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Analytical Methods Section

Routine microbiological assay for carnitine activity in biological fluids and tissues

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A practical method for measuring acid-soluble and total (acid- and alkaline-soluble) carnitine growth activity for a carnitine-specific mutant of the enteric yeast *Torulopsis bovina* in biological fluids and tissues is described. It grows at 37°C in a simple, cheap, chemically defined medium; its response threshold to carnitine is 100 pg ml^{-1}. Growth is measured as absorbance units with any turbidometer. Carnitine contents in human and rat fluids and tissues so measured accord with those obtained after multi-step extraction methods, enzyme procedures or radiochemical procedures.

INTRODUCTION

Carnitine (3-hydroxy-4-*N*-trimethylaminobutyric acid) is ubiquitous in higher animals. By acylation with coenzyme A, it transports long-chain fatty acids into the matrix compartment of mitochondria, enabling their β-oxidation (Bremer, 1983; Rebouche & Paulson, 1986), thereby permitting those fatty acids to serve as a major energy source. Pathologic manifestations of carnitine deficits are well documented (Rebouche & Engel, 1984; Gilbert, 1985; Stumpf *et al.*, 1985).

Many assay methods have been reported for carnitine using radio-enzymes or other radio-labeled substrates, radio-counters, HPLC, ion-exchange binding elution coupled with reversed-phase chromatography, or spectroscopy (Cederblad & Lindstedt, 1972; McGarry & Foster, 1976; Borum, 1987; Sekas & Paul, 1989; Borum, 1990; Deufel, 1990). These assays are awkward for routine use. A spontaneous carnitine-requiring mutant strain of the choline-auxotrophic yeast *Torulopsis bovina* was proposed for carnitine assay (Travassos & Sales, 1974); this methodology proved erratic in routine use with clinical materials. This paper reports on a practical routine procedure for assaying carnitines in biologic fluids and tissues by their growth promotion of *T. bovina*.

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MATERIALS AND METHODS

Maintenance of *T. bovina*

T. bovina is in the American Type Culture Collection (No. 26014), Rockville, MD and Centraalbureau von Schimmel Cultures (No. 2760), Delft, Holland. For maintenance, the culture is grown in maintenance broth for 24 h at 37°C until use (see below). One drop of the 4°C-stored subculture serves for inoculating new stock cultures (as above) every week. The maintenance medium is one part basal medium (choline omitted) (Table 1) + one part distilled water + 30 ng dl^{-1} of L-carnitine; it is distributed as 10 ml in 20 × 125 mm screwcapped tubes, autoclaved 20 min at 118–120°C, and then stored at 4°C. The medium is stable indefinitely.

Standards

Synthetic L-carnitine HCl (Sigma Chemical Co., St. Louis, MO) serves as standard. (The gravimetric factor for L-carnitine as L-carnitine HCl is 1·23) L-Carnitine HCl (123 mg) is dissolved in distilled water (100 ml). Ten-fold dilutions of this stock solution are made serially with water to provide carnitine working standards: 1, 10 and 100 ng ml^{-1}. Standards are stable almost indefinitely when stored at 4°C. All solutions, and basal medium are kept at 4°C with a few drops of volatile

Table 1. Basal medium for assay of carnitine activity with *T. bovina*[a]

Constituent	Concentration
Glucose	20·0 g
L-Asparagine. H_2O	1·0 g
KH_2PO_4	1·0 g
$MgSO_4$. $7H_2O$	0·5 g
NaCl	1·0 g
Citric acid. H_2O	1·0 g
KNO_3	1·0 g
NH_4HCO_3	1·0 g
L-Arginine HCl	1·0 g
Metal Mix[b]	0·1 g
Biotin[c]	40 μg
Ca pantothenate[c]	4 mg
Thiamin. HCl[c]	4 mg
Pyridoxine. HCl[c]	4 mg
Pyridoxal. HCl[c]	4 mg
Pyridoxamine. 2 HCl[c]	4 mg
Nicotinic acid[c]	4 mg
Inositol[c]	10 mg
Choline bitartrate[c]	2 mg
Tween 80 (polyoxyethylene sorbitan monooleate)	0·001 ml (v/v)
	Distilled water to 500 ml (double strength)

[a] pH 4·5 (use 4N H_2SO_4 to adjust pH).
[b] $Fe(NH_4)_2(SO_4)_2$. $6H_2O$ (4·2 g); $MnSO_4$. H_2O (1·6 g); $ZnSO_4$. $7H_2O$ (2·2 g); $(NH_4)_6MO_7O_{24}$. $4H_2O$ (0·36 g); $CuSO_4$. $5H_2O$ (0·16 g); H_3BO_3 (0·06 g); $CoSO_4$. $7H_2O$ (0·05 g); NH_4VO_3 (0·05 g); $NiSO_4$. $6H_2O$ (0·05 g); and CrK $(SO_4)_2$. $12H_2O$ (0·1 g). Prepared as a fine-ground powder; the stipulated mix suffices for 50 liters of medium.
[c] Added from stock solutions (mg ml^{-1}).

preservative (one part chlorobenzene; one part 1,2 dichloroethane; two parts *n*-butyl chloride). The preservative volatilizes away upon autoclaving. A standard curve is prepared (Table 2). A blank (Table 2, no. 1)—2·5 ml of basal medium plus 2·5 ml distilled water—is included to allow for carnitine introduced with the inoculum. Growth of *T. bovina* (ordinate) against carnitine (abscissa) is plotted on semi-log paper (Keuffel & Esser, no. 46–5490, 3 cycle); values of unknowns are calculated from the standard curve.

Table 2. Preparation of the standard carnitine curve[a]

Flask	Content (ng per 5 ml)	Carnitine standard addition	Basal medium[b]	Distilled water
1	0	0	2·5	2·5
2	0·5	0·5 ml of 1·0 ng ml^{-1}	2·5	2·0
3	1·5	1·5 ml of 1·0 ng ml^{-1}	2·5	1·0
4	5	0·5 ml of 10 ng ml^{-1}	2·5	2·0
5	15	1·5 ml of 10 ng ml^{-1}	2·5	1·0
6	50	0·5 ml of 100 ng ml^{-1}	2·5	2·0

[a] Figure 1 shows a typical curve.
[b] See Table 1.

Assaying carnitine activity

The basal medium for specifically assaying carnitine (Table 1), completely defined, is dispensed 2·5 ml per 25-ml borosilicate micro-Fernbach flask (Fisher Scientific Co., Springfield, NJ) provided with a 25-mm aluminum cap (Fisher). The solution to be assayed is added and the volume brought to 5 ml with water (Table 3). The capped flasks containing standards and specimen solution are placed in a 2-litre Pyrex utility tray and autoclaved (121°C) for 20 min, covered with an inverted tray after removal from the autoclave and cooled to room temperature to prepare for *T. bovina* inoculation. For preparation of the inoculum one adds 2 ml of a stored week-old culture (see above) into fresh maintenance medium and incubates for 6 h at 37°C. One millilitre of this culture is added to sterile water (5 ml); a drop of this cell suspension is then added aseptically to each flask. The flasks are re-covered with a tray, sealed with masking tape, and incubated at 37°C; full growth is reached within 20–24 h. Growth turbidity is measured with any turbidometer at 660 nm, and is expressable in linear absorbance units.

Biological fluids and tissues used for carnitine assay

Fluids

Blood is collected in Vacutainers containing EDTA as anticoagulant (Becton-Dickinson, Rutherford, NJ). Plasma is pipetted away from red blood cells (rbc) after centrifuging the whole blood. Rbc are washed three times with saline; the washes are discarded; one millilitre of packed cells is used for assay. Bile to be assayed is collected via drainage tube after cholecystectomy. An aliquot of a collected 24-h urine is used for the assay; a random morning urine is valueless. Also, calculation of micronutrients based on creatinine excretion is an unreliable index, notably for patients with decreased protein intake which will lower renal plasma flow and creatinine clearance (Vestergard & Leverett, 1958).

Tissues

A fresh tissue biopsy, obtained by needle or autopsy, is freed from coagulum and adhering tissue, sliced, and washed thrice with distilled water to lyse all rbc. A weighed portion of tissue, suspended in enough distilled water for assay, is homogenized in a tissue grinder; it may be stored frozen at –20°C until assay as in Table 3.

Human

Blood, plasma, urine, spinal fluid before surgery, and rbc were obtained (*vide supra*) from healthy volunteers after obtaining informed consent (ages 24 to 55) without history of liver, kidney, heart, muscle or gastrointestinal disease. Tissue specimens obtained at autopsy were from people killed by accident; none had evidenced disease.

Table 3. Extraction and assay of acid-soluble and total carnitine activity in rat and human fluid and tissues

Acid-soluble carnitine activity in fluids and tissues

Fluids

(1) To fluid (1 ml) add 2·5% trichloroacetic acid (TCA) (9 ml).
(2) Mix well to precipitate protein and release carnitine. Centrifuge off debris, save supernatant.
(3) Dilute supernatant with distilled water to 2000. Greater dilutions may be needed for urine. e.g. to 4000; less dilution for CSF, e.g. to 200.
(4) Assay in multiple concentrations as below.

Tissues

(1) Homogenize tissues with distilled water to yield a concentration of 10 mg tissue per ml.
(2) To 1 ml of homogenate, add 9·0 ml of 2·5% TCA; dilution corresponds to 1·0 mg tissue per ml. Mix well.
(3) Centrifuge off debris; save supernatant.
(4) When necessary, further dilutions of supernatant are made with distilled water, e.g. heart dilute to 0·025 mg tissue per ml; liver dilute to 0·01 mg tissue per ml.
(5) Assay diluted supernatant extract in multiple concentration as below.

Total carnitine activity in fluids and tissues

Fluids

(1) To fluid (0·5 ml) add 0·5 N KOH (1 ml).
(2) Autoclave for 10 min at 121°C, 15 psi to hydrolyze long-chain acyl carnitines.
(3) Add distilled water (8·5 ml). Mix and centrifuge; save supernatant. Dilution of fluid is now 20-fold.
(4) Supernatants from fluids are diluted further with water, e.g. to 2000, or 4000 for urine.
(5) Assay diluted supernatant in multiple concentrations as below.

Tissues

(1) To 0·5 millilitre of homogenized tissue (10 mg ml^{-1}) add 0·5 N KOH (1 ml).
(2) Follow (2) and (3) above for fluids.
(3) Further dilutions of supernatant with water from step (3) are always necessary for tissue; the extract depends on the tissue used, e.g. heart, dilute to 0·025 mg tissue per ml; liver dilute to 0·01 mg tissue per ml.
(4) Assay diluted supernatant extract in multiple concentration as below.

Addition of carnitine extracts from fluids and tissues for assay

Flask (1–6 is standard curve–Table 2)	*Basal medium (ml) (Table 1)*	*Supernatant (ml) of unknowns*	*Water (ml)*
7	2·5	1·0	1·5
8	2·5	1·5	1·0
9	2·5	2·0	0·5

Rat

Male Wistar rats (Charles River) were kept in metabolic cages and fed a standard commercial rodent chow diet (Purina) and water ad lib until weighing approximately 250 g. Urine was collected for 24 h, then rats were decapitated and exsanguinated. Whole blood was used for assay. Organs for assay were excised, washed three times with water and kept at –20°C.

Extraction of carnitines

As denoted here and by others (Fuller & Hoppel, 1988; Borum, 1990; Deufel, 1990), when specimens are treated with acid before alkaline hydrolysis, acid-soluble carnitines (free and short-chain) can be extracted for determination. Alkaline hydrolysis, coupled with subsequent acid hydrolysis, will solubilize all carnitines, e.g. free, short-chains plus long-chain acyl carnitines to yield total carnitines present in the specimen; as designated here, those carnitines active in promoting *T. bovina* growth can then be determined. Since the authors, as well as many other investigators, do not know the exact molecularity of all the carnitines liberated by the procedure used here, the term 'carnitine activity' was chosen to comprise carnitines that are active for growing *T. bovina*. Extraction and preparations for assay of such carnitines in biological fluids are detailed in Table 3. Extraction of acid-soluble carnitines is with 2·5% trichloroacetic acid (TCA) (Table 3); this extract contains free and short-chain (less than 10 carbon) acyl carnitines (Fuller & Hopple, 1988; Borum, 1990). Extraction for total carnitines is first carried out with alkaline hydrolysis using 0·5N KOH (Table 3). As shown here and by others, alkaline hydrolysis liberates carnitine from long-chain (more than 10 carbon length) acyl carnitines, e.g. palmitoylcarnitine (Travassos & Sales, 1974; Fuller & Hoppel, 1988; Borum, 1990). After alkaline hydrolysis, 2·5% TCA is added to the

mixture to solubilize remaining acid-soluble carnitines for assay with *T. bovina* (Table 3). The extreme dilutions used in the carnitine assay (Table 3) obviates interfering substances, e.g. drugs. A control solution containing 5 μg of L-carnitine is added to each assay procedure to check recovery of carnitine. Recovery of palmitoylcarnitine (Sigma Chemical, St. Louis, MO), equimolar with 5 μg L-carnitine, is also added to monitor the effectiveness of the alkaline hydrolysis for obtaining carnitine from long-chain acylated carnitines for utilization by *T. bovina*.

Calculations for carnitine content

Fluids

a = Concentration of carnitine standard (per ml) (Table 2) derived from sample absorbance from the standard curve, e.g. the position along the abscissa at which the 'unknown' absorbance coincides with the standard curve; b = final dilution of the sample (Table 3); c = total volume in assay flasks, e.g. 5 ml (Table 3); d = ml (volume) sample used for test (Table 3); e = ml (volume) of diluted sample added to assay flasks 'supernatant' (Table 3);

$\frac{a \times b \times c}{d \times e}$ = the concentration of carnitine activity in sample per ml.

For urine

Multiply by total 24 h volume; express as carnitine activity per 24 h sample.

Tissues

a = Concentration of carnitine (per ml) (Table 3) derived from sample absorbance; b = total volume in assay flask, e.g. 5 ml; c = concentration of tissue extract (mg ml^{-1}) after dilution (Table 3); d = ml of diluted sample added to assay flask (Table 3).

$\frac{a \times b}{c \times d}$ = the concentration of carnitine activity per milligram of sample.

RESULTS AND DISCUSSION

This assay is designed to measure only those carnitines active for *T. bovina* growth, i.e. carnitine 'activity'. As mentioned, neither the authors nor others specifically know the exact molar proportions of the various carnitines liberated from fluids and tissues under the specified test conditions; such *T. bovina* growth stimulation elicited by various carnitines must therefore be denoted as being specifically due to carnitine 'action'. A standard curve for measuring L-carnitine growth activity (means ± SD for 20 different assays) is shown in Fig. 1; results (Table 4) are listed as mean ± SD. *T. bovina* responds to as little as 100 pg ml^{-1} of L-carnitine; in the assay, growth of the blank is negligible without carnitine. As noted, the high dilution of the fluids and tissues as assayed eliminates interferences of drugs and precursors of carnitine synthesis, e.g. butyrobetaine, lysine and methionine. With the procedures in Table 3, *T. bovina* responds to representative short-chain carnitines, e.g. acetylcarnitine and also to long-chain carnitines, e.g. palmitoyl-, myristoyl-, stearylcarnitine, but only after alkaline hydrolysis. Recovery of 5 μg ml^{-1} of aforementioned carnitines as equimolar with respect to L-carnitine, was 90–110%. The *T. bovina* growth promoting activity of other short- or long-chain acyl carnitines could not be assayed: they are not commercially available. As noted, *T. bovina* does not respond to long-chain acyl carnitines without alkaline hydrolysis to liberate the carnitines; these apparently are unavailable to the yeast (Emaus & Beiber, 1983). It is therefore necessary to hydrolyze the long-chain acyl carnitines with alkali and heat (Table 3) to make them available to *T. bovina*. Activities of acid-soluble carnitine and long-chain acyl carnitine can thus be differentiated here, e.g. subtracting the acid-soluble carnitines from the total carnitines (Table 4) will yield a value for alkaline-soluble carnitines. Such differentiations may quickly identify clinical problems associated with acid–alkali carnitine imbalances and signal further investigations, with more complex procedures, to pinpoint the specific carnitines that elicit the imbalance if deemed necessary; this has been done (Duran *et al.*, 1990). Results with *T. bovina* (Table 4) parallel those obtained in biologic fluids and tissues by others using multi-step extraction methods, enzyme-based procedures, radiochemicals and radio-counters (Cederblad & Lindstedt, 1972; Mitchell, 1978; Borum *et al.*, 1985;

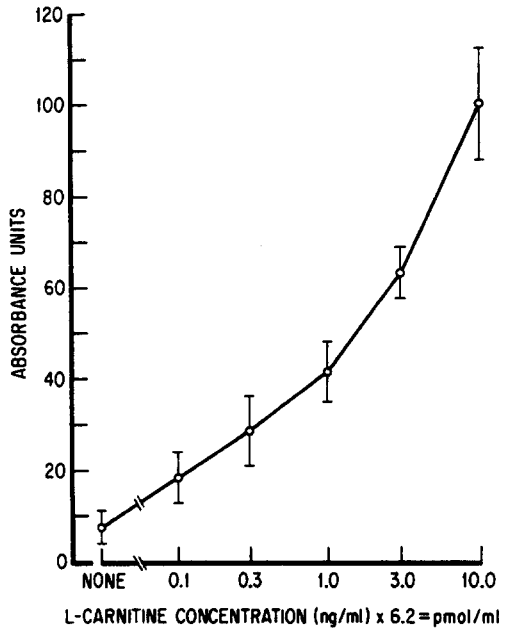

Fig. 1. Standard reference of *T. bovina* to L-carnitine (mean ± SD; 20 determinations for each point).

Table 4. Acid-Soluble/Total Carnitine Activity in Human and Fluids and Wet-Weight Tissues[a]

	Human		Rat	
Fluids (μg ml^{-1})				
Whole blood	8·3 ± 2·7 / 10·2 ± 3·4	(32)	7·4 ± 1·0 / 10·9 ± 0·7	(12)
Plasma	6·6 ± 0·8 / 7·7 ± 0·9	(214)	—	
Cells	7·3 ± 3·0 / 9·2 ± 4·2	(42)	—	
Bile	1·5 ± 0·3 / 1·9 ± 0·1	(8)	—	
Cerebrospinal fluid (ng ml^{-1})	512 ± 178 / 689 ± 332	(11)	—	
Urine (mg per 24 h)	25 ± 9 / 40 ± 10	(16)	1·5 ± 0·3 / 1·6 ± 0·4	(12)
Tissue (ng mg^{-1})				
Heart	93 ± 28 / 194 ± 71	(6)	188 ± 80 / 253 ± 106	(12)
Muscle	535 ± 180 / 905 ± 271	(6)	201 ± 86 / 291 ± 92	(12)
Liver	90 ± 31 / 146 ± 54	(6)	78 ± 17 / 110 ± 31	(12)
Kidney	—		53 ± 6 / 67 ± 17	(12)
Brain	—		16 ± 4 / 21 ± 6	(12)
Pancreas	—		8 ± 3 / 11 ± 3	(12)

[a] Numbers in parentheses indicate number of specimens tested.

Carter *et al.*, 1986; Fuller & Hoppel, 1988; Sekas & Paul, 1989; Borum, 1990; Deufel, 1990; Haeckel *et al.*, 1990); hence are valid.

Equipment and materials for the *T. bovina* method are cheaper than those for the aforementioned alternative methods and may be present in most conventional or hospital microbiological laboratories. Carnitine-activity surveys can thus be carried out much like those well established for clinical activity of folates, B_{12} and other vitamins (Baker *et al.*, 1967; Baker & Frank, 1968; Baker *et al.* 1975, 1979; Sauberlich, 1984; Baker *et al.*, 1987; Stokstad *et al.*, 1988) even though one does not know to which specific carnitines the yeast is actually responding. This was achieved originally by documenting clinical folate activity imbalances (Baker *et al.*, 1959; Herbert, 1961; Waters & Mollin, 1961; Chanarin & Berry, 1964). At that time one did not know the molecular identity of the folate which activated growth of *Lactobacillus casei*; however the main *L. casei* growth activity produced by the 'unknown' folates matched the clinical folate status. Similarly, with *T. bovina*, one can carry out carnitine surveys on a large-scale to delineate carnitine imbalances associated with acid-soluble or alkaline hydrolyzable carnitines involved in pathologic states (Engel, 1980; Rebouche & Engel, 1984; Stumpf *et al.*, 1985) without necessarily knowing the actual carnitines the yeast was responding to—a significant consideration because carnitine dyscrasias can be life-threatening (Kendler, 1986). High performance liquid chromatography and GC–MS methods can allow determination of specific carnitines but are not practical for large-scale routine assays (Borum, 1990) at this preliminary stage of clinical knowledge. In the authors' studies, using *T. bovina*, it was found that mainly acid-soluble liver carnitines were decreased in patients with alcoholic liver disease and cirrhosis, and this confirms work by others (Rudman *et al.* 1974). The authors are attempting to document that such a carnitine decrease contributes to production of fatty liver; the effects of carnitine therapy are being considered.

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