A RAPID AND SENSITIVE ASSAY OF γ-BUTYROBETAINE HYDROXYLASE

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1. Introduction

γ-Butyrobetaine hydroxylase (EC 1.14.11.1) is the terminal enzyme in the pathway of the biosynthesis of carnitine [1–3]. In the rat γ-butyrobetaine is converted to carnitine only in the liver and the testis [4]. The synthesis of carnitine from γ-butyrobetaine by a soluble fraction of rat liver was demonstrated in 1961 [5]. However, this enzyme has not been extensively purified. A partial explanation for the lack of purification of this enzyme is the unavailability of a sensitive rapid assay.

Two methods currently exist for the assay of γ-butyrobetaine hydroxylase; the use of:

(1) Methyl-labelled γ-butyrobetaine as a substrate, and the separation of the carnitine formed from the unreacted γ-butyrobetaine by Dowex 50 chromatography [1];
(2) γ-[2,3-^{3}H]Butyrobetaine as substrate and the sublimation of water from the reaction with the subsequent measurement of the tritium released [6,7].

The method utilizing Dowex 50 for separation is sensitive but requires a 12 cm column for each sample and a relatively long time for the separation. The second method is much more rapid but cannot be used if the amount of carnitine produced is <10 nmol.

Here we demonstrate a rapid, sensitive method for the assay of γ-butyrobetaine hydroxylase.

2. Materials and methods

γ-Butyrobetaine was prepared as in [8]. Rats (Sprague-Dawley strain) were purchased from King Animal Labs., Madison, WI, and fed Purina rat chow ad libitum. Ovine muscle was obtained from a freshly slaughtered animal at the Institute for Muscle Biology at this university.

Acetyl [1-^{14}C]coenzyme A was purchased from either New England Nuclear, Boston, MA, or Amersham, Chicago, IL. Otuska Pharmaceut. Co., Osaka, donated the L-carnitine. All other chemicals and enzymes were of the highest purity available from commercial sources.

2.1. *Preparation of extracts*

Livers and muscle were homogenized in 2 vol. buffer [250 mM mannitol, 70 mM sucrose, 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid), 1 mM EGTA [ethylene bis(oxyethylenenitrilo)]-tetra-acetic acid (pH 7.4) with KOH] with a PT 10-35 Polytron homogenizer fitted with a PT 10ST generator.

The homogenates were centrifuged for 15 min at 28 700 × *g*; the resulting supernatant fractions were centrifuged at 105 000 × *g* for 1 h. The high speed supernatant fractions were dialyzed against 2 l 0.1 M potassium phosphate buffer (pH 7.0) overnight at 5°C to remove salts and especially carnitine from the extracts. These samples were used for determination of γ-butyrobetaine hydroxylase activity in the liver of male rats and in the ovine muscle.

The dialyzed, 45–55% saturated ammonium sulfate fraction of the high-speed supernatant from rat liver was used for determining the various parameters of the assay. The protein concentration in each of the samples was determined by the biuret method.

2.2. *Method of assay*

The principle of the assay was to carry out the hydroxylation of γ-butyrobetaine using unlabeled substrates and then to determine the carnitine produced using a modification of the two radioactive enzyme assays in [9,10].

Reactions were carried out in 1.5 ml Eppendorf centrifuge tubes in 0.2 ml containing 10 mM TES

[*N*-((trishydroxymethyl)methyl)-2-aminoethanesulphonic acid] buffer (pH 7.2); 200 μM ferrous sulfate; 2 mM ascorbate; 4 mM α-ketoglutarate; 10 mM γ-butyrobetaine; catalase (EC 1.11.1.6) (10 μg protein) and sufficient γ-butyrobetaine hydroxylase. This reaction mixture was a modification of that in [1]. The reaction is initiated by the addition of the γ-butyrobetaine. Incubation was for ≤1 h in a water bath at 37°C. Reactions were terminated by inserting the centrifuge tube into a boiling water (90°C) bath for 5 min.

The acetylation of carnitine is carried out in the centrifuge tube used for the γ-butyrobetaine reaction and allowed to proceed at room temperature for 1 h. For reactions that produce >600 pmol carnitine an appropriate aliquot is transferred to another centrifuge tube for the carnitine assay. The reaction conditions are those of [9] with the substitution of 0.1 M TES buffer (pH 7.2) for the phosphate buffer. Each reaction mixture contains 0.2 ml, 0.1 M TES (pH 7.2) and 6 mM NEM (*N*-ethyl maleimide). Initiation of the reaction was by the addition of a mixture of acetyl-CoA:carnitine *O*-acetyl-transferase (EC 2.3.1.7) (5 μg protein) and sufficient acetyl [1-^{14}C]coenzyme A. The reaction is terminated by the addition of 0.6 ml Dowex 50X8-400 slurry as in [10], vortexed, stored on ice for 10 min, vortexed and centrifuged at 8000 × *g* for 2 min in an Eppendorf centrifuge (model 3200). Of the 0.8 ml final vol., 0.2 ml is counted in a phase-combining cocktail in mini-vials in a liquid scintillation spectrophotometer. The NEM reacts with free coenzyme A to pull the reaction of carnitine acetyl transferase to completion in the direction of acetylcarnitine production.

3. Results and discussion

The effects of increasing amounts of γ-butyrobetaine hydroxylase (dialyzed 45–55% saturated ammonium sulfate fraction of 105 000 × *g* rat liver supernatant) on the time-dependent conversion of γ-butyrobetaine to carnitine is shown in fig.1. Where necessary an aliquot of the γ-butyrobetaine hydroxylase incubation was used for the carnitine assay. In all other cases the entire incubation was assayed for carnitine. The assay is linear over a wide range of amounts of added enzyme. The data in fig.2 indicate that the rate of carnitine production is linear for up to 2 h incubation. Thus, time of incubation and enzyme concentrations can be easily adjusted to obtain maximum sensitivity in the shortest time period. Note that at 0 incubation time, 57 pmol carnitine was detected by the carnitine assay method. This small but significant amount of carnitine was found in the enzyme preparation used for this assay. It is postulated that some proteins have an affinity for carnitine which prevents

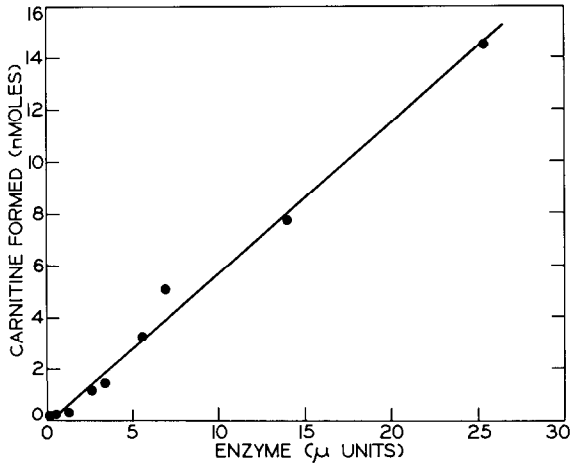

Fig.1. The influence of γ-butyrobetaine hydroxylase concentration on the conversion of γ-butyrobetaine to carnitine. Reaction mixture as in section 2.2. One micro unit of enzyme activity is equal to 1 pmol carnitine formed/min. Incubation of γ-butyrobetaine hydroxylase was for 1 h.

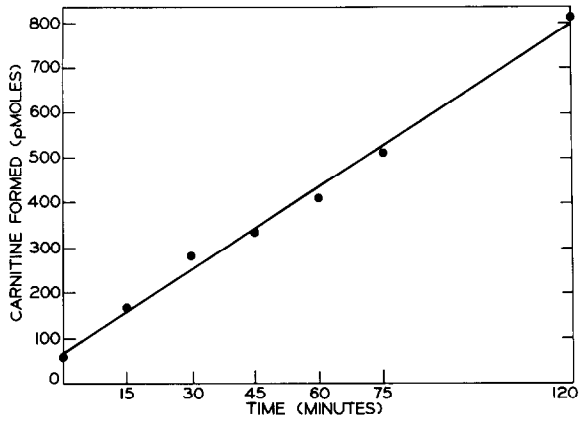

Fig.2. Time-dependent conversion of γ-butyrobetaine to carnitine by γ-butyrobetaine hydroxylase. Reaction mixture as in section 2.2. An aliquot of the dialyzed 45–55% ammonium sulfate fraction of the high speed supernatant containing 6.2 μunits γ-butyrobetaine hydroxylase was added to each reaction mixture.

Table 1
Substrate requirements of γ-butyrobetaine hydroxylase

Reactions	Carnitine produced (pmol)	% Control
Complete system[a]	362	100
Boiled enzyme[b]	0	0
Complete system		
+1 mM NEM[c]	193	53
+5 mM EtOH	323	89
-KCl	396	110
$-MgCl_2$	384	110
-Nicotinamide	377	100
$-FeSO_4$	109	30
-Ascorbate	38	11
-Catalase	254	70
-α-Ketoglutarate	0	0
-γ-Butyrobetaine	0	0

[a] The complete reaction mixture of 0.2 ml consisted of 10 mM TES (pH 7.2); 20 mM KCl; 200 mM nicotinamide; 200 μM $FeSO_4$; 5 mM $MgCl_2$; 2 mM ascorbate; 4 mM α-ketoglutarate; 10 mM γ-butyrobetaine and catalase (10 mg protein)

[b] The dialyzed 45–55% ammonium sulfate fraction of the 105 000 × *g* supernatant was boiled prior to the 37°C incubation

[c] NEM (*N*-ethyl maleimide) was added prior to the start of the 37°C incubation

All incubations were for 1 h at 37°C

these carnitine molecules from being completely removed by dialysis. However, the carnitines are probably released upon denaturation of the protein in the boiling step making them available as substrate for the carnitine assay.

Table 1 shows the effects of various substrates and cofactors on γ-butyrobetaine hydroxylase activity. The γ-butyrobetaine hydroxylase preparations must be dialyzed to remove:

(1) Any carnitine which would result in a high background in the carnitine assay;
(2) Coenzyme A which may react with any endogenous carnitine transferase enzymes to convert carnitine to acylcarnitines which would result in underestimation of the carnitine produced by γ-butyrobetaine hydroxylase.

If NEM is included in the reaction only a 47% reduction is observed in the production of carnitine. This incomplete inhibition of γ-butyrobetaine hydroxylase requires the termination of this reaction by a boiling water bath prior to the carnitine assay. Several of the cofactors, such as KCl, nicotinamide, $MgCl_2$ or catalase are not required for full activity. The effect of catalase cannot be completely ruled out because it may be present in the dialyzed supernatants or ammonium sulfate-precipitated protein fractions used in these experiments. These data do not support the substrate or cofactor requirements for γ-butyrobetaine hydroxylase reported [9]. γ-Butyrobetaine hydroxylase activity in male rat (140–160 g wt) liver (dialyzed 105 000 × *g* supernatant) was 9.12 nmol carnitine formed . mg protein^{-1} . h^{-1}. We were unable to detect γ-butyrobetaine hydroxylase activity in ovine forearm muscle at 10 times the protein concentration used for rat liver even with this extremely sensitive assay. Thus, our data confirm [12], but do not substantiate the report [13] that indicates small quantities of this enzyme are present in sheep muscle.

Acknowledgements

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