation of its substrate with retention of configuration. Identical conclusions could be drawn from similar data obtained with these compounds by using the bacterial γ-butyrobetaine hydroxylase (data not shown).

Incubation of [3(*S*)-3-^2H]-γ-butyrobetaine with calf liver or *Pseudomonas* sp AK1 γ-butyrobetaine hydroxylase resulted in the production of carnitine that provided the ^1H NMR spectra shown in Figure 2. Since the C_3 proton resonance of carnitine is close to the large HOD peak, the splitting patterns of the C_2 and C_4 methylene protons were used to assess the extent of deuteration at C_3. Compared to standard L-carnitine (Figure 2A), the enzymatically generated carnitine obtained by hydroxylation of [3(*S*)-3-^2H]-γ-butyrobetaine by either the calf liver or *Pseudomonas* γ-butyrobetaine hydroxylase (Panels B and C of Figure 2, respectively) appears to be fully deuterated at C_3. The chemial shift position of all resonances are pH dependent, and spectra obtained at pH 2 showed quantitatively similar results.

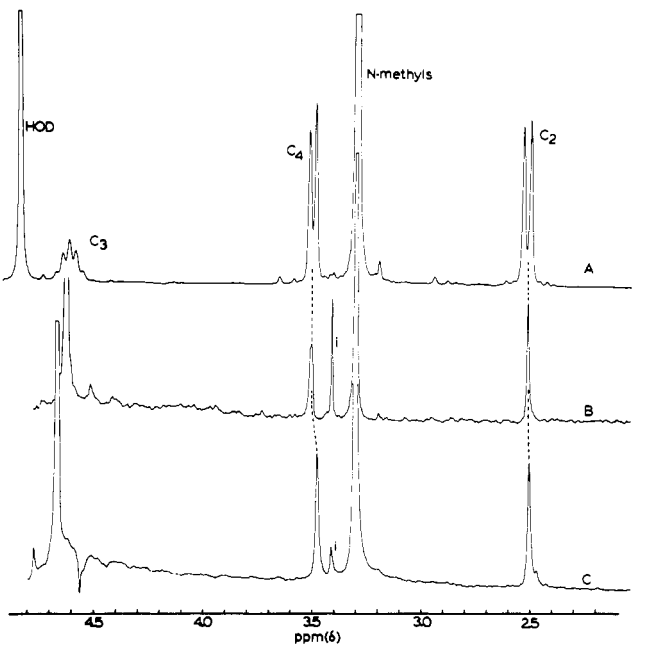


FIGURE 2: ^1H NMR spectra of (A) L-[3-^1H]carnitine (four scans, pH 9.21), (B) product of calf liver γ-butyrobetaine hydroxylase acting on [*methyl*-^3H,3(*S*)-3-^2H]-γ-butyrobetaine as substrate (120 scans, pH 9.18), and (C) product of *Pseudomonas* sp AK1 γ-butyrobetaine hydroxylase acting on [*methyl*-^3H,3(*S*)-3-^2H]-γ-butyrobetaine as substrate (16 scans, pH 9.02). The *N*-methyl proton peak was used to align the spectra. Citrate, carried over in the purification of these compounds, is the impurity designated by i.

To critically distinguish between absolute stereospecificity and a small degree of stereoselectivity in the hydroxylation reaction, we determined for the calf liver hydroxylase incubated with [*methyl*-^{14}C,3(*R*)-3-^3H]-γ-butyrobetaine the percentage of tritium retained in the product [*methyl*-^{14}C]-L-carnitine as a function of percent reaction. As seen in Table I, despite large increases in the integrated $^3H/^{14}C$ ratios of the unreacted substrate at values of $f > 0.9$, the ratios of counts in the tritium and carbon-14 channels for the isolated L-carnitine remained essentially invariant as a function of percentage reaction. In order to place limits on *pro-S* hydrogen abstraction, we assumed that a pure carbon-14 label would result in a maximal spillover of 13% of counts in the tritium channel.[3] Correction

[3] A value of at least 13% spillover was required to calculate nonnegative numbers for the amount of ^3H retained in the product.

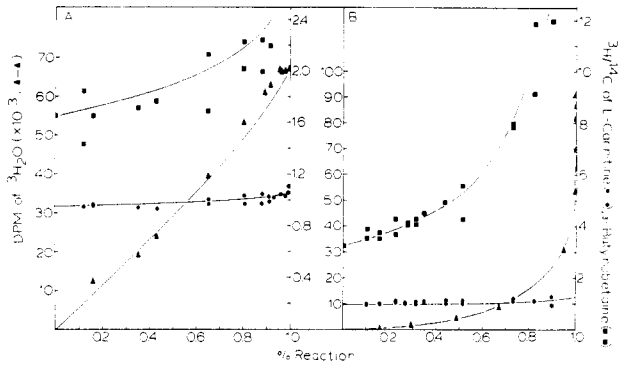


FIGURE 3: Substrate and product analysis of [*methyl*-^{14}C,3(*RS*)-3-^{3}H]-γ-butyrobetaine hydroxylation by *Pseudomonas* sp AK1 (A) and calf liver (B) γ-butyrobetaine hydroxylases. At the time points indicated, aliquots were removed and analyzed for $^{3}H_2O$ (▲), and the labeled carnitine (●) and γ-butyrobetaine (■) were isolated as described in the text. In panel A, the curves are fits of the data to eq 1 and 2 assuming $^{T}(V/K)$ primary = 1.25 (▲ and ■) and to eq 3 assuming $^{T}(V/K)$ secondary = 1.10 (●). In panel B, the curves are fits of the data to $^{T}(V/K)$ primary = 15 (▲ and ■) and $^{T}(V/K)$ secondary = 1.31 (●).

of the tritium counts for such a spillover allowed us to calculate the maximum percentage of ^{3}H counts retained in the product [*methyl*-^{14}C]-L-carnitine. These values were then compared to those predicted for various intrinsic geometrical selectivities and a $^{T}(V/K)$ of 15. As can be seen from the results presented in Table I, reasonable correlations between the observed and predicted percentages of ^{3}H retained in the product are obtained by assuming 0.033% *pro-S* abstraction. For comparison, a 0.1% *pro-S* selectivity would yield 9.7% tritium retention in L-carnitine at 99.8% reaction.

Results substantiating the absolute stereospecificity of the hydroxylation reaction were obtained for both the bacterial and calf liver enzymes in a parallel experiment with the racemate [*methyl*-^{14}C,3(*RS*)-3-^{3}H]-γ-butyrobetaine. Thus as seen in Figure 3A, the $^{3}H/^{14}C$ ratio of the isolated L-carnitine produced by the bacterial enzyme was essentially invariant over the entire course of the reaction. From the shape of the curve fitting the experimental data, a normal α-secondary ^{3}H isotope effect of 1.10 was calculated. For the calf liver enzyme (Figure 3B), the $^{3}H/^{14}C$ ratios for L-carnitine again remained essentially constant as a function of the percent reaction; the fitted curve is a plot of the expected $^{3}H/^{14}C$ ratios in the product calculated for an α-secondary kinetic tritium isotope effect of 1.31.

Discussion

Aliphatic hydroxylation reactions exhibit an impressive constancy of stereochemistry considering the variety of substrates hydroxylated and the nature of the cofactors required for such hydroxylations [for reviews, see Hanson & Rose (1975) and Rose & Hanson (1976)]. Thus, steroid (Hayano et al., 1959) and fatty acid (Morris, 1970) hydroxylations proceed with retention of configuration, as does the hydroxylation of dopamine catalyzed by dopamine β-hydroxylase (Battersby et al., 1974; Taylor, 1974). Of the α-ketoglutarate-coupled hydroxylations, only the stereochemistry of hydroxylation at C_4 of peptidyl proline by prolyl hydroxylase has been demonstrated to occur with retention of configuration (Fujita et al., 1964). A notable exception to the stereochemical consistency of aliphatic hydroxylation is the cytochrome P-450 catalyzed hydroxylation of camphor as revealed by the recent stereochemical analysis of Gelb et al. (1982). In that case, the oxidant in the reaction can remove either the exo or endo hydrogen atom and yet uniquely produces an exo alcohol product. The intrinsic endo/exo hydrogen atom selection is in turn influenced by the isotopic nature of the endo and exo substituents as a small intramolecular isotope effect was demonstrated for the hydroxylation of the enantiotopic 5-deuteriocamphors.

To determine the stereochemistry of aliphatic hydroxylation catalyzed by γ-butyrobetaine hydroxylase, the rates and extent of ^{3}H loss into the solvent upon enzymatic hydroxylation of both [3(*S*)-3-^{3}H]- and [3(*R*)-3-^{3}H]-γ-butyrobetaines were compared to the corresponding release of ^{3}H from the racemate, i.e., [3(*RS*)-3-^{3}H]-γ-butyrobetaine. As seen in Figure 1A, when [3(*S*)-3-^{3}H]-γ-butyrobetaine was incubated with the calf liver hydroxylase preparation, the amount of carnitine formed, calculated from the amount of ^{3}H released and recovered as ^{3}HOH, was less than $^{1}/_{100}$th of that measured by enzymatic assay. When the opposite enantiomer was used (Figure 1B), the discrepancy between the amount of carnitine calculated from the ^{3}H release data and by enzymatic determination could be fully accounted for by the primary tritium kinetic isotope effect (Blanchard & Englard, 1983). In view of the previously established *R* configuration of the product L-carnitine, these results defined the hydroxylation sequence as proceeding with retention of configuration and further suggested a high degree of stereospecificity for the reaction.[4]

The suggestion of a stereospecific mechanism of hydroxylation gained further support from results obtained in experiments using [3(*S*)-3-^{2}H]-γ-butyrobetaine as substrate that allowed us to determine the isotopic composition of enzymatically generated carnitines. The stereospecific synthesis of [3(*S*)-3-^{2}H]-γ-butyrobetaine was achieved with a high degree of isotopic labeling (>99 atom % excess), and within the limits of the NMR analysis, results were as expected for the chirally-labeled glutamate intermediates (see Materials & Methods).[5] The conversion of [3(*S*)-3-^{2}H]-L-glutamate to [3(*S*)-3-^{2}H]-γ-butyrobetaine occurred without loss of deuterium content and presumably also without loss of chiral purity. As seen in Figure 2 for the isolated carnitines derived from [3(*S*)-3-^{2}H]-γ-butyrobetaine, no splitting of the C_2 or C_4 methylene protons by a C_3 hydrogen atom was observed. Thus, within the limits of the method and the instrumentation, the hydroxylation sequence must have proceeded with a high degree of stereospecificity. Because of the nonvolatile nature of carnitine, mass spectrometric analysis of the deuterium content of the product was not feasible. Further, the quantitatively similar spectra obtained for the L-carnitine products from either the calf liver or *Pseudomonas* γ-butyrobetaine hydroxylase reactions, in spite of the large difference in the primary isotope effects exhibited by the two enzymes (Blanchard & Englard, 1983), support our conclusion that

[4] Comparison of the extent of detritiation obtained for the two isomers of [3-^{3}H]-γ-butyrobetaine, corrected both for the difference in substrate specific radioactivity and for relative enzymatic activity, revealed that ^{3}H release from [3(*S*)-3-^{3}H]-γ-butyrobetaine was 5.9% of that obtained with the 3(*R*)-3-^{3}H isomer. We interpret our observation of a slight loss of ^{3}H from [3(*S*)-3-^{3}H]-γ-butyrobetaine during hydroxylation to reflect the presence of a small amount of [3(*R*)-3-^{3}H]-γ-butyrobetaine (5–7%) as a result of enolization of the intermediate [3(*S*)-3-^{3}H]-α-ketoglutarate. In that synthesis, NADPH was *not* added initially; the reductive amination of α-ketoglutarate was therefore solely dependent on the NADPH generated by the enzymatic oxidative decarboxylation of the starting isocitrate. Similar enolization with loss of chiral purity has been observed in the synthesis of [3(*S*)-3-^{2}H]-L-malate from enzymatically generated [3(*S*)-3-^{2}H]oxaloacetate (Blanchard & Cleland, 1980).

[5] Loss of chiral purity, presumably by enolization of the intermediate [3(*S*)-3-^{2}H]-α-ketoglutarate, was minimized by performing the oxidative decarboxylation of isocitrate in D_2O with a large excess of glutamate dehydrogenase *and* in the presence of initially added NADPH.

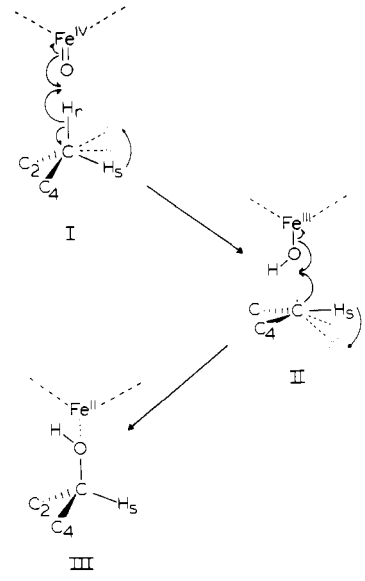
both enzymes catalyze hydroxylation with retention of configuration and by a stereospecific process.[6]

A more sensitive method of probing the question of absolute stereospecificity involved analysis of product specific radioactivity as a function of percent reaction (Table I). With enantiomerically pure [*methyl*-^{14}C,3(*R*)-3-^{3}H]-γ-butyrobetaine as substrate for the calf liver hydroxylase, any retention of tritium in the product would reflect a stereoselective hydrogen abstraction process. For calf liver γ-butyrobetaine hydroxylase, the sensitivity of the method would be further enhanced by the large primary tritium kinetic isotope effect of ~15 as previously determined for that enzyme (Blanchard & Englard, 1983). Thus, if a 94:6 *pro-R* to *pro-S* stereoselective process were operating,[7] the 15-fold intermolecular isotope effect for *pro-R* hydrogen abstraction would result in a reduction of the *pro-R* to *pro-S* ratio to approximately 1:1. Despite the technical difficulties inherent in counting small amounts of residual tritium in the L-carnitine product that is also labeled with carbon-14, we calculated that starting with a 1:1 ^{3}H to ^{14}C ratio in the substrate the analysis of ^{3}H retained in the product sets limits of *pro-S* hydrogen abstraction at less than 0.04%. This represents an upper limit, considering the assumption of only a 13% spillover of ^{14}C counts into the ^{3}H channel.

To reinforce the conclusion of 99.9% stereospecificity based on the interpretation of the results obtained for the hydroxylation of [*methyl*-^{14}C,3(*R*)-3-^{3}H]-γ-butyrobetaine by the calf liver enzyme, in another experiment we used the racemate [*methyl*-^{14}C,3(*RS*)-3-^{3}H]-γ-butyrobetaine as substrate. This allowed us to determine more accurately the percentage of ^{3}H retained in the hydroxylated product as a function of percent reaction. For that substrate, with either the bacterial or mammalian enzyme, significant levels of ^{3}H from the *pro-S* position should appear in the product L-carnitine and thus ensure a more quantitative measurement of that isotope as determined by the dual-label counting procedure. As seen in Figure 3A, the ^{3}H/^{14}C ratio of L-carnitine produced by the bacterial hydroxylase remained essentially constant between 0 and 100% reaction. Since this enzyme exhibits a very small tritium isotope effect (<1.5) and the ^{3}H/^{14}C ratio of the remaining substrate therefore increases only slightly, we would not expect a significant increase in the product ^{3}H/^{14}C ratio even if some *pro-S* hydrogen atom abstraction were occurring. We interpret the slight rise in this ratio at $f > 0.8$ to be the result of an α-secondary tritium kinetic isotope effect of 1.10. This value was obtained by assuming an intrinsic α-secondary tritium kinetic isotope effect of 1.31 (see below) and a forward commitment of 2.1 for this enzyme, calculated from secondary kinetic isotope effect data previously reported (Blanchard & Englard, 1983).

In contrast to that situation, the large primary tritium kinetic isotope effect exhibited by the calf liver enzyme, resulting in a steeply increasing ^{3}H/^{14}C ratio of the remaining substrate, should result in a substantial increase in the ^{3}H/^{14}C ratio of the isolated product at high percentages of reaction. As seen in Figure 3B, no such increase was observed, and the slight increase in the product ^{3}H/^{14}C ratio could be fully accounted for by an α-secondary tritium kinetic isotope effect of 1.31. This value agrees with the independently obtained value of 1.31 previously reported for the calf liver enzyme (Blanchard & Englard, 1983).

Scheme II: Proposed Topographical Arrangement of Ferryl–Oxo Oxidant and Substrate at Active Site of γ-Butyrobetaine Hydroxylase



Our proposal of a radical mechanism for the reaction catalyzed by γ-butyrobetaine hydroxylase and the stereochemical results reported here at first seem contradictory. Thus, for hydroxylations catalyzed by P-450$_{CAM}$ (Gelb et al., 1982) and P-450$_{LM2}$ (Groves et al., 1978), the *lack* of stereospecificity has been used most convincingly to argue for a radical mechanism. We have previously drawn heavily on both chemical precedents (Groves & McClusky, 1976; Groves & Van Der Puy, 1976) and the enzymatic studies mentioned above for support of such a radical mechanism for the reaction catalyzed by γ-butyrobetaine hydroxylase. A more recent study, however, has pointed out large differences in the regioselectivity of hydroxylation of alicyclic hydrocarbons by P-450$_{CAM}$ and P-450$_{LM2}$ (White et al., 1984). Although both reactions proceed through radical intermediates, P-450$_{CAM}$ yields a single hydroxylated product for each substrate, while P-450$_{LM2}$ yields two or more isomeric products. Thus, while the substrate is topologically constrained in P-450$_{CAM}$, considerable movement of the substrate is allowed in the P-450$_{LM2}$ active site, so that the product distribution reflects the chemical reactivity of the various hydroxylatable positions.

We now propose that a further topological constraint occurs during the hydroxylation of γ-butyrobetaine by γ-butyrobetaine hydroxylase. We suggest that the group abstracting the hydrogen atom and the group adding the oxygen atom are located in the same topographical orientation, and that they are chemically identical. In the cytochrome P-450$_{CAM}$ system, the lack of stereospecificity was used to argue for the nonequivalence of the species abstracting hydrogen and that adding oxygen (Gelb et al., 1982). Since, a priori, differences in the bond strengths of the C$_{3(R)}$ and C$_{3(S)}$ carbon–hydrogen bonds are unlikely, we conclude that the ferryl–oxo species is positioned adjacent to the 3(*R*) hydrogen atom and that the 3(*S*) hydrogen atom is shielded from the oxidant (Scheme II). Hydrogen atom abstraction leads to formation of a planar carbon radical, which is firmly anchored by charged moieties (carboxylate and quaternary amine) at both ends of the sub-

[6] As one reviewer commented, if a stereoselective process were operating to give 94% *pro-R* hydrogen removal and 6% *pro-S* removal, this effect would be counterbalanced in the case of the liver hydroxylase by the large primary deuterium kinetic isotope effect of ~6.5. In this case, one would only expect to see approximately 1% protiocarnitine. On the other hand, the bacterial enzyme would give the full 6% protiocarnitine product, because of the very small isotope effect.

[7] Based on the extent of ^{3}H release from [3(*S*)-3-^{3}H]-γ-butyrobetaine compared to that of [3(*R*)-3-^{3}H]-γ-butyrobetaine in the experiment shown in Figure 1 (cf. footnote 4).

strate and not free to move in the active site. Addition of the hydroxyl radical to the *re* face of the carbon radical results in formation of (*R*)-carnitine. This topographical arrangement predicts both the observed retention of configuration and the stereospecificity of hydrogen atom abstraction, while allowing for the intermediacy of a carbon radical.

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Chorismate Mutase–Prephenate Dehydrogenase from *Escherichia coli*: Positive Cooperativity with Substrates and Inhibitors

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ABSTRACT: Investigations have been made at pH 6.0 of the effect of chorismate and adamantane derivatives on the mutase and dehydrogenase activities of hydroxyphenylpyruvate synthase from *Escherichia coli*. When used over a wide range of concentrations, chorismate 5,6-epoxide, chorismate 5,6-diol, adamantane-1,3-diacetate, adamantane-1-acetate, adamantane-1-carboxylate, and adamantane-1-phosphonate give rise to nonlinear plots of the reciprocal of the initial velocity of each reaction as a function of the inhibitor concentration. The inhibitors do not induce the enzyme to undergo polymerization and have only a small effect on the $s_{20,w}$ value of the enzyme as determined by using sucrose density gradient centrifugation. At low substrate concentration, low concentrations of adamantane-1-acetate cause activation of both the mutase and dehydrogenase activities while at higher concentrations this compound functions as an inhibitor. When chorismate and prephenate are varied over a wide range of concentrations, double-reciprocal plots of the data indicate that the reactions exhibit positive cooperativity. The addition of albumin eliminates the cooperative interactions associated with substrates but has little effect on those associated with inhibitors.

The immediate precursor for the biosynthesis of tyrosine in the Enterobacteriacae is 4-hydroxyphenylpyruvate whose formation from chorismate (eq 1) is catalyzed by a bifunctional enzyme that possesses chorismate mutase (EC 5.4.99.5) and prephenate dehydrogenase (EC 1.3.1.12) activities. The enzyme, which has also been referred to as hydroxyphenylpyruvate synthase (Christopherson et al., 1983), is a dimeric

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