γ-Butyrobetaine Hydroxylase: Primary and Secondary Tritium Kinetic Isotope Effects†

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ABSTRACT: Primary and secondary tritium kinetic isotope effects have been determined for the reactions catalyzed by purified preparations of γ-butyrobetaine hydroxylase obtained from *Pseudomonas* sp AK 1 and from calf liver. With [*methyl*-^{14}C,(3*R*)-3-^{3}H]-γ-butyrobetaine as substrate, the bacterial hydroxylase was found to exhibit a primary $^{T}(V/K)$ of 1.3–1.5. This value was determined from measurements of either the specific activity of the medium $^{3}H_2O$ or of the ratio of $^{3}H/^{14}C$ in the residual γ-butyrobetaine. Under identical conditions of analysis, the calf liver enzyme exhibited a primary $^{T}(V/K)$ of ~15. With [*methyl*-^{14}C,(4*R*)-4-^{3}H]-γ-butyrobetaine as substrate, a β-secondary $^{T}(V/K)$ of 1.10 has been determined for the calf liver hydroxylase; this supports the existence in the reaction mechanism of an sp^2-hybridized transition state. A large normal value of 1.31 for the α-secondary $^{T}(V/K)$, as derived from measurements with [*methyl*-^{14}C,2,3-^{3}H]-γ-butyrobetaine, suggests that the motions of the primary and α-secondary hydrogens are coupled in the C–H cleavage step and resulting synchronous rehybridization. A chemical mechanism involving homolytic cleavage of the C–H bond at the position undergoing hydroxylation is proposed and discussed.

The enzyme γ-butyrobetaine hydroxylase [4-trimethylaminobutyrate,2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1] catalyzes the aliphatic hydroxylation of γ-butyrobetaine to form L-carnitine (eq 1). The

$$\gamma\text{-butyrobetaine} + O_2 + \alpha\text{-ketoglutarate} \rightarrow \text{L-carnitine} + CO_2 + \text{succinate} \quad (1)$$

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 review, see Abbott & Udenfriend (1974)]. The dioxygenase nature of the reaction has been demonstrated with $^{18}O_2$ labeling (Lindblad et al., 1969), and hydroxylation has been shown to proceed with displacement of one of the two C_3 hydrogen atoms and with retention of configuration (Englard & Midelfort, 1978). 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. Under certain conditions, however, the enzyme catalyzes α-ketoglutarate decarboxylation in the absence of γ-butyrobetaine hydroxylation (Holme et al., 1982); such uncoupling of decarboxylation of α-ketoglutarate from hydroxylation has also been reported for other representatives of this class of enzymes such as prolyl hydroxylase (Counts et al., 1978; Rao & Adams, 1978) and thymine 7-hydroxylase (Hsu et al., 1981; Holme & Lindstedt, 1982). Significant hydroxylation of γ-butyrobetaine occurs only in the presence of a reductant such as ascorbate, and addition of catalase to the assay system both stimulates the maximum velocity of the reaction and protects the enzyme against hydrogen peroxide inactivation (Blanchard et al., 1982).

The mechanism by which the α-ketoglutarate-coupled dioxygenases catalyze hydroxylation at unactivated aliphatic carbon atoms remains unknown. Mechanisms that have been proposed include (1) a carbanion mechanism (Holme et al., 1968; Lindblad et al., 1969), (2) an oxenoid mechanism involving persuccinic acid as the oxygen-inserting reagent (Hamilton, 1971), and (3) a radical mechanism in which an intermediate ferryl–oxo intermediate abstracts a hydrogen atom from the position undergoing hydroxylation (Siegel, 1979; Holme & Lindstedt, 1982). We report here on the primary and secondary tritium kinetic isotope effects for the reactions catalyzed by both the bacterial and calf liver γ-butyrobetaine hydroxylases. The differences in the observed isotope effects exhibited by the two enzymes and the implication of these results regarding the mechanism of hydroxylation are discussed.

Materials and Methods

γ-Butyrobetaine hydroxylase, isolated and purified from *Pseudomonas* sp AK 1 (Lindstedt et al., 1970, 1977), was generously provided by Dr. Göran Lindstedt (University of Gothenburg, Gothenburg, Sweden). The enzyme was homogeneous as determined by SDS–polyacrylamide gel electrophoresis and had a specific activity of 21 units/mg. 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, aconitase, isocitrate dehydrogenase, and glutamate dehydrogenase were purchased from Sigma Chemical Co. NADP was obtained from Boehringer-Mannheim. [^{14}C]-Methyl iodide (58 mCi/mmol) was purchased from Amersham International Ltd. $^{3}H_2O$ (100 mCi/mL) and 4-amino-[2,3(N)-^{3}H]butyrate (27.2 Ci/mmol) were products from New England Nuclear. Methyl iodide (puriss) and the proton sponge 1,8-bis(dimethylamino)naphthalene were purchased from Tridom-Fluka and Aldrich Chemical Co., respectively. All other chemicals and reagents were of the highest purity commercially avilable.

[(4*R*)-4-^{3}H]-γ-Butyrobetaine was prepared by the exhaustive N-methylation of 4-amino[(4*R*)-4-^{3}H]butyric acid. The latter compound was obtained by enzymatic decarboxylation of L-glutamate in $^{3}H_2O$, a reaction that proceeds

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0006-2960/83/0422-5922$01.50/0

stereospecifically with retention of configuration (Yamada & O'Leary, 1978). Progress of the glutamate decarboxylase reaction was monitored with ninhydrin spray reagent after the chromotographic 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 the decarboxylation of L-glutamate, 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 0–3 N HCl gradient, and the fractions containing the stereospecifically labeled 4-amino[(4*R*)-4-^{3}H]butyrate were pooled and concentrated by rotary evaporation. This material was then subjected to exhaustive methylation as described previously (Englard et al., 1978), and the quaternarized product was purified chromatographically on Dowex 50 (H^+). Elution of the column was carried out with a linear 0–5.8 N HCl gradient, peak radioactive fractions emerging at the position of elution of γ-butyrobetaine were pooled, and HCl was removed by repeated rotary evaporation. [(4*R*)-4-^{3}H]-γ-Butyrobetaine (0.87 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 ion-exchange chromatography on Technicon type C resin (LaBadie et al., 1976).

[(3*R*)-3-^{3}H]-γ-Butyrobetaine was prepared by methylation of 4-amino[(3*R*)-3-^{3}H]butyric acid. The latter compound was obtained through a stereospecific synthesis involving the following sequence of enzymatic reactions (Englard & Midelfort, 1978): *cis*-aconitate + ^3H_2O → *threo*-D_s-[(3*R*)-3-^{3}H]isocitrate → [(3*R*)-3-^{3}H]-α-ketoglutarate → L-[(3*R*)-3-^{3}H]glutamate → 4-amino[(3*R*)-3-^{3}H]butyrate. The enzymatically prepared 4-amino[(3*R*)-3-^{3}H]butyric acid was purified as described above and its specific radioactivity determined to be 1.63 μCi/μmol. In control experiments using large quantities of the bacterial hydroxylase, greater than 98% of the radioactivity initially present in [(3*R*)-3-^{3}H]-γ-butyrobetaine was released into water.

[*methyl*-^{14}C]-γ-Butyrobetaine (1.07 μCi/μmol) and [2,3-(N)-^{3}H]-γ-butyrobetaine (4.6 μCi/μmol) were synthesized by exhaustive methylation of 4-aminobutyric acid with [^{14}C]-methyl iodide and of 4-amino[2,3(N)-^{3}H]butyric acid with unlabeled methyl iodide (Englard et al., 1978) and purified by ion-exchange chromatography as described above. In control experiments using large quantities of the bacterial hydroxylase, 24.8% of the radioactivity initially present in [2,3(N)-^{3}H]-γ-butyrobetaine was released into water. Oxalylglycine was prepared according to Visconti (1946) in 40% yield.

Initial Velocity Measurements and Isotope-Exchange Studies. γ-Butyrobetaine hydroxylase activity was routinely assayed at 34 °C essentially as described previously (Englard et al., 1978). The standard assay system contained in 0.5 mL 100 mM Hepes[1] plus 15 mM K_2HPO_4, pH 7.7, 3 mM α-ketoglutarate, 15 mM sodium ascorbate, 1.2 mM $Fe(NH_4)_2(SO_4)_2$, 1.4–2.0 mg/mL catalase (65 000 units/mg), and either 0.4 or 2.4 mM [2,3(N)-^{3}H]-γ-butyrobetaine for the calf liver and bacterial hydroxylase assays, respectively. These assay mixtures were air saturated ($[O_2]$ = 240 μM), and incubations were carried out for 1 h with vigorous reciprocal shaking. Reactions were initiated by addition of either 0.8 μg of *Pseudomonas* sp AK 1 γ-butyrobetaine hydroxylase or 10–20 μg of the calf liver hydroxylase and terminated by addition of trichloroacetic acid (TCA) to a final concentration of 5%. ^3H_2O was separated from the unreacted γ-butyrobetaine and its hydroxylated product by applying the deproteinized solutions to columns (0.5 × 2.2 cm) of Dowex 50 (H^+) and washing the resin with H_2O. Samples of the water effluent in 10 mL of hydrofluor (National Diagnostics, Somerville, NJ) were counted in an LKB Rack Beta liquid scintillation counter.

Isotope-exchange studies were carried out under identical conditions of assay except that the [2,3(N)-^{3}H]-γ-butyrobetaine was replaced with unlabeled γ-butyrobetaine and each 0.5-mL reaction mixture contained 20 μCi of ^3H_2O. At various time points (corresponding to approximately 5, 10, 15, 20, 30, and 60% conversion of substrate into product), aliquots were removed, acidified, and applied to 0.5 × 5 cm columns of Dowex 50 (H^+). The columns were washed with water until the effluent was free of any radioactivity, and the residual γ-butyrobetaine (plus the carnitine formed) was then eluted with 2 × 2 mL of 4 N HCl. These fractions were counted for radioactivity after removal of the HCl by rotary evaporation.

Isotope exchange was also measured with both [(3*R*)-3-^{3}H]- and [2,3(N)-^{3}H]-γ-butyrobetaine in the presence of oxalylglycine, a linear competititive inhibitor with respect to α-ketoglutarate (K_i = 200 μM). The composition of the assays and the method of analysis were identical with those described above for measurement of ^3H_2O release into the reaction medium.

Isotope-Effect Studies. For the isotope-effect studies, single reaction mixtures were prepared that contained the following components added at the indicated concentrations: 100 mM Hepes plus 15 mM K_2HPO_4, pH 7.7, 5 mM α-ketoglutarate, 15 mM sodium ascorbate, 1.2 mM $Fe(NH_4)_2(SO_4)_2$, 1.4–2.0 mg/mL catalase, 100–250 μM of the appropriately labeled [^{3}H]-γ-butyrobetaine, 14 μM [*methyl*-^{14}C]-γ-butyrobetaine, and sufficient enzyme to achieve complete conversion to carnitine in 4–5 h. Aliquots (0.25–0.50 mL) were removed at various intervals, and TCA was added to a final concentration of 5%. Precipitated protein was removed by centrifugation, and small aliquots of the deproteinized solutions were analyzed for ^3H_2O by the Dowex 50 (H^+) minicolumn method as described above. The remainder of the deproteinized solutions was applied to Technicon type C resin, and the ^{14}C,^{3}H-labeled L-carnitine and γ-butyrobetaine were separated with a sodium citrate/sodium hydroxide pH gradient (LaBadie et al., 1976). L-Carnitine and γ-butyrobetaine were well resolved and eluted at pH values of 4.5–4.6 and 5.1–5.2, respectively. Aliquots (1 or 2 mL) of the peak fractions in 15 mL of Hydrofluor scintillation fluid were counted for ^{3}H and ^{14}C content at 40–50% and 75–90% efficiencies, respectively. Each sample was counted a minimum of 5 times, and 5 × 10^4 dpm of ^{14}C was allowed to accumulate each time. $^3H/^{14}C$ ratios were calculated after subtraction of appropriate blank values for each channel. These ratios were used for the calculation of isotope effects.

Data Analysis. The tritium isotope effects reported here are V/K effects (Northrop, 1975), and the nomenclature used is that of Northrop (1977). Primary $^T(V/K)$ values were determined in two ways. In the first method, we measured the specific activity of the ^3H_2O released from the C_{3R} position, and used

$$^T(V/K) = \log (1.0 - f)/\log (1.0 - DPM_f/DPM_0) \quad (2)$$

where f is the fractional percent conversion of γ-butyrobetaine

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

to carnitine, DPM_f is the dpm of ^3H_2O at f, and DPM_0 is the dpm of ^3H_2O at 100% conversion. This equation does not require that the dpm of ^3H_2O be corrected for the percentage of the complete reaction and dilution of the released ^3H into the unlabeled medium water. The other method measured the $^3H/^{14}C$ ratio of the remaining and reisolated γ-butyrobetaine as a function of percent reaction, and $^T(V/K)$ was calculated from (Melander, 1960)

$$^T(V/K) = 1.0/[1.0 + \log (R_f/R_0)/\log (1.0 - f)] \quad (3)$$

where R_f is the $^3H/^{14}C$ ratio of γ-butyrobetaine at fractional percent conversion f and R_0 is the $^3H/^{14}C$ ratio of γ-butyrobetaine at 0% conversion.

Secondary isotope effects were calculated from

$$^T(V/K) = \log (1.0 - f)/\log (1.0 - fR_f/R_0) \quad (4)$$

where R_f and R_0 are the $^3H/^{14}C$ ratios of the isolated L-carnitine at fractional percentages of completion of the reaction and at complete conversion, respectively.

Results

Isotope-Exchange Studies. Exchange studies with unlabeled substrates capable of undergoing hydroxylation were performed in the presence of ^3H_2O to ensure that the observed enzymatically catalyzed release of ^3H from the C_{3R} position of γ-butyrobetaine into the medium H_2O was the result of net L-carnitine formation rather than a consequence of an unproductive reaction cycle leading to ^3H release. For both the bacterial and calf liver enzymes, no back-exchange of ^3H from solvent into substrate could be demonstrated (data not shown) at specific activities of ^3H_2O that would have allowed us to observe 2% back-exchange with a ^3H isotope effect of 10.

A similar lack of exchange was demonstrated under conditions of "uncoupling", i.e., when the cosubstrate α-ketoglutarate was replaced by oxalylglycine, an analogue that cannot undergo decarboxylation and inhibits the hydroxylation reaction competitively with respect to α-ketoglutarate. This was determined by measuring the release of tritium from suitably labeled substrates into the medium. Thus, no release of ^3H from [(3*R*)-3-^{3}H]-γ-butyrobetaine into the aqueous medium occured with either the bacterial or mammalian enzyme in the presence of oxalylglycine (data not shown). Identical data were obtained with [2,3(N)-^{3}H]-γ-butyrobetaine.

Primary Isotope Effects. Our previous studies with γ-butyrobetaine hydroxylase isolated from mammalian liver sources indicated a significant ^3H discrimination at the carbon position undergoing hydroxylation (Englard et al., 1978; Kondo et al., 1981). Because of the low specific activity of the mammalian enzyme relative to that of the *Pseudomonas* sp AK 1 hydroxylase, we initiated the present studies by measuring the primary isotope effects on the hydroxylation reaction catalyzed by the bacterial enzyme. When [*methyl*-^{14}C,(3*R*)-3-^{3}H]-γ-butyrobetaine was used as substrate and release of ^3H_2O was measured at low and at 100% conversion of substrate into product, the data in Table I were obtained. An average value of $^T(V/K) = 1.54$ was found for the reaction catalyzed by the bacterial hydroxylase.

The primary tritium kinetic isotope effect was also determined from $^3H/^{14}C$ ratios of the reisolated γ-butyrobetaine. As seen in Figure 1A, the experimental points yield an average $^T(V/K)$ value of 1.30, which is in substantial agreement with the value obtained by measuring the ^3H content of the medium water. By use of this same labeled compound (that is, [*methyl*-^{14}C,(3*R*)-3-^{3}H]-γ-butyrobetaine) as substrate with the purified calf liver hydroxylase, the primary tritium kinetic isotope effect as measured by analysis of the $^3H/^{14}C$ ratio of the reisolated γ-butyrobetaine was determined to be 15 (Figure 1B). This latter value is in excellent agreement with the value obtained by measuring the radioactivity of the medium ^3H_2O as a function of percent substrate conversion. The magnitude of the primary isotope effect as measured in this manner is also unaffected by the absolute initial concentration of α-ketoglutarate in the reaction mixture. Thus, as shown in Table II, when the concentration of α-ketoglutarate was varied in the range from $0.12K_m$ to $6K_m$, there was no significant change in the determined value for $^T(V/K)$.

Table I: Primary Tritium Kinetic Isotope Exhibited by *Pseudomonas* sp AK 1 γ-Butyrobetaine Hydroxylase[a]

f (% rxn)[b]	dpm of ^3H_2O	R_f(corr) ($\times 10^{-5}$)[c]	$^T(V/K)$ for [(3*R*)-3-^{3}H]-γ-butyrobetaine[d]
1.2 (1 min)	2 890	2.426	1.559
2.4 (2 min)	5 930	2.487	1.524
3.6 (3 min)	8 930	2.498	1.521
4.8 (4 min)	11 790	2.472	1.540
6.0 (5 min)	14 640	2.456	1.554
7.2 (6 min)	17 690	2.474	1.546
8.4 (7 min)	20 240	2.425	1.581
9.6 (8 min)	23 760	2.492	1.542
10.8 (9 min)	27 040	2.521	1.527
100.0	377 500[e]	3.775	mean: 1.544 ± 0.019

[a] Experimental methods are described in the text. [b] Calculated from % reaction ([^{14}C]carnitine/^{14}C total) at 10 min (f = 0.12) assuming linear reaction in the first 10 min. [c] R_f(corr) = dpm of $^3H_2O/f$. [d] Calculated from eq 2. [e] Average of three separate control experiments with 10× enzyme.

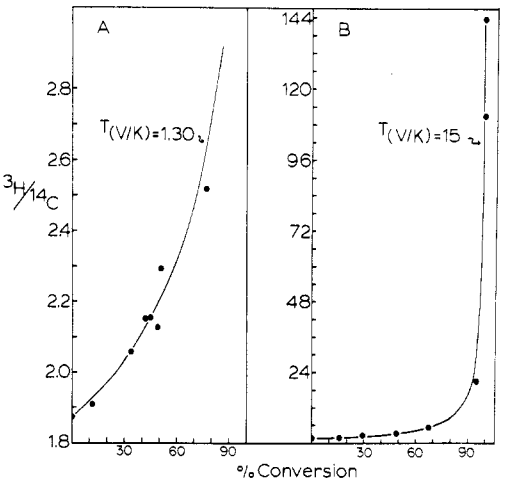


FIGURE 1: Primary $^T(V/K)$ measured by analyzing the $^3H/^{14}C$ ratio of unreacted γ-butyrobetaine. The bacterial (panel A) and calf liver (panel B) γ-butyrobetaine hydroxylases were incubated with the complete system containing [*methyl*-^{14}C,(3*R*)-3-^{3}H]-γ-butyrobetaine, and at the intervals indicated, aliquots were removed, and γ-butyrobetaine was isolated and counted. The experimental points are shown, and the lines are fits of the data to eq 3 with $^T(V/K)$ = 1.3 and 15, respectively.

Secondary Tritium Kinetic Isotope Effects. Because of the large $^T(V/K)$ value for the primary isotope effect obtained with the calf liver enzyme, we examined the kinetic β-secondary tritium isotope effects for the hydroxylation reaction catalyzed by that enzyme. Using [*methyl*-^{14}C,(4*R*)-4-^{3}H]-γ-butyrobetaine as substrate and measuring the $^3H/^{14}C$ ratio of the isolated L-carnitine formed, we determined the β-secondary $^T(V/K)$ to be 1.105 (Table III).

When [*methyl*-^{14}C,2,3(N)-^{3}H]-γ-butyrobetaine was used to measure the secondary isotope effects for the hydroxylation

Table II: Dependence of Primary Tritium Kinetic Isotope Effect Exhibited by Calf Liver Hydroxylase on Concentration of α-Ketoglutarate[a]

α-KG (mM)	f (% rxn)[b]	dpm of ^3H_2O		$^T(V/K)$ for [(3*R*)-3-^{3}H]-γ-butyrobetaine[c]
5	0.16	130		13.34
	0.33	240		14.56
	0.49	320		16.40
	0.66	520		13.49
	0.83	600		14.35
	100.00[d]	1.053×10^6	mean:	14.43 ± 1.22
1	0.15	120		13.89
	0.31	230		14.19
	0.47	440		11.18
	0.62	530		12.47
	0.78	630		13.11
	100.00[d]	1.054×10^6	mean:	12.97 ± 1.21
0.5	0.14	90		16.23
	0.28	200		15.27
	0.43	350		12.99
	0.57	440		13.65
	0.72	600		12.76
	100.00[d]	1.054×10^6	mean:	14.18 ± 1.51
0.1	0.19	120		17.43
	0.38	330		12.48
	0.58	490		12.58
	0.77	670		12.10
	0.96	870		11.75
	100.00[d]	1.054×10^6	mean:	13.33 ± 2.35

[a] Experimental methods are described in the text. [b] Calculated from % reaction ([^{14}C]carnitine/^{14}C total) assuming linear reaction in the first 10 min. [c] Calculated from eq 2. [d] 100% conversion was obtained by the addition of 4 μg of bacterial hydroxylase at 12 min.

Table III: β-Secondary $^T(V/K)$ Exhibited by Calf Liver γ-Butyrobetaine Hydroxylase[a]

f (% rxn)[b]	^{3}H/^{14}C for carnitine		$^T(V/K)$ of [(4*R*)-4-^{3}H]-γ-butyrobetaine[c]
2.6	2.872		1.034
5.4	2.669		1.115
7.0	2.673		1.114
8.7	2.694		1.106
11.1	2.610		1.145
14.7	2.589		1.158
18.1	2.804		1.064
19.8	2.719		1.102
100.00	2.967[d]	mean:	1.105 ± 0.040

[a] Experimental methods are described in the text. [b] Calculated from ([^{14}C]carnitine/^{14}C total). [c] Calculated from eq 4. [d] Average of four 100% conversion values.

reactions catalyzed by both the bacterial and calf liver enzymes, the data in Figure 2 were obtained. The bacterial hydroxylase exhibited a $^T(V/K)$ of 1.21 (Figure 2A), and the calf liver enzyme exhibited a $^T(V/K)$ of 1.59 (Figure 2B). These values are a combination of both an α-secondary effect (at C_3) and of two β-effects (at C_2). The β-kinetic secondary tritium isotope effects can be factored out by assuming that the β-effect at C_4 is equal to the β-effect at C_2,[2] and thus, for the calf liver hydroxylase for which the β-secondary effect at C_4 has been determined, the α-secondary effect can be calculated to be 1.302 (=1.59/1.105[2]).

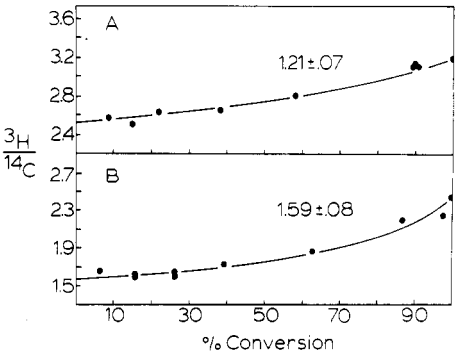


FIGURE 2: Secondary $^T(V/K)$ measured by analyzing the ^{3}H/^{14}C ratio of L-carnitine. The bacterial (panel A) and calf liver (panel B) γ-butyrobetaine hydroxylases were incubated in the complete system containing [*methyl*-^{14}C,2,3(N)-^{3}H]-γ-butyrobetaine, and at the intervals indicated, aliquots were removed, and L-carnitine was isolated and counted. The experimental points are shown, and the lines are fits of the data to eq 4, with $^T(V/K)$ = 1.21 and 1.59, respectively.

Discussion

Isotope effects as determined for enzyme-catalyzed reactions are powerful probes for assessing chemical mechanisms and transition-state structures. To date, there have been few reports on the use of this method to analyze the reactions catalyzed by α-ketoglutarate-coupled dioxygenases. For the hydroxylation of [1-^{14}C,2,3-^{3}H]-γ-butyrobetaine by a crude ammonium sulfate fraction of rat liver, Lindstedt (1967) reported an increase of 6.7-fold in the ^{3}H/^{14}C ratio of the residual substrate at 92% conversion into L-carnitine. These data can be used to calculate a value of 4.05 for the primary $^T(V/K)$ (eq 3). More recently, inter- and intramolecular $^T(V/K)$ values of 1.0 and 6.5, respectively, were reported for the α-ketoglutarate-linked hydroxylation of [7-*methyl*-^{3}H]thymine by thymine 7-hydroxylase (Holme & Lindstedt, 1982). The synthesis in our laboratory of several stereospecifically labeled tritiated species of γ-butyrobetaine has allowed us to determine primary and secondary isotope effects on the enzyme-catalyzed hydroxylation. The method is ideally suited to the study of α-ketoglutarate-coupled dioxygenases, in particular γ-butyrobetaine hydroxylase, due to their low catalytic turnover and lack of a sensitive continuous assay method.

Isotope Exchange. These studies were undertaken to determine whether isotope exchange occured between the C_{3R} position of γ-butyrobetaine and solvent since the effect of such an exchange would be to reduce the observed isotope effect (Robinson & Rose, 1972). Neither the bacterial nor the calf liver hydroxylase exhibited demonstrable back-exchange from solvent into substrate. Furthermore, oxalylglycine, an analogue of α-ketoglutarate that cannot undergo decarboxylation, did not promote release of ^{3}H from the C_{3R} position of γ-butyrobetaine. This point is significant since nonhydroxylatable analogues of γ-butyrobetaine have been shown to induce the "uncoupled" decarboxylation of α-ketoglutarate (Holme et al., 1982). It thus appears that either the decarboxylation reaction precedes the C–H bond cleavage step or, alternatively, if C–H bond cleavage occurs prior to α-ketoglutarate decarboxylation, then that hydrogen atom is unable to exchange with the solvent until a subsequent irreversible step.

Primary Kinetic Isotope Effects. The bacterial γ-butyrobetaine hydroxylase exhibits a very small, normal tritium kinetic isotope effect when measured either by determination of the extent of ^{3}H released into the aqueous medium to yield ^3H_2O [$^T(V/K)$ = 1.54 ± 0.02, Table I] or by measurement of the ^{3}H/^{14}C ratio of the remaining γ-butyrobetaine [$^T(V/K)$

[2] The data of Cook et al. (1980) demonstrate that essentially identical β-secondary equilibrium isotope effects are observed for the conversion of L-malate to [(3*R*)-3-^{2}H]oxaloacetate (0.945) and for the conversion of [2,3,3,4,4-$^2H_5$]glutamate to [3,3,4,4-$^2H_4$]-α-ketoglutarate (0.948/deuterium). β-Secondary equilibrium isotope effects are apparently unaffected by the nature of adjoining functionalities (i.e., γ-substituents) and support our assumption of equality of effects at C_2 and C_4 of γ-butyrobetaine.

= 1.30, Figure 1A]. The isotope effects obtained by the two methods are in substantial agreement.

The primary tritium kinetic isotope effect exhibited by the calf liver hydroxylase, determined by measuring the $^3H/^{14}C$ ratio of unreacted γ-butyrobetaine, was approximately 15 (Figure 1B). Similar large values were obtained by measuring the specific activity of ^3H_2O (Table II). The agreement between the two methods is excellent considering the very different sources of possible error in the two methods.[3]

The equation that describes the expression of a tritium isotope effect is (Northrop, 1975)

$$^T(V/K) = (^Tk_{chem} + c_f + c_r{}^TK_{eq})/(1.0 + c_f + c_r) \quad (5)$$

where $^Tk_{chem}$ is the intrinsic tritium kinetic isotope effect, $^TK_{eq}$ is the tritium equilibrium isotope effect, and c_f and c_r are the forward and reverse commitment factors, respectively, for γ-butyrobetaine. In the case of γ-butyrobetaine hydroxylase, where there is a single isotope-sensitive step, c_f will be the ratio of the rate constant for the carbon–hydrogen bond cleavage step to the net rate constant for the release of γ-butyrobetaine from the enzyme. The reverse commitment, c_r, determined by the competitive method used here, is calculated for the first irreversible step, which may be the first product-release step, or some other irreversible step occurring before C-H bond cleavage. The measured $^T(V/K)$ will be smaller than $^Tk_{chem}$ if either c_f or c_r is large. In the case of α-ketoglutarate-coupled dioxygenases, we assume that the reverse commitment is negligible because of the irreversibility of the overall reaction and the presence of a number of irreversible steps, including the decarboxylation of α-ketoglutarate and the cleavage of the O–O bond.[4] We therefore conclude that the forward commitments to a large extent are responsible for the values of $^T(V/K)$ exhibited by the hydroxylases. If we assume, however, that the calf liver enzyme has *no* forward commitment, then the experimentally determined primary $^T(V/K)$ value corresponds to the intrinsic primary tritium kinetic isotope effect, $^Tk_{chem}$. If the commitment is in fact zero, and thus $^T(V/K) = {}^Tk_{chem}$, then the Swain–Schaad equation (Swain et al., 1958) allows us to calculate the equivalent $^Dk_{chem}$ of 6.55, which compares favorably with reported intrinsic primary deuterium isotope effects on enzyme-catalyzed reactions of between 5 and 8.5.[5] While these calculations are rigorously correct only for the case where there are no forward (or reverse) commitments, the magnitude of the primary $^T(V/K)$ exhibited by the calf liver hydroxylase supports the assumption that the calf liver has no, or at least very small, commitments.[6] The primary kinetic isotope effect exhibited by the bacterial enzyme is small (1.3–1.5), whereas the secondary kinetic isotope effect (see below) exhibited by that enzyme is relatively large. Thus, while the bacterial enzyme certainly has a larger forward commitment than the calf liver enzyme on the basis of both primary and secondary isotope effect data, additional differences in the transition states and/or chemical mechanisms of the two enzymes may account for the expression of a very small primary kinetic isotope effect by the bacterial enzyme.

The primary kinetic isotope effect exhibited by the calf liver γ-butyrobetaine hydroxylase is independent of the concentration of α-ketoglutarate (Table II). The possibility of distinguishing between kinetic mechanisms by examination of the consequences of varying the cosubstrate concentration on an isotope effect has been discussed by Klinman (1980) for dopamine β-hydroxylase and by Cook & Cleland (1981) for alcohol dehydrogenase. Lack of an observed effect of changing α-ketoglutarate concentrations on the primary isotope effect as determined for calf liver γ-butyrobetaine hydroxylase accords with an ordered Ter Ter mechanism, as has been proposed for several other α-ketoglutarate linked dioxygenases. Thus, for prolyl hydroxylase (Myllyla et al., 1977), lysyl hydroxylase (Puistola et al., 1980), and thymine 7-hydroxylase (Holme & Lindstedt, 1982), the steady-state kinetic evidence supports a mechanism in which α-ketoglutarate is bound to the enzyme first, followed by the subsequent binding of either the substrate that undergoes hydroxylation or molecular oxygen. Our results also support such an ordered sequence in which α-ketoglutarate binds to the enzyme before γ-butyrobetaine.[7] On the basis of the presently available data, we cannot distinguish between an ordered sequence in which O_2 binding precedes the binding of γ-butyrobetaine or vice versa. The dependence of the primary isotope effect on the concentration of O_2, or the lack of it, would discriminate between these two possibilities.

Secondary Tritium Isotope Effect. In view of the small magnitude of β-secondary tritium kinetic isotope effects, this effect was measured with the calf liver hydroxylase because of the initial evidence suggesting a relatively low commitment for this enzyme. As seen in Table III, a β-secondary $^T(V/K)$ value of 1.105 was determined with [*methyl*-^{14}C,(4*R*)-4-^{3}H]-γ-butyrobetaine as substrate. Again, if one assumes that $c_f = 0$, and thus that the Swain–Schaad equation is valid, then the equivalent calculated deuterium effect would be 1.072, which can be compared to the reported average β-secondary deuterium equilibrium isotope effects of 1.057 per deuterium

[3] The measurement of specific radioactivities, or $^3H/^{14}C$ ratios, of remaining substrates suffers from an insensitivity in distinguishing between large isotope effects, as Cleland (1982) has discussed. The change in the $^3H/^{14}C$ ratio as the reaction proceeds is very small at low percentages of conversion and then increases steeply, approaching infinity as f approaches 1.0. Since the largest changes in the $^3H/^{14}C$ ratio, and the ability to distinguish between large isotope effects, occur at high percentages of conversion, it is important to obtain as much data as possible in this region. Difficulties in the precise determination of kinetic isotope effects arise from three factors: (1) the inability to precisely determine f at high percentages of conversion, (2) the increasingly small amounts of total radioactivity, and (3) the sharply increasing $^3H/^{14}C$ ratios that make these ratios difficult to measure. Because of this insensitivity, we also determined the primary tritium kinetic isotope effect by measuring the product, ^3H_2O. Although it is difficult to precisely determine f at very low percentages of conversion, this method relies less on the precise determination of f than on the ability to accurately determine the specific activity of the product. When one couples a very low percentage of total reaction with a large isotope effect, the total radioactivity to be determined, as ^3H_2O, is very small, and it is important to count these samples for long times to obtain statistically significant specific activities (in some cases, individual samples may have to be counted for as long as 2 h).

[4] This is certainly true for the external reverse commitment, that is, the commitment factors for the products; however, internal reverse commitments (that is, re-formation of the C–H bond) would also lower $^T(V/K)$. These internal commitments are probably negligible as well due to our inability to demonstrate any back-exchange of ^3H from solvent into substrate and the strong oxidizing nature of the metal–oxo complex.

[5] Values for $^Dk_{chem}$ reported include 5–8 for malic enzyme (Schimerlik et al., 1977), 5.7 for yeast alcohol dehydrogenase (Cook & Cleland, 1981b), 6.3 for liver alcohol dehydrogenase (Cook & Cleland, 1981c), 7.4 for ethanolamine deaminase (Weisblat & Babior, 1971), 7–8.5 for the enolase-catalyzed elimination of HCl from chlorolactate (Stubbe & Abeles, 1980), and 8.4 for L-α-hydroxyacid oxidase (Cromartie & Walsh, 1975).

[6] For $^Dk_{chem}$ = 7, 8, 9, and 10, the corresponding forward commitments would be 0.08, 0.26, 0.44, and 0.62, respectively.

[7] Both the Bi Bi Uni Uni and Bi Uni Uni Bi Ping-Pong mechanisms would also predict that the primary $^T(V/K)$ should be independent of α-ketoglutarate concentrations; however, the kinetic mechanisms determined for prolyl hydroxylase, lysyl hydroxylase, and thymine 7-hydroxylase argue against such a kinetic mechanism for γ-butyrobetaine hydroxylase.

for sp^3–sp^2 conversions (Cook et al., 1980). The magnitude and direction of the secondary kinetic effect determined for the reaction catalyzed by calf liver γ-butyrobetaine hydroxylase indicate that C_3 is completely sp^2 hybridized in the transition state.[8]

The experimentally determined value for the β-secondary kinetic isotope effect was used to dissect out the β-secondary effects from the multiple effects[9] derived from studies using [*methyl*-^{14}C,2,3(N)-^{3}H]-γ-butyrobetaine as substrate. Thus, one can calculate from the data obtained with the calf liver hydroxylase a value for the α-secondary tritium kinetic isotope effect at C_3 of 1.31. Again, assuming that $c_f = 0$, we can calculate an α-secondary deuterium kinetic isotope effect of 1.21. The equilibrium isotope effect would be expected to be inverse, with the heavier isotope enriching in the alcohol product ($^DK_{eq} = 0.85$; Cook et al., 1980). We interpret this large normal α-secondary kinetic effect to be the result of coupled motions of the C_{3R} and C_{3S} hydrogen atoms during the C_3–H_R cleavage step and synchronous hybridization change. As Cleland (1982) has discussed, in such cases the out of plane bending mode of the α-hydrogen atom becomes part of the reaction coordinate motion, and in the resulting transition state, a real vibrational frequency is partially or completely absent. Such a large normal α-secondary kinetic isotope effect has been observed with formate dehydrogenase (^Dk with [4-^{2}H]DPN = 1.22; Cook et al., 1981), where there are no commitments (Blanchard & Cleland, 1980a) and the experimentally determined value therefore represents the intrinsic isotope effect. Similarly, for yeast alcohol dehydrogenase, where the intrinsic α-secondary kinetic isotope effect can be calculated from the observed effects obtained with suitably deuterated species of NAD^+ and from the known commitment factors, values for ^Dk of 1.22 for [4-^{2}H]NAD and 1.38 for [4-^{2}H]NADH have been reported (Cook et al., 1981). Although in these latter cases the hybridization changes are the result of the formation of a planar carbonium ion at C_4 of the nicotinamide moiety of the NAD coenzyme, the direction and magnitude of the α-secondary kinetic isotope effect should be independent of the chemical nature of the sp^2-hybridized intermediate as long as bond-breaking and rehybridization changes occur synchronously. Thus, heterolytic bond cleavage to yield a hydride ion and a carbonium ion and homolytic cleavage to yield a hydrogen atom and a carbon radical should both exhibit coupled atomic motions resulting in large, normal α-secondary kinetic isotope effects. Since similar data has not been obtained with the bacterial hydroxylase, we cannot perform these calculations for this enzyme.

Chemical Mechanism. The data in the present study do not accord with either a carbanion or an "oxenoid" insertion mechanism for the reaction catalyzed by γ-butyrobetaine hydroxylase. Carbanions are generally thought to remain sp^3 hybridized unless stabilization can be afforded by adjacent functionalities (Cram, 1965), such as an α-carbonyl moiety, as has been proposed for fumarase (Blanchard & Cleland, 1980b) on the basis of the strong inhibition exerted by 3-nitro-2-hydroxypropionic acid (Porter & Bright, 1980). Our results indicate that for the enzymatically catalyzed hydroxylation of γ-butyrobetaine to L-carnitine, C_3 is sp^2 hybridized in the transition state; and since the methylene carbon undergoing hydroxylation is insulated by flanking methylene carbons, it is unlikely that resonance or inductive stabilization of a putative carbanion occurs. Further, the pK_a of such a methylene group would be expected to be extremely high.[10] Indeed, such energetic considerations led Hamilton to propose the alternative oxenoid mechanism (Hamilton, 1971). This latter mechanism, however, also is not supported by the present results that suggest hybridization changes occurring at C_3 in the transition state in the course of γ-butyrobetaine hydroxylation. A reaction mechanism involving oxygen atom insertion would not be expected to result in any significant change in hybridization at C_3. In fact, such a reaction sequence would be predicted to exhibit inverse secondary isotope effects due to the stiffer bonding of ^{3}H in the alcoholic product relative to that in the substrate (Cook et al., 1980).

Accordingly, only the proposed mechanisms for γ-butyrobetaine hydroxylase involving either a carbonium ion or a carbon radical intermediate accord with the experimental results indicating that the carbon atom undergoing hydroxylation is sp^2 hybridized in the transition state. Although the available isotope-effect data do not distinguish between a heterolytic and homolytic carbon–hydrogen cleavage for the γ-butyrobetaine hydroxylase reaction, chemical considerations and enzymological precedents would favor a homolytic cleavage sequence. Thus, studies on the hydroxylation of hydrocarbon substrates by the P-450$_{cam}$ support a radical mechanism (Gelb et al., 1982), and these reactions have been reported to exhibit large intramolecular deuterium isotope effects of ~11 (Hjelmeland et al., 1977; Groves et al., 1978). Furthermore, to our knowledge, there is no known enzymological precedent for carbonium ion intermediacy in iron-dependent hydroxylation reactions.

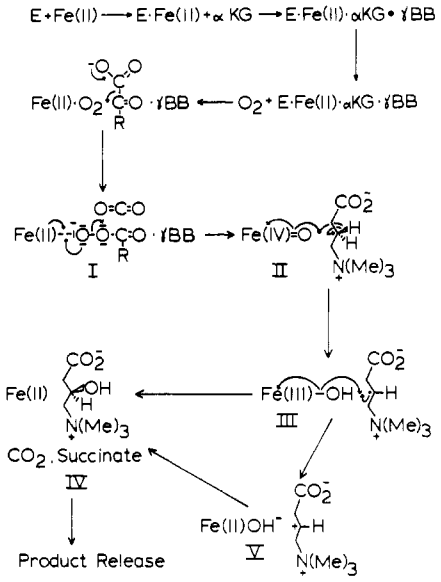
During catalysis, an oxidant capable of removing the hydrogen atom from C_3 of γ-butyrobetaine must be generated. Such an oxidant, in the form of an iron–oxo complex, may arise either from an initial decarboxylation of α-ketoglutarate to form succinic peracid (Hamilton, 1971), followed by its decomposition into the oxidizing complex, or more directly from concerted decarboxylation of α-ketoglutarate coupled to oxygen–oxygen bond cleavage (Siegel, 1979). Both Sligar (Gelb et al., 1982) and Groves (Groves et al., 1978) have proposed the perferryl–oxo complex ($Fe{=}O^V$) as the hydrogen atom abstracting oxidant in the P-450$_{cam}$-catalyzed oxidation of camphor, while model studies on the oxidation of cyclo-

[8] The value of 1.072 is significantly larger than the reported measured $^DK_{eq}$ values of 1.057 for β-secondary deuterium equilibrium isotope effects on sp^3 to sp^2 conversions (Cook et al., 1980). However, these values of 1.057/deuterium are equilibrium isotope effects for the conversion of alcohols to ketones, whereas the kinetic β-secondary isotope effect that we have measured reflects the conversion of an sp^3-hybridized methylene position to an sp^2 center of unknown charge and/or valency (we propose that the chemical identity of this center is a carbon radical, see Chemical Mechanism below). No literature values for β-secondary equilibrium isotope effects for either enzymatic or chemical transformations of this type are available, and thus no limits can be placed on such β-secondary kinetic isotope effects. Halevi (1963) has discussed such normal β-secondary kinetic isotope effects on radical formation in the context of the hyperconjugative effects normally used to explain secondary kinetic isotope effects. Although no charge is developed in the formation of radicals, extensive delocalization would result in hyperconjugative effects that would yield normal (as opposed to inverse) β-secondary kinetic isotope effects.

[9] Although the compound used is labeled at multiple positions, the method of preparation (reduction of crotonylbetaine with $^3H_2$) precludes the introduction of ^{3}H atoms at both C_{3R} and C_{3S}. Further, the specific activity of the compound used is not sufficiently high that a significant portion of the molecules contain more than a single ^{3}H atom, and the observed effect can therefore be considered to be the product of individual effects at each position. The presence of isotopically labeled hydrogen at both the primary and α-secondary positions would result in a reduction of the α-secondary effect, as has been observed with formate dehydrogenase (J. Hermes and W. W. Cleland, unpublished observations).

[10] The pK_a's of methane and C_2 of propane are 40 and 44 on the McEwen–Streitweiser–Applequist–Dessy (MSAD) scale (Cram, 1965).

Scheme I: Proposed Mechanism for γ-Butyrobetaine Hydroxylase



hexanol with Fe(II)/H_2O_2 support a ferryl–oxo complex (Fe=O^{IV}) as the oxidant (Groves & McClusky, 1976; Groves & Van Der Puy, 1976). Both the ferryl- and perferryl–oxo species are strong one-electron oxidants.[11] However, the ferryl–oxo complex would appear to be the preferred species in α-ketoglutarate-linked hydroxylation systems because the iron would then remain in the ferrous state at the completion of the reaction cycle. This would be in accord with observations reported for lysyl hydroxylase (Puistola et al., 1980) and prolyl hydroxylase (Myllyla et al., 1978; DeJong et al., 1982), indicating that in the initial stages of the reaction, ascorbate is not required to achieve demonstrable hydroxylation.

Several features of the mechanism previously postulated for prolyl hydroxylase (Hamilton, 1971; Siegel, 1979) are depicted in Scheme I and are elaborated on in the context of the present data pertaining to the mechanism of γ–butyrobetaine hydroxylase. After the binding of α-ketoglutarate, γ-butyrobetaine, and molecular oxygen to the ferrous–enzyme complex, α-ketoglutarate is decarboxylated with reduction of the ferrous-ligated oxygen molecule to form the ferryl–oxo complex (II), perhaps through the intermediacy of a ferrous–persuccinate complex (I). For calf liver hydroxylase, γ-butyrobetaine must be freely dissociable throughout these chemical steps to account for the large observed primary kinetic isotope effect. The ferryl–oxo complex abstracts a hydrogen atom (C_{3R}) in the rate-determining step to form the sp^2-hybridized carbon radical and the ferric–hydroxyl radical complex (III). Radical recombination results in the formation of the hydroxylated product, L-carnitine (IV). It should be noted here that the observed stereochemistry (that is, retention of configuration; Englard & Midelfort, 1978) is the result of topological, rather than mechanistic, constraints. An alternative pathway involving a second oxidation at C_3 of γ-butyrobetaine to form the carbonium ion and the ferrous–hydroxyl anion complex (V) has been proposed (Siegel, 1979). Our data are moot on this point, since both intermediates are sp^2 hybridized and differ only in oxidation state. Introduction of a second oxidation step, however, does not appear to be mechanistically essential since radical recombination would be expected to be thermodynamically and kinetically efficient. If indeed a second oxidation does occur, it must proceed rapidly relative to the initial hydrogen atom abstraction, since that step is rate determining in the reaction sequence through the first product-release step for the calf liver γ-butyrobetaine hydroxylase.

[11] Higher oxidation states of chromium and manganese rapidly oxidize hydrocarbons in solution. The oxidation of diphenylmethane by dichromate (Cr^{VI}) proceeds with a deuterium isotope effect of 6.6, and the relative rates of Cr^{VI} oxidation of primary, secondary, and tertiary carbon positions in hydrocarbons parallel their rate of reaction with hydrogen atom abstractors (Wiberg & Evans, 1960). Even more convincing data exist for hydrogen atom abstraction from hydrocarbons during oxidation by higher oxidation states of Mn, such as permanganate, $Mn^{VII}O_4^-$ (Wiberg, 1965).

Conclusions

Although both the bacterial and calf liver γ-butyrobetaine hydroxylases catalyze the same reaction by using the same substrates and cofactors and hydroxylate γ-butyrobetaine with identical stereochemistry, the two enzymes are not immunologically cross-reactive (Kondo et al., 1981), have vastly different turnover numbers, and exhibit different primary and secondary kinetic isotope effects. We cannot distinguish between possibilities in which the two enzymes catalyze hydroxylation via different chemical mechanisms or via different transition states; however, it seems more likely that two enzymes accomplish hydroxylation by using the same mechanism. The proof of this latter point and the independent determination of intrinsic isotope effects and commitment factors for the two enzymes are currently under investigation. These studies again point to the power of the analysis of kinetic isotope effects to determine the chemical mechanism by which enzymes accomplish catalysis.

Registry No. [(4*R*)-4-^{3}H]-γ-Butyrobetaine, 87518-57-8; [(3*R*)-3-^{3}H]-γ-butyrobetaine, 87518-58-9; [2,3(N)-^{3}H]-γ-butyrobetaine, 87583-22-0; [*methyl*-^{14}C]-γ-butyrobetaine, 87518-59-0; 4-amino[(4*R*)-4-^{3}H]butyric acid, 60033-17-2; 4-amino[(3*R*)-3-^{3}H]-butyric acid, 87518-60-3; 4-aminobutyric acid, 56-12-2; 4-amino-[2,3(N)-^{3}H]butyric acid, 87583-23-1; γ-butyrobetaine hydroxylase, 9045-31-2.

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