A simplified method for the measurement of γ-butyrobetaine hydroxylase activity

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Summary A method for the assay of γ-butyrobetaine hydroxylase activity is described. The procedure is based on the measurement of ^3H_2O formed from [2,3-^{3}H]γ-butyrobetaine. The formation of ^3H_2O was essentially linear with time of incubation and enzyme concentration. Despite a significant isotope effect that causes the extent of hydroxylation to be underestimated, an appropriately determined correction factor permits one to relate quantitatively the degree of detritiation to the amount of carnitine formed. The assay is simple, rapid, specific, accurate, highly reproducible, and relatively sensitive. Its reliability and convenience represent an improvement over existing methods based on the tedious and time-consuming enzymatic radioisotopic determination of the carnitine formed or on the coupled decarboxylation of [1-^{14}C]α-ketoglutarate, a method that cannot be used in crude extracts.

Supplementary key words [2,3-^{3}H]γ-butyrobetaine · 2,2′-bipyridine-HCl · carnitine · ferrous ammonium sulfate · ascorbic acid · α-ketoglutaric acid · kidney γ-butyrobetaine hydroxylase.

γ-Butyrobetaine hydroxylase (γ-butyrobetaine, 2-oxoglutarate dioxygenase; EC 1.14.11.1) catalyzes

the last step in the carnitine biosynthetic pathway. Partially purified and essentially homogeneous preparations of the enzyme have been obtained from rat liver and *Pseudomonas* sp. AK1, respectively, and the cofactor requirements, kinetic, and physical properties have been examined in some detail (1–4). As do all other α-ketoglutarate-coupled dioxygenase reactions described to date, γ-butyrobetaine hydroxylase requires the participation of molecular oxygen, ferrous ions, and a reducing agent such as ascorbic acid (for reviews see 5, 6).

A radioisotopic assay for carnitine, first described by Cederblad and Lindstedt (7), subsequently modified by Bøhmer, Rydning, and Solberg (8), and then more recently simplified and improved by McGarry and Foster (9) and Parvin and Pande (10) to yield a linear response, permits the measurement of carnitine in the nanomole to picomole range. The determination involves incubation of a sample containing carnitine with [1-^{14}C]acetyl CoA and excess highly purified carnitine acetyltransferase. The reaction

$$\text{acetyl CoA} + \text{carnitine} \rightleftharpoons \text{acetylcarnitine} + \text{CoASH}$$

is carried out in the presence of either sodium tetrathionate, oxidized glutathione or *N*-ethylmaleimide to pull the reaction toward completion. The labeled acetylcarnitine is separated from the unreacted [1-^{14}C]-acetyl CoA by its selective adsorption on Dowex 1-H^+ or on charcoal, and the isotope content of the supernatant fluid is determined (9, 10). Though highly sensitive for carnitine, this method is tedious and time consuming for routine assay of γ-butyrobetaine hydroxylase. An alternative method of assay, widely used for α-ketoglutarate-coupled dioxygenases (5, 6) and employed for measuring the activity of γ-butyrobetaine hydroxylase from *Pseudomonas* sp AK1 (3, 4), is based on the stoichiometric decarboxylation during hydroxylation of [1-^{14}C]α-ketoglutarate of known specific activity. This method, however, is unreliable for crude extracts or partially purified preparations where α-ketoglutarate could undergo appreciable nonspecific decarboxylation (11).

In view of the limitations of the existing assays for γ-butyrobetaine hydroxylase, a new procedure was required to permit the rapid and accurate determination of enzyme activity in numerous samples. Accordingly, an assay was designed in which hydroxylation of γ-butyrobetaine to carnitine is measured by detritiation of [2,3-^{3}H]γ-butyrobetaine and release of the tritium into the aqueous medium. In principle, measurement of tritiated water formed from [2,3-^{3}H]γ-butyrobetaine during the hydroxylation reaction is similar to procedures devised for assaying protocollagen prolyl 4-hydroxylase (12), lysyl hydroxylase (13), and prolyl 3-hydroxylase (14). The method described in the present report is simple, reproducible, and allows rapid measurement of γ-butyrobetaine hydroxylase activity in crude tissue extracts.

Methods and materials

Preparation of kidney and liver cell-free extracts and of partially purified calf liver enzyme. Sprague-Dawley rats (Charles River Breeding Laboratory) and H.A.R.E. rabbits (Marland Farms) were used. Animals were killed and their livers and kidneys were removed and placed on ice. Weighed portions of each tissue were dispersed and homogenized in exactly 2 volumes of 20 mM Tris-HCl, pH 7.5, containing 2 mM monothioglycerol; for this purpose, a PT 10-35 Polytron homogenizer adapted with a PT 10ST saw-tooth generator was used. Two 15-sec bursts at an instrument setting of 8 were applied and the homogenates so obtained were centrifuged for 1 hr at 105,000 *g* in a Spinco Model L preparative ultracentrifuge. The supernatants were decanted through glass wool to remove gross fat particles and stored on ice until assayed. Partially purified γ-butyrobetaine hydroxylase was prepared from a 215,000 *g* supernatant of a calf liver homogenate. The procedure of purification was essentially that described by Lindstedt and Lindstedt (2) for the corresponding enzyme from rat liver. Fractionation was carried through the hydroxylapatite chromatography step, and the most active fractions were pooled to yield a preparation with a specific activity of 4–6 nmol per min per mg protein under the specified conditions of assay.

Preparation of [2,3-^{3}H]γ-butyrobetaine. [2,3-^{3}H]γ-Butyrobetaine ([2,3-^{3}H]4-*N*-trimethylaminobutyric acid) was synthesized from methyl iodide and [2,3-^{3}H]-4-aminobutyric acid (approximately 1 mCi/mmol) using the method initially described by Mazzetti and Lemmon for the synthesis of D,L-carnitine (15) and conditions as subsequently modified by Cox and Hoppel for the synthesis of 6-*N*-trimethyl-2-*N*-acetyl-L-lysine (16). The product of the methylation reaction, after treatment with Dowex-1 resin (Biorad AG 1-X8, 200–400 mesh; OH^- form) to remove an excess of anions, was applied to columns (1.2 × 49 cm) of Dowex-50 (Biorad AG 50W-X8, 200–400 mesh) equilibrated with 0.1 N HCl. The columns were first washed with 0.1 N HCl, collecting 2-column volumes of effluent, followed by a linear gradient using 250 ml each of 0.1 N and 3.0 N HCl. Under these conditions, at a flow rate of 50 ml/hr, 4-aminobutyric acid emerged as a sharp peak at an elution volume of 270–296 ml; it was clearly separated from γ-butyrobetaine which emerged later as a slightly broader peak at an elution volume of 353–383 ml. The identity and radiochem-

ical purity of the chromatographically fractionated γ-butyrobetaine was further established by thin-layer chromatography using the solvent systems described by Cox and Hoppel (17). γ-Butyrobetaine concentration was quantitatively determined as its periodide by the method of Wall et al. (18). The preparation of [2,3-^{3}H]γ-butyrobetaine used in the present study had a sp act of 2.58×10^6 dpm/μmol.

Assay of γ-butyrobetaine hydroxylase activity. The reaction mixtures for assaying γ-butyrobetaine hydroxylase activity, in a final volume of 509 μl, contained: Tris-HCl, at pH 7.7, 25 μmol; $Fe(NH_4)_2SO_4$, 1 μmol; sodium ascorbate, 7.5 μmol; α-ketoglutarate, 1.25 μmol; potassium chloride, 10 μmol; catalase, 1.5 mg; and [2,3-^{3}H]γ-butyrobetaine, 0.59 μmol (1.51×10^6 dpm). The assay components, including the crude extracts to be assayed for hydroxylase activity, were added to 16 × 100-mm tubes immersed in an ice bath. Prior to initiation of reactions by addition of labeled substrate, the assay mixtures were preincubated for exactly 3 min in a shaking water bath at 37°C. Unless otherwise noted, incubations were carried out for 1 hr and the reactions were terminated by addition of 100 μl of 0.5 M 2,2′-bipyridine-HCl. Water was collected from the assay mixtures in the following manner. The reaction mixture was transferred to the side-bulb of a Thunberg tube and frozen by immersion of the bulb in a dry ice–acetone mixture. The tube was evacuated under high vacuum and, after 1 min, sealed off from the atmosphere by rotating the side-bulb to the closed position. The body of the tube was then immersed in a dry ice–acetone mixture until all of the medium water from the side-bulb was collected by sublimation in the immersed portion of the Thunberg tube. Samples of sublimate were counted in aliquots of 400 μl using 5 ml of Yorktown Hydromix scintillation fluid. Radioactivity was measured at 30% efficiency in a Searle Model 6880 Mark III liquid scintillation system. Unless otherwise indicated, activity is expressed as total dpm of ^3H_2O released to the aqueous medium of the assay mixture per hour.

In the experiment designed to correlate the release of tritiated water with the amount of carnitine formed, the latter was quantified using aliquots of the 2,2′-bipyridine inactivated assay mixtures, by the method of McGarry and Foster (9). Protein concentration was determined by the method of Lowry et al. (19) with crystalline bovine serum albumin as a standard.

Materials. [2,3-^{3}H]4-Aminobutyric acid was obtained from New England Nuclear Corp. Unlabeled 4-aminobutyric acid and methyliodide (distilled and stabilized with silver wool), both Fluka AG, Buchs SG products, were purchased from Tridom Chemical, Inc. D-L-Carnitine was a product from Aldrich Chemical Company, Inc. 2,2′-Bipyridine-HCl was obtained from G. F. Smith Chemical Co.; sodium ascorbate from Sigma Chemical Co.; and sodium tetrathionate from ICN Pharmaceuticals, Inc. Carnitine acetyltransferase, catalase (a water suspension, stabilized with 0.01% alkylbenzyldimethyl ammonium chloride) and α-ketoglutaric acid were purchased from Boehringer Mannheim Biochemicals. Other chemical reagents used were highly purified commercial preparations.

Results and discussion

The results summarized in **Table 1** show that, with the complete system containing all of the components necessary to elicit α-ketoglutarate-dependent dioxygenase activity (cf. references 5 and 6), high-speed supernatants of both rat liver and rabbit kidney homogenates catalyzed maximal release of tritium from [2,3-^{3}H]γ-butyrobetaine. Omission of the enzyme, of ascorbate, of both ascorbate and ferrous ions, or of α-ketoglutarate, caused considerable decreases in the extent of detritiation. The residual activities observed in the absence of these components, and the more significant residual activity noted when ferrous ions were not added, can be accounted for by the presence of small and variable amounts of these components in the undialyzed crude extracts used in the assay system. The unusual and pronounced enhancing effect of catalase shown for the rabbit kidney extract, under the specified conditions of assay presently employed (in particular, the high sodium ascorbate concentrations), have been noted previously with partially purified preparations of rat liver γ-butyrobetaine hydroxylase (1, 2). The data presented in Table 1 on the cofactor requirements for [2,3-^{3}H]-γ-butyrobetaine detritiation by both the rat liver and rabbit kidney preparations are consistent with an α-ketoglutarate-dependent dioxygenase type of hydroxylation reaction in which γ-butyrobetaine is converted to carnitine as has been reported previously for the corresponding reactions in rat liver and *Pseudomonas* sp AK1 as assayed by either direct measurement of carnitine or by decarboxylation of [1-^{14}C]α-ketoglutarate (1–4).

With rat liver extract as the source for γ-butyrobetaine hydroxylase, the release of tritium from [2,3-^{3}H]γ-butyrobetaine into the assay medium was essentially linear with time of incubation up to 90 min (**Fig. 1*A***) and with enzyme concentration (**Fig. 1*B***). The assay was also found to be highly reproducible as shown by the data summarized in **Table 2.** Thus, for the rat liver enzyme, duplicate assays performed on different days, with the extract stored during the interim at −20°C, each determination fell within 3.3% of the average value obtained for the four assays. For the rab-

TABLE 1. Requirements for ^3H_2O formation from $(CH_3)N^+CH_2C^3H_2C^3H_2COOH$ in the γ-butyrobetaine hydroxylase reaction

Tissue Extract Assayed	Omission(s) from Complete System[a]	[^{3}H]Water Formation	
		dpm	% of Control
Rat liver[b]	None	12,440	
	Enzyme	409	3.3
	Ferrous ammonium sulfate	3,811	31
	Sodium ascorbate	2,117	17
	Ferrous ammonium sulfate and sodium ascorbate	628	5.1
	α-Ketoglutarate	1,445	12
Rabbit kidney[b,c]	None	16,793	
	Ferrous ammonium sulfate	4,590	27
	Sodium ascorbate	918	5.5
	Ferrous ammonium sulfate and sodium ascorbate	700	4.2
	α-Ketoglutarate	1,806	11
	Catalase	5,980	36

[a] Defined in the text.

[b] Each assay was carried out with 200 μl of a high-speed supernatant of either rat liver or rabbit kidney homogenate containing 6.6 mg and 4.9 mg of protein, respectively.

[c] For the rabbit kidney extract experiment, the incubation period was extended to 2 hr.

bit, considering the significant difference in the protein concentration of the extracts obtained from the individual kidneys, one notes an excellent correlation in comparing the respective specific activities (the deviation is no more than 1.2% of the average value obtained from both determinations).

For the detritiation assays devised for the measurement of protocollagen prolyl 4-hydroxylase (12), lysyl hydroxylase (13), and prolyl 3-hydroxylase (14), a correspondence has been reported with formation of 4-hydroxyproline, 5-hydroxylysine, and 3-hydroxyproline, respectively. On the other hand, Lindstedt reported a 6.7-fold increase in the ^3H:^{14}C ratio of residual γ-butyrobetaine after 92% conversion of [1-^{14}C, 2,3-^{3}H]γ-butyrobetaine into carnitine by a crude rat liver hydroxylase preparation; no isotope effect, however, was noted when the substrate was [1-^{14}C, *methyl*-^{3}H]γ-butyrobetaine (1).

These observations, suggesting that the rate-limiting step in γ-butyrobetaine hydroxylation is probably the dissociation of the carbon-bound hydrogen atom, prompted us to examine the quantitative rela-

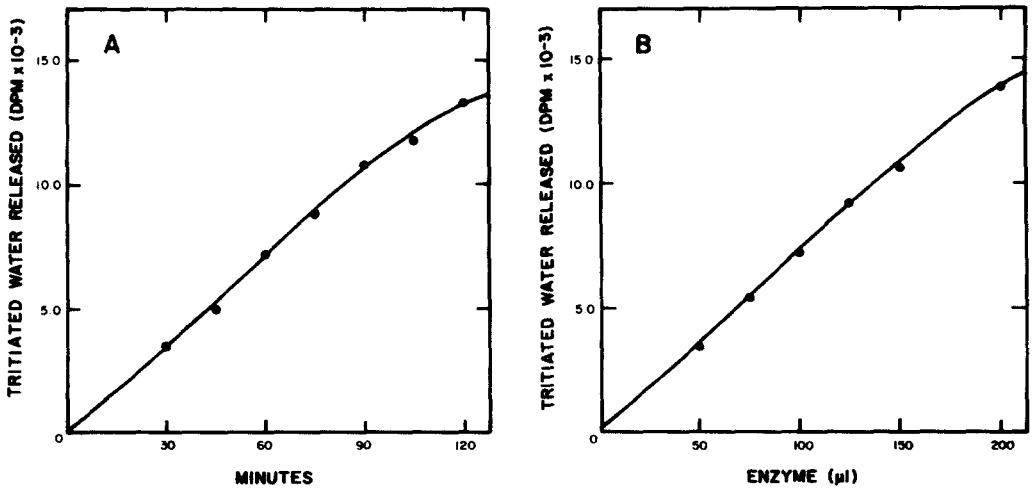

Fig. 1. Formation of ^3H_2O from [2,3-^{3}H]γ-butyrobetaine by 105,000 *g* supernatant of rat liver homogenate as a function of time (*A*) and enzyme concentration (*B*). The extract contained 32.9 mg of protein per ml and the assay conditions are as described under Methods and Materials. In the graph depicting the time course of the reaction, incubations were carried out for different lengths of time with 100 μl of extract (*A*). Incubations with varying aliquots of extract were for 1 hr (*B*).

TABLE 2. Reproducibility of assay for γ-butyrobetaine hydroxylase by detritiation of [2,3-^{3}H]γ-butyrobetaine

Tissue	Nature of Extract	Assay	Enzyme Activity: dpm of ^3H_2O Released[a]	Enzyme Activity: dpm of ^3H_2O Released per mg protein
Rat liver	A. Freshly prepared high speed supernatant	1	13,347	2,028
		2	13,466	2,047
	B. Above supernatant stored frozen at −20°C for 3 days	1	13,036	1,981
		2	13,876	2,109
Rabbit kidney	A. Freshly prepared high speed supernatant[b]	1	13,014	2,656
	B. Freshly prepared high speed supernatant of the other kidney from the same rabbit[c]	1	10,683	2,593

[a] Defined in the text.
[b] 200 μl of extract containing 4.9 mg of protein assayed.
[c] 200 μl of extract containing 4.1 mg of protein assayed.

tionship between the release of tritium and the formation of carnitine in the present assay procedure. The substrate introduced in our assay system has four of its hydrogen atoms labeled (at C-2 and C-3); on hydroxylation only one of these hydrogen atoms (at C-3) is displaced stereospecifically (20). Accordingly, with a sp act of 2.58×10^6 dpm/μmol for the [2,3-^{3}H]γ-butyrobetaine used in this study, the pertinent specific activity in terms of the detritiation assay for measurement of hydroxylase activity is 6.45×10^5 dpm per μatom of H. Barring an isotope effect that would favor the preferential hydroxylation of unlabeled γ-butyrobetaine molecules, the release of 645 dpm of ^3H_2O into the reaction medium should then be equivalent stoichiometrically to 1 nmol of carnitine formed. This is the basis of the calculation involved in the experiment summarized in **Fig. 2,** designed to relate the degree of detritiation of γ-butyrobetaine with the amount of carnitine formed as measured quantitatively by an independent, alternative procedure. As may be noted in Fig. 2, although the release of tritium from [2,3-^{3}H]γ-butyrobetaine was proportional to carnitine formation over the entire range of time examined, the significant isotope effect previously reported for this reaction (1) caused the detritiation method to underestimate the extent of hydroxylation by a factor of nearly 10. The isotope effect noted in the detritiation of γ-butyrobetaine as it undergoes hydroxylation, although decreasing the sensitivity of the method, in no way affects its usefulness as a routine assay, particularly since a proper correction factor can be introduced that stoichiometrically relates the degree of detritiation with the amount of carnitine formed. With the availability of scintillation spectrometers that operate with counting efficiencies of over 40% for tritium, the specific activity of our substrate allows us by the detritiation procedure, yet, to detect accurately at least, 5 nmol of carnitine formed.

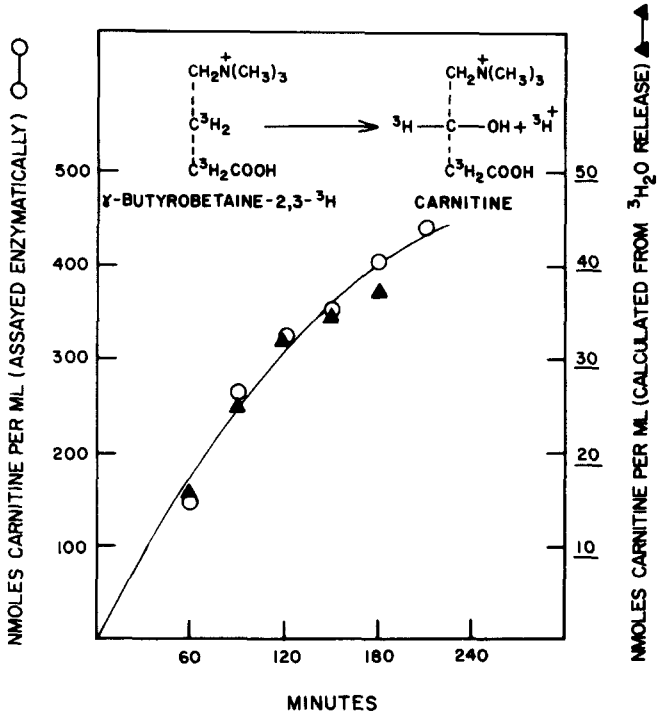

Fig. 2. Relationship between ^3H_2O release from [2,3-^{3}H]γ-butyrobetaine and formation of carnitine. The reaction mixture (5.0 ml) contained: Tris-HCl, at pH 7.7, 50 mM; Fe $(NH_4)_2SO_4$, 2 mM; sodium ascorbate, 15 mM; α-ketoglutarate, 2.5 mM; potassium chloride, 20 mM; catalase, 15 mg; 7.75 mg of partially purified calf liver γ-butyrobetaine hydroxylase; and 1.5 mM [2,3-^{3}H]γ-butyrobetaine (total dpm = 1.94×10^7) to initiate the reaction after a 3-min preincubation of the reaction mixture at 37°C. At the indicated time intervals, exactly 500-μl aliquots were removed and added to 100 μl of 0.5 M 2,2′-bipyridine-HCl and the tubes were placed in an ice bath. After all samples were collected, suitable aliquots were removed for the radioisotopic enzymatic determination of carnitine (9), using the appropriate counting program of the liquid scintillation spectrometer to discriminate the ^{14}C-labeled acetyl carnitine from the residual ^{3}H in the carnitine moiety derived from the [2,3-^{3}H]γ-butyrobetaine. Water was collected from the remainder of each sample as described under Methods and Materials. The basis of the calculation for the quantitative estimation of the carnitine formed from the extent of ^{3}H released into the medium is discussed in the text.

The method presented here devised for assay of γ-butyrobetaine hydroxylase is simple, direct, specific, and accurate and is sufficiently sensitive to allow measurement of this enzyme in crude extracts. Indeed, the method has been used to assay the enzyme in extrahepatic tissues from a number of different mammalian organisms (21) and, as shown in this report, was successfully applied to measure the relatively high levels of γ-butyrobetaine hydroxylase activity in rabbit kidney tissue.

Carnitine is known to have a central role in the proper metabolic utilization and synthesis of fatty acids (for reviews see 22–25). In the rat, the species most extensively studied, tissues such as adipose tissue, heart, kidney, and skeletal muscle catalyze the entire sequence of reactions from ε-*N*-trimethyl-L-lysine to γ-butyrobetaine, but have been reported not to hydroxylate the latter compound to carnitine (1, 26–28). Consistent with these observations, a suggestion has been made that extrahepatically synthesized γ-butyrobetaine is converted to carnitine exclusively in the liver, and that the carnitine so formed is subsequently and rapidly transported to other organs (27, 29). Of great interest is the finding reported here and elsewhere (21) that γ-butyrobetaine hydroxylase activity indeed is present in kidney of rabbit and of other mammalian species, and that the activity is as great or greater than the hydroxylase present in the corresponding livers of the same animals. Relevant to these findings is the report that ovine kidney and muscle tissues have a relatively low but significant capacity to synthesize carnitine from γ-butyrobetaine (30). These observations argue against the prevailing generalization, based on studies carried out almost exclusively with the rat, that the liver is the exclusive or primary site of carnitine synthesis for both use in the liver and transport to other tissues.

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