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γ-Butyrobetaine in tissues and serum of fed and starved rats determined by an enzymic radioisotopic procedure

Henri NOËL, Rehana PARVIN* and Shri V. PANDE†
Laboratory of Intermediary Metabolism, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal H2W 1R7, Quebec, Canada

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A method for the determination of picomole quantities of γ-butyrobetaine and its application for the determination of γ-butyrobetaine distribution in tissues are described. The method is based on the quantitative conversion of γ-butyrobetaine into carnitine by using a 50–60%-satd.-$(NH_4)_2SO_4$ fraction of rat liver supernatant as the source of γ-butyrobetaine hydroxylase [4-trimethylaminobutyrate,2-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1]; the carnitine formed is then measured enzymically. The mean γ-butyrobetaine content, as nmol/g wet wt. of tissue, ranged from a low of 4.6 in livers to a high of 12.3 in hearts of normal fed male adult rats. Starvation for 48h did not affect the γ-butyrobetaine concentration in serum, liver and brain, but that in skeletal muscles, kidney and heart was increased. These data are in line with the present views that most tissues are able to produce γ-butyrobetaine, and show that starvation enhances the synthesis and/or the retention of this compound in many tissues. The observed high affinity of γ-butyrobetaine hydroxylase for γ-butyrobetaine (K_m 7 μM), the high activity of this enzyme and the low concentration of γ-butyrobetaine in liver indicate that γ-butyrobetaine availability is one of the factors that normally limit carnitine synthesis.

γ-Butyrobetaine is the immediate precursor of carnitine and is itself derived, in several steps, from 6-*N*-trimethyl-lysine. Much work on the distribution of the related enzyme activities and on the conversion of tracer precursors, in whole animals as well as in experiments *in vitro*, has led to the notion that most tissues are capable of converting their endogenous trimethyl-lysine into γ-butyrobetaine, which is then released into the circulation. In addition, in tissues having γ-butyrobetaine hydroxylase activity, mainly liver in the rat, this exogenous γ-butyrobetaine, along with that produced *de novo*, is then converted into carnitine (Cox & Hoppel, 1973, 1974; Haigler & Broquist, 1974; Henderson *et al.*, 1982; Rebouche, 1982). We describe below that the determination of γ-butyrobetaine distribution in tissues and serum of rats supports the above notion further; these analyses have now become possible because of the development of a sensitive method for γ-butyrobetaine determination. Details of this assay procedure are also described below.

* Deceased July 1982.
† To whom reprint requests should be addressed.

Experimental

Materials

Deoxycarnitine hydrochloride was purchased from Calbiochem. Ox liver catalase (EC 1.11.1.6, 65000units/mg) and pigeon breast muscle carnitine acetyltransferase (EC 2.3.1.7, 80units/mg) were from Boehringer Mannheim. [1-^{14}C]Acetyl-CoA (sp. radioactivity 55Ci/mol) was obtained from Amersham and was dissolved in 10mM-sodium acetate buffer, pH5.4. Other chemicals were from Sigma [ascorbate, α-oxoglutarate (monosodium salt), EDTA], Boehringer {Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], oxidized glutathione}, P-L Biochemicals (unlabelled acetyl-CoA) and Baker (ferrous ammonium sulphate). Bio-Rad provided the AG 50W-X8 (H^+ form) and AG 2-X8 (Cl^- form) 200–400-mesh resins.

Animals

Twelve male Sprague–Dawley rats weighing 200–300g (Charles River Breeding Laboratories) were acclimated to the animal room for 1 week. Six rats continued to receive food; the others were starved for 48h.

Preparation of extracts for γ-butyrobetaine assay

Animals were killed by decapitation and the blood was collected. Tissues were quickly removed and immediately frozen between tongs of aluminium plates precooled in liquid N_2. The frozen tissues were either processed at once for $HClO_4$ extraction or kept at −80°C until use. The frozen tissues were powdered in a mortar cooled in liquid N_2, and the powder was transferred to a homogenizer tube; 3.5vol. of 0.6M-$HClO_4$ was added per g of tissue and the mixture was homogenized in a Potter–Elvehjem homogenizer with a tight-fitting pestle. The slurry was centrifuged at 25000*g* for 10min at 4°C, and the supernatant kept. The pellet was re-extracted once with 2.5vol. of 0.3M-$HClO_4$. After centrifugation as above, the supernatants were combined. Serum was similarly extracted by adding 3.5ml of 0.6M-$HClO_4$/ml of serum and by washing the pellet with 0.3M-$HClO_4$ as described for the tissues. $HClO_4$ extracts were kept at −80°C until use.

Isolation of γ-butyrobetaine fraction

$HClO_4$ extracts were thawed; 10M-KOH was added to make the solution 0.3M with respect to excess alkali. After incubation at 56°C for 20min to hydrolyse acylcarnitines, the solution was acidified again with $HClO_4$ to give a final excess acid concentration of 0.3M. The mixture was chilled and the $KClO_4$ formed was eliminated by centrifugation at 6000*g* for 10min at 0°C. A portion of the clear supernatant, corresponding to 0.3–0.7mg wet wt. of tissue, was adjusted to 5ml with 0.3M-$HClO_4$; the sample was then applied to a column (7mm × 160mm) of AG 50W-X8 (H^+ form) resin. The column was developed with 37ml of 1M-HCl (to elute carnitine), and then with 2M-HCl. γ-Butyrobetaine appeared between 40 and 52ml of effluent. The pooled γ-butyrobetaine fractions were evaporated to dryness with a rotary evaporator; the residues were dissolved in 10ml of water, evaporated again and then extracted in 5ml of ethanol. After centrifugation (6000*g*, 10min) to remove the precipitated salts and evaporation of the solvent, the residues were finally dissolved in 10mM-potassium phosphate buffer, pH7.8 (1ml/0.3–0.6g of wet wt. of tissue), for γ-butyrobetaine assay as described below.

Assay of γ-butyrobetaine

γ-Butyrobetaine was first converted into carnitine by incubation with an excess of a partially purified hydroxylase preparation, and the carnitine subsequently determined by coupling to carnitine acetyltransferase by using radiolabelled acetyl-CoA (Parvin & Pande, 1977).

Buffered γ-butyrobetaine hydroxylase-catalase premix. A 50–60%-satd.-$(NH_4)_2SO_4$ fraction of rat liver supernatant, prepared as described elsewhere (Daveluy *et al.*, 1982) and dialysed extensively against 25mM-potassium phosphate (pH7.4) containing 75mM-KCl, 0.1mM-dithiothreitol and 0.05mM-EDTA provided γ-butyrobetaine hydroxylase of specific activity 2–4munits when assayed at non-inhibitory γ-butyrobetaine concentration (0.1mM) (Pande & Parvin, 1980). The enzyme was stored at −20°C; no activity loss occurred after 5 months. An enzyme mixture for 50 assays was prepared fresh by mixing 150μl of the γ-butyrobetaine hydroxylase preparation (about 30munits), 50μl of catalase (65000 units, 1.0mg of protein), 50μl of 100mM-potassium phosphate, pH6.7, and 250μl of water.

Buffered ascorbate/α-oxoglutarate mixture. Reagent for 50 tubes was prepared before use by mixing 500μl of 37.5mM-ascorbate (monosodium salt, freshly dissolved), 375μl of 25mM-α-oxoglutarate (monosodium salt, freshly dissolved) and 125μl of 1M-potassium phosphate, pH6.7.

Salt-depleted carnitine acetyltransferase. On the day of use, 150μl of the stock enzyme suspension in $(NH_4)_2SO_4$ was centrifuged for 10min at 12000*g* and the supernatant was aspirated off. The pellet was dissolved in 250μl of 10mM-Hepes/KOH, pH7.6, and provided enzyme for 50 tubes.

Assay of γ-butyrobetaine. This was performed in 1.5ml Eppendorf tubes. The tubes, kept in ice, received, in the order mentioned, sample to be analysed containing 0.1–1nmol of γ-butyrobetaine, water to give a volume of 85μl, 20μl of the buffered ascorbate/α-oxoglutarate mixture, 10μl of the buffered γ-butyrobetaine hydroxylase/catalase mixture and 10μl of 6.25mM-ferrous ammonium sulphate (freshly dissolved). Addition of ferrous ammonium sulphate to successive tubes was made at 30s intervals. Immediately after receiving ferrous ammonium sulphate, the contents were mixed and the tubes were transferred to a 30°C bath. Control tubes lacking the γ-butyrobetaine hydroxylase/catalase mixture were set up for each sample volume to allow correction for endogenous carnitine present. After 30min incubation with shaking at 30°C, 25μl of 1.6M-KOH was added to each tube at 30s intervals and, after mixing, the tubes were transferred to an ice/water bath. Each control tube now received the buffered γ-butyrobetaine hydroxylase/catalase mixture. All tubes were capped, incubated at 56°C for 20min to inactivate the enzyme and ascorbate, returned to the ice/water bath for a few minutes, and then centrifuged for 30s in an Eppendorf 3200 centrifuge to bring all fluid droplets to the bottom of the tubes. Each tube, kept in the ice bath, then received, with mixing, 35μl of a neutralizing mixture [this reagent for 50 assay tubes is prepared by mixing 1ml of 1M-Hepes as free acid, 400μl of 0.5M-oxidized gluta-

thione, 10μl of 100mM-EDTA and sufficient predetermined volume of 2M-H_3PO_4 (a few additional tubes are included to allow this titration for each batch of reagents) and water to give a volume to 1750μl; 35μl of this reagent when added to the assay tubes should give a final pH of 7.6±0.1)], 10μl of [^{14}C]acetyl-CoA (2nmol, 20nCi) and 5μl of salt-depleted carnitine acetyltransferase. The last addition was made to the successive tubes at 30s intervals and, immediately after mixing, the tubes were transferred to a 30°C bath. Then 30min later, about 290mg of dry AG 2-X8 resin (Cl^- form, 200–400 mesh) and 600μl of water were added to each tube. [To enable convenient addition of the resin at 30s intervals, a plastic 1ml 40–80-unit insulin syringe (0.6mm inner diameter; Becton, Dickinson and Co., Rutherford, NJ, U.S.A.) was cut at the 0 mark to eliminate the narrow end, and the resin was filled to the volume mark corresponding to 0.5ml through the cut end by pressing the inverted syringe in a 100ml beaker containing the dry resin with plunger held at the 0.5ml mark; the resin was then added to the assay tubes by depressing the plunger.] The tubes were mechanically shaken for 10min at room temperature, briefly centrifuged (12000*g* for 30s), then 0.4ml of the supernatant was mixed with 3.2ml of Beckman Ready-Solv EP scintillant and the radioactivity was measured.

Results and discussion

We mentioned previously that under appropriate conditions γ-butyrobetaine hydroxylase enabled a quantitative conversion of γ-butyrobetaine into carnitine, which, on coupling to carnitine acetyltransferase, allowed assay of γ-butyrobetaine (Parvin & Pande, 1979; Pande & Parvin, 1980; Daveluy *et al.*, 1982). Fig. 1 shows that, under the selected optimum conditions, details of which are described in the Experimental section, the method allowed measurement of picomole quantities of γ-butyrobetaine and exhibited excellent linearity with increasing amounts (up to 1.2nmol) of the standard solution.

When tissue extracts were analysed, however, the assay procedure showed limited, albeit acceptable, linearity with the sample size (Fig. 2, curve *A*) only when ferrous ammonium sulphate was added separately and as the last component, but not when this compound was included in the buffered ascorbate/α-oxoglutarate mixture (Fig. 2, curve *B*). It is noteworthy that trimethyl-lysine hydroxylase has been shown to be completely inactivated when ferrous ammonium sulphate is added before α-oxoglutarate and ascorbate (Henderson *et al.*, 1982); the same appears to apply for the γ-butyrobetaine hydroxylase activity (Rebouche & Engel, 1980). Moreover, this deviation from linearity was found

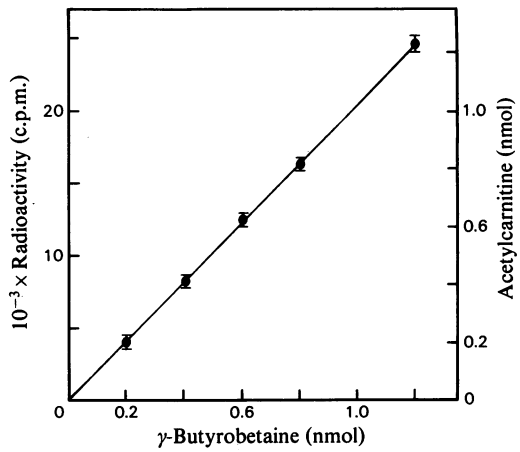

Fig. 1. *Standard curve for γ-butyrobetaine*
Conditions were exactly as described in the Experimental section. The values shown are means±S.E.M. for $n = 4$–6. The coefficient of variation for 0.2 and 0.4nmol standards of γ-butyrobetaine, analysed over a 1-month period, was 3.6 and 3.7% ($n = 19$ each) respectively.

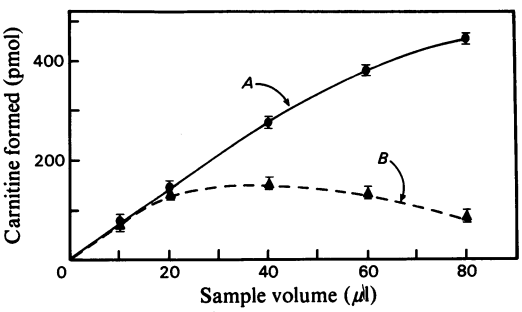

Fig. 2. *Linearity during γ-butyrobetaine analyses of tissue extracts as affected by the manner of addition of ferrous ammonium sulphate*
Conditions for curve *A* were exactly as described in the Experimental section, where ferrous ammonium sulphate was added separately and as the last component. Conditions for curve *B* were identical, except that the same quantity of ferrous ammonium sulphate was included in the chilled buffered α-oxoglutarate/ascorbate reagent, which was prepared about 15min before use. A sample volume of 10μl corresponded to extract derived from 30mg wet wt. of heart. For more details, see the text. The values shown are means±S.E.M. for $n = 4$–6.

to worsen with aging of the chilled ferrous ammonium sulphate-containing mixture (results not shown). This was found to be true with extracts of all the tissues and serum examined. Since this limitation was not realized with the standard solution of γ-butyrobetaine, some component present in the processed tissue extracts was causing this

non-linearity. No attempt was made to characterize this phenomenon further, since under the conditions described recoveries of added γ-butyrobetaine were excellent (95–99%) when these analyses were performed with those sample sizes that gave a linear response in the assay procedure. As a consequence, when analysing tissue γ-butyrobetaine by the present procedure, various sample sizes were included and γ-butyrobetaine was estimated from those that came within the linear range of the assay procedure.

In our outlined procedure, two separate enzyme-incubation steps are used. In the first incubation, γ-butyrobetaine is converted into carnitine, which is subsequently converted into acetylcarnitine. The possibility that the assay may be simplified by combining these two steps was precluded by the opposing requirements of the two enzymes necessary for quantitative conversion. Thus, as described previously (Parvin & Pande, 1977), quantitative conversion of carnitine into acetylcarnitine requires the presence of thiol-removing agents, but these agents were found to be inhibitory for the γ-butyrobetaine hydroxylase activity. This simplification was also precluded by the presence of a contaminating acetyl-CoA hydrolase activity in the preparations of γ-butyrobetaine hydroxylase used. Alkalinization of the reaction mixture after incubation with the hydroxylase served to destroy this acetyl-CoA hydrolase activity as well as most of the ascorbate present. Excess ascorbate otherwise was found to decrease the amount of [^{14}C]acetyl-CoA available for acetylcarnitine formation, owing to the progression of a non-enzymic reaction between ascorbate and acetyl-CoA. Formation of this radioactive compound goes unnoticed in the Cl^--exchange resin method of carnitine determination recommended above, because that ^{14}C-labelled product, like [^{14}C]acetyl-CoA itself, is retained on the resin. However, when instead of resin the charcoal method (Parvin & Pande, 1977) is used for the separation of acetyl-CoA from acetylcarnitine, interference results because the labelled product formed from the interaction of [^{14}C]acetyl-CoA and ascorbate is not retained by charcoal.

Since γ-butyrobetaine in tissue extracts constituted only a small fraction of the carnitine present, analyses by the present procedure required that the samples be first freed from the bulk of the carnitine present. The conditions selected, described in the Experimental section, were those that enabled this to be accomplished in a short time and with a minimum eluent volume. Recovery experiments with mixtures of 10 μmol of carnitine and 0.1 μmol of γ-butyrobetaine showed that the recommended 2M-HCl eluent contained 97–99% of γ-butyrobetaine, with less than 1% of the carnitine. The presence of this residual carnitine required that a correction be applied for it, and this necessitated inclusion of control tubes in which γ-butyrobetaine hydroxylase was added only after alkalinization of the samples; this method also allowed correction for a small quantity of carnitine that remains associated with the γ-butyrobetaine hydroxylase preparation despite extensive dialysis.

The possibility that the tissue γ-butyrobetaine values obtained by the present procedure may include compounds other than γ-butyrobetaine is unlikely, because both γ-butyrobetaine hydroxylase (Lindstedt, 1967) and carnitine acetyltransferase (Fritz & Schultz, 1965; Daveluy *et al.*, 1982) are fairly substrate-specific enzymes; thus the specificity of the present assay, which utilizes both enzymes, should be even greater. Besides, we found that the determination of γ-butyrobetaine in tissue extracts requires the absolute presence of γ-butyrobetaine hydroxylase, ascorbate and α-oxoglutarate. Omission of any one of these components lowered the values to those of controls, indicating that spurious compounds were not being picked up as γ-butyrobetaine. To ascertain that the coupled reactions of γ-butyrobetaine hydroxylase and carnitine acetyltransferase gave rise solely to acetylcarnitine, the reaction mixture obtained after incubation with a kidney extract was subjected to Dowex AG 50W-X8 (NH_4^+ form) chromatography, as described by Bohmer & Bremer (1968). Under these conditions, a single peak of ^{14}C radioactivity was obtained (results not shown), with an elution volume (2–3 bed vol.) characteristic of that of authentic acetylcarnitine (Daveluy *et al.*, 1982).

Much higher 'apparent γ-butyrobetaine' content was observed with the tissue extracts, especially of brain and of liver mitochondria, when these extracts were directly analysed (i.e. without the intervention of the H^+-exchanger step) and when, after the conversion of carnitine into [^{14}C]acetylcarnitine, charcoal was added to remove excess [^{14}C]acetyl-CoA. This interference was traced to the presence of a contaminating glutamate–oxaloacetate transaminase (EC 2.6.1.1) activity in the partially purified γ-butyrobetaine hydroxylase preparation and a weak citrate synthase (EC 4.1.3.7) activity in the crystalline carnitine acetyltransferase. Thus, during incubation with γ-butyrobetaine hydroxylase and α-oxoglutarate, aspartate of tissue extracts gave rise to oxaloacetate, which, on incubation with carnitine acetyltransferase and [^{14}C]-acetyl-CoA, produced [^{14}C]citrate; this compound, being non-adsorbable to charcoal, then falsely contributed to the final γ-butyrobetaine values. Such an interference was not observed with the procedure described here, because (*a*) the use of Cl^--exchanger resin in place of charcoal ensures

Table 1. *γ-Butyrobetaine content in tissues and serum of normal and 48h-starved adult male rats*

Values shown are means ± S.E.M. for six rats in each group: *significantly different ($P <$ or $\ll 0.05$) from the fed value.

	γ-Butyrobetaine content (nmol/g wet wt.)	
	Fed	Starved
Skeletal muscles	8.8 ± 0.75	11.3 ± 0.55*
Heart	12.3 ± 0.74	20.3 ± 0.84*
Brain	8.0 ± 0.32	7.5 ± 0.61
Kidney	10.0 ± 1.30	14.2 ± 0.93*
Liver	4.6 ± 0.25	5.0 ± 0.36
Serum†	0.84 ± 0.06	0.95 ± 0.05

† nmol/ml.

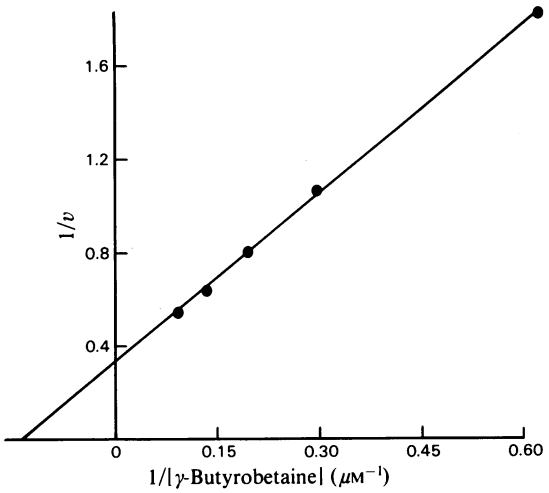

Fig. 3 *Double-reciprocal plot of the effect of γ-butyrobetaine concentration on the activity of γ-butyrobetaine hydroxylase*

The reaction system (final volume 125 μl) contained 20mM-potassium phosphate, pH6.7, 5mM-ascorbate, 1.5mM-α-oxoglutarate, 0.5mM-ferrous ammonium sulphate, 20mM-KCl, 40μg of catalase, γ-butyrobetaine as shown and 11μg of the rat liver hydroxylase preparation. Incubations were for 10min at 30°C. Reactions were stopped by the addition of KOH, and samples processed exactly as described for γ-butyrobetaine assay in the Experimental section. Velocity (v) is expressed as nmol of carnitine formed/min per mg of protein. The values shown are means ± S.E.M. for $n = 4$–6. The calculated K_m is 7.3μM.

that possible interfering anions such as citrate, acetate etc. are retained on the resin, and (*b*) processing of tissue extracts through H^+-exchanger columns removed both aspartate and carnitine, which are eluted before the peak of γ-butyrobetaine.

Although the presence of γ-butyrobetaine as a naturally occurring metabolite in urine is known since 1910 (see Reinwein & Thielmann, 1924; Broekhuysen & Deltour, 1961), evidence for its presence in tissues and serum of rats has rested on the detection of radioactive γ-butyrobetaine derived from administered radioactive precursors (Cox & Hoppel, 1973; Haigler & Broquist, 1974; Frenkel & Carter, 1980). The sensitivity of the present procedure allowed demonstration and quantification of γ-butyrobetaine in tissues and serum of rats (Table 1). The γ-butyrobetaine content of the tissues examined (except brain) and that of serum corresponded to 1–2% of their known (Marquis & Fritz, 1965; Pearson & Tubbs, 1967; Parvin & Pande, 1979) carnitine values; in brain the corresponding percentage was about 16, owing to the low carnitine content of this tissue. Among the tissues analysed, the lowest γ-butyrobetaine content was found in liver (Table 1), which is in line with the present views that, whereas most tissues are producers of γ-butyrobetaine, liver is the major site of γ-butyrobetaine hydroxylation in the rat. The much lower γ-butyrobetaine concentration in serum compared with that in tissues shows that, as is known for carnitine, a marked concentration gradient exists also for γ-butyrobetaine across the plasma membrane of the tissues. Again, like carnitine, the major quantity of the body's γ-butyrobetaine appears to reside in muscles, because of the high muscle mass in the body.

Starvation for 48h increases hepatic carnitine, expressed per g wet wt. of tissue, without affecting the carnitine content of heart and kidney (Brass & Hoppel, 1978; Parvin & Pande, 1979). The effect of a similar starvation on the γ-butyrobetaine content was different, in that the values in liver were not affected, whereas those for heart, skeletal muscles and kidney were increased (Table 1). Starvation is known to increase carnitine retention in the body, because its concentration in urine declines despite relatively unchanged serum carnitine values (Brass & Hoppel, 1978; Parvin & Pande, 1979); the same applies for γ-butyrobetaine also, inasmuch as starvation did not afffect the serum γ-butyrobetaine concentration (Table 1), although under the same conditions the urinary γ-butyrobetaine excretion is markedly decreased (Parvin & Pande, 1979).

It is curious that, among the tissues examined, the highest γ-butyrobetaine content was found in heart and that the starvation-induced enhancement was also maximal (65%) in this organ (Table 1). Why most tissues have retained the entire enzymic machinery for the conversion of endogenous trimethyl-lysine into γ-butyrobetaine when the responsibility for the ultimate production of carni-

tine, an essential metabolite for most tissues, has been relegated to certain organs only is a matter of conjecture. Could it be that γ-butyrobetaine or one of its immediate precursors has some additional metabolic role within the tissue of origin, besides that of acting as a carnitine precursor?

Inasmuch as systemic carnitine deficiency disease is now regarded as a heterogeneous entity, the possibility that different synthetic and/or transport steps may be affected in different individuals needs consideration. Development of sensitive, simple and rapid methods of assaying enzymes and precursors of carnitine synthesis would thus be helpful and, in this regard, we wish to point out: (*a*) that the present analytical method permits also a very sensitive assay of γ-butyrobetaine hydroxylase, which, as mentioned previously (Pande & Parvin, 1980), can be performed with as little as 10 μg of rat liver supernatant, a higher sensitivity that that of the similar Carter & Stratman (1980) procedure; (*b*) that for optimal enzyme activity γ-butyrobetaine concentration should be near 100 μM and not >1 mM, as commonly used (Lindstedt & Lindstedt, 1970; Englard *et al.*, 1978; Carter & Stratman, 1980), owing to marked inhibition by excess substrate (Pande & Parvin, 1980), a property that is expressed in intact liver cells as well (Christiansen & Bremer, 1976); (*c*) that K_m values of rat liver γ-butyrobetaine hydroxylase obtained by the present method, of 7.3 μM for γ-butyrobetaine (Fig. 3), 38 μM for α-oxoglutarate and about 0.5 mM for ascorbate, are much lower than values reported by others (50 μM for γ-butyrobetaine and 500 μM for α-oxoglutarate) who used a less-sensitive assay procedure (Lindstedt, 1967; Lindstedt & Lindstedt, 1970). The specific activity of γ-butyrobetaine hydroxylase in whole homogenates of rat liver, when assayed with 100 μM-γ-butyrobetaine at 37°C, was about 170 nmol of carnitine formed/min per g wet wt. of tissue, from which the capacity for carnitine production by rat liver was calculated as 783 μmol/24h per 100 g body wt. The corresponding value of net carnitine synthesis in the body is, however, only about 2 μmol/24h per 100 g body wt. (Cederblad & Lindstedt, 1976). It is clear, therefore, that, for determining the rates of carnitine synthesis in rats, availability of γ-butyrobetaine is likely to be more important than changes in the amount of γ-butyrobetaine hydroxylase. Besides, other data (Sandor *et al.*, 1983) indicate that in the liver of the guinea pig carnitine synthesis may be limited by the availability of ascorbate as well.

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