An improved and simplified radioisotopic assay for the determination of free and esterified carnitine

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Summary The radioisotopic assay for carnitine first described by Cederblad and Lindstedt (*Clin. Chim. Acta.* **37:** 235–243, 1972) and modified by Bøhmer et al (*Clin. Chim. Acta.* **57:** 55–61, 1974) has been improved and simplified. As a result, the assay yields a linear response over a wide range of carnitine concentrations without the need for excessive amounts of labeled acetyl-CoA. In addition, it will measure very small quantities of carnitine even in the presence of excess acylcarnitine. The method allows rapid determination of free and esterified carnitine in small volumes of plasma (50 μl is sufficient) without the need for prior deproteinization of the samples.

Supplementary key words tetrathionate · acylcarnitine · acetylcarnitine · acetylcarnitine transferase · plasma carnitine

Because of the relative insensitivity of existing spectrophotometric techniques for the measurement of carnitine (1, 2), Cederblad and Lindstedt (3) introduced a radioisotopic method designed to detect picomole quantities of this compound in small volumes of plasma. Basically, the initial step was incubation of a neutralized perchloric acid extract of plasma with [1-^{14}C]acetyl-CoA of known specific activity and carnitine acetyltransferase (E.C. 2·3·1·7). The labeled acetylcarnitine formed according to the reaction

L-carnitine + acetyl-CoA

$$\rightleftharpoons \text{acetyl-L-carnitine} + \text{CoASH} \quad [1]$$

Abbreviation: DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.

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was separated from the unreacted [1-^{14}C]acetyl-CoA by passing the mixture through a column of anion exchange resin and determining the isotope content of the effluent fluid.

A serious limitation of the assay as outlined above stems from the ready reversibility of reaction *1* (4) which results in nonlinear standard curves unless a considerable excess of acetyl-CoA relative to the carnitine concentration is used. Bøhmer, Rydning, and Solbery (5) partially resolved the problem by including 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in the assay mixture to trap the CoASH released in reaction *1*, but even so obtained a 1:1 relationship between the amount of carnitine present and acetylcarnitine formed only if the ratio of [acetyl-CoA]:[carnitine] exceeded 5:1.

The present report describes an alternative modification of the Cederblad-Lindstedt technique that overcomes the problem of nonlinear responses discussed above and that possesses a number of additional advantages over the methods previously described (3, 5).

Experimental procedure

Assay for free carnitine. Assays were carried out in disposable Kimble glass culture tubes (13 × 100 mm). The basic reaction mixture contained, in a volume of 1.05 ml, 120 μmole of Tris-HCl buffer, pH 7.3; 2 μmole of sodium tetrathionate; 25 nmole (0.025 μCi) of [1-^{14}C]acetyl-CoA, and the sample to be analyzed; in the case of plasma this sample was generally 0.05 ml. Three carnitine standards (2.5, 5.0 and 10 nmole) were routinely included with each series of test samples. Reactions were initiated by addition of 5 μl (1 U) of a carnitine acetyltransferase suspension and the mixture was allowed to stand for 30 min at room temperature. At this time 0.3 ml of a continuously stirred slurry of Dowex 1-X10 anion exchange resin, containing 0.22 ml of water as determined by drying to constant weight, was added using an automatic pipette of wide bore (Selectapette, Clay Adams, Parsippany, N.J.). The mixture was then agitated with vortex and placed in ice. This step was repeated twice at 10 min intervals, after which the tubes were centrifuged and 0.5 ml of the supernatant fluid was mixed with 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and assayed for radioactivity in a liquid scintillation counter. An enzyme blank allowed correction for nonspecific supernatant radioactivity. In this system the quantity of [1-^{14}C]acetylcarnitine formed (not adsorbed to the resin) was stoichiometrically related to the amount of carnitine present.

Assay for total (free plus esterfied) carnitine. The procedure was identical with that described above except that the sample, usually 0.05 ml in volume, was added to 0.1 ml of 1M Tris base and 0.05 ml of 0.4N KOH (pH of mixture approx. 13) and allowed to stand for 1 hr at 37°C in order to hydrolyze any acylcarnitines present. Thereafter, 0.2 ml of 0.575 N HCl was added to return the pH to 7.3, followed by the remainder of the components listed in the assay for free carnitine.

Variations in the above procedures are indicated in the text.

Materials. L-carnitine was obtained from Gibco, Grand Island, N. Y. D,L-[methyl-^{14}C]carnitine was from Amersham/Searle, Arlington Heights, Ill. Acetyl-L-carnitine and acetyl-CoA were prepared by the methods of Bremer (6) and Stadtman (7), respectively. The concentrations of stock solutions of carnitine and acetyl-CoA were determined using the DTNB assay described by Marquis and Fritz (8). The acetylcarnitine solution was assayed in the same way after prior alkaline hydrolysis. [1-^{14}C]Acetyl-CoA (specific activity 55 mCi per mmole) was from New England Nuclear, Boston, Mass. Sodium tetrathionate was obtained from ICN Pharmaceuticals, Inc., Plainview, N. Y. Dowex 1-X10 anion exchange resin in its Cl^- form (200–400 mesh) was from Baker Chemical Co., Phillipsburg, N.J. The resin was treated with 1 N HCl, filtered under vacuum and washed with water until the filtrate had a neutral pH. Carnitine acetyltransferase was from Boehringer Mannheim Corp., N. Y.

Results and discussion

Determination of optimal conditions for the radioisotopic assay of carnitine. As indicated above, reaction *1* is readily reversible with an equilibrium constant of 1.67 in the direction of acetylcarnitine and CoASH formation (4). Accordingly, in the assay described by Cederblad and Lindstedt (3) a nonlinear response would be predicted unless the concentration of acetyl-CoA relative to carnitine is kept extremely high. Moreover, if the mixture also contains unlabeled acetylcarnitine in significant amounts major errors can be expected due to equilibration of label between the pools of acetyl-CoA and acetylcarnitine (3). Both problems, however, should be surmountable provided that the CoASH released in reaction *1* could be rapidly removed from the system. It was felt that a useful agent for this purpose might be sodium tetrathionate, a disulfide compound that oxidizes CoASH through reaction with its sulfhydryl group. In contrast to DTNB, tetrathionate does not inhibit the carnitine acetyltransferase enzyme (4, 9, 10).

To test this possibility a series of tubes containing a

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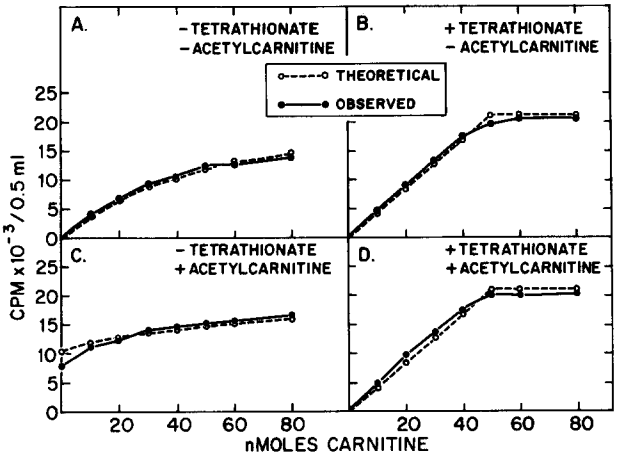

Fig. 1. Effect of tetrathionate on the assay for carnitine in the absence and presence of acetylcarnitine. Reactions were carried out as described in the assay for free carnitine under Experimental Procedure with the following modifications: 50 nmole and 52,660 cpm of [1-^{14}C]acetyl-CoA were used; tetrathionate was omitted in the experiments of panels A and C, and acetylcarnitine (36 nmole) was included in the experiments of panels C and D; carnitine was present in the amounts indicated. Reactions were initiated with 0.5 U of carnitine acetyltransferase and were terminated after 60 min by the addition of 0.2 ml of a Dowex 1-X10 slurry containing 0.13 ml of water.

fixed amount of [1-^{14}C]acetyl-CoA[1] received different amounts of carnitine such that the concentration range of the latter would extend from below to significantly above the concentration of acetyl-CoA. A fixed quantity of unlabeled acetylcarnitine was added to a similar series of tubes. Enzyme was then added and the reactions were allowed to proceed either in the absence or presence of tetrathionate. Since the initial concentrations of reactants and products were known it was possible to calculate the expected yield of radioactivity associated with the [1-^{14}C]acetylcarnitine formed on the assumption that, in the absence of tetrathionate, reactions would proceed only to a point of equilibrium, whereas in the presence of the CoASH trapping agent they would be forced to completion.

Excellent agreement was obtained between the theoretically predicted and the observed values in all four series of incubations (**Fig. 1**). As expected, in the absence of tetrathionate (panel A) the response of the assay to increasing carnitine concentrations was nonlinear. A more serious problem was evident when the assay was run in the absence of tetrathionate but with acetylcarnitine present (panel C). In this circumstance the recovery of label in acetylcarnitine was nearly independent of carnitine concentration over the range studied, rendering the assay valueless. In contrast, when tetrathionate was included the relationship between radioactivity recovered as acetylcarnitine and the amount of carnitine present was essentially linear until the quantity of carnitine equaled that of the [1-^{14}C]acetyl-CoA present, i.e., 50 nmoles (panels B and D). At this point all of the label initially present as acetyl-CoA had been converted into acetylcarnitine, with the result that the addition of excess carnitine produced no further response. The fact that identical results were obtained whether or not unlabeled acetylcarnitine was present indicates that the assay will accurately determine free carnitine even in the presence of excessive amounts of short chain acylcarnitines.

To test the time course of the assay a second series of experiments was done as depicted in **Fig. 2.** Panel A shows that even with the largest amount of carnitine used (42 nmole) the production of [1-^{14}C]acetylcarnitine ceased between 30 and 40 min. Further evidence that the inclusion of tetrathionate had pulled the reactions to completion is provided in panel B, where the radioactivity associated with acetylcarnitine at the 60 min time point is plotted against the quantity of carnitine initially present. Not only is the relationship linear, but extrapolation of the line to a point on the abscissa equivalent to 56 nmole, i.e., the amount of acetyl-CoA used in this case, gives an intersect on the ordinate of 21,600 cpm per 0.5 ml of supernatant fluid. Since 53,000 cpm were added as [1-^{14}C]acetyl-CoA and the total fluid volume was 1.18 ml after addition of the Dowex slurry (1.05 ml reaction

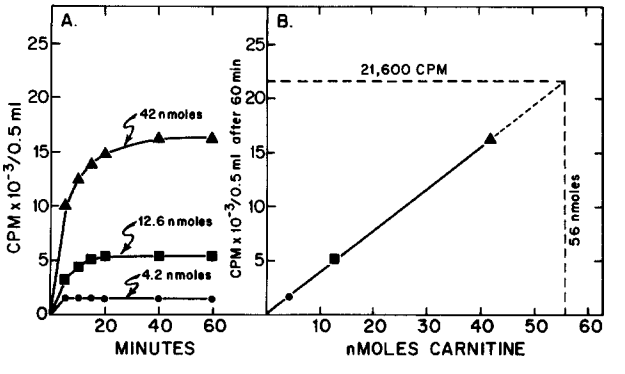

Fig. 2. Time course of the carnitine assay. Reactions were carried out as described in the assay for free carnitine under Experimental Procedure with the following modifications: 56 nmole and 53,000 cpm of [1-^{14}C]acetyl-CoA were used; carnitine was present in the amounts shown. Reactions were initiated with 0.5 U of carnitine acetyltransferase and were terminated at the indicated times by the addition of 0.2 ml of a Dowex 1-X10 slurry containing 0.13 ml of water. In panel B the radioactivity present in the supernatant fluid at the 60 min time point of panel A has been plotted against the carnitine content.

[1] The acetyl-CoA used was contaminated by free CoASH to the extent of 2–3%. While this was of no consequence in the standard assay, which contained an excess of tetrathionate, it was responsible for the formation of labeled acetylcarnitine observed in the experiments of Fig. 1C under circumstances where no carnitine was added to the reaction mixture.

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TABLE 1. Assay for carnitine in untreated human plasma in the presence of added carnitine or acetylcarnitine

Additions to Assay Mixture			Carnitine Found	
50 μl Plasma	5 nmole Carnitine	3.6 nmole Acetylcarnitine	Free	Total
			nmole	
+	−	−	2.5	3.0
−	+	−	5.0	5.1
−	−	+	0	3.7
+	+	−	7.1	7.8
+	−	+	2.7	6.3
+	+	+	7.5	11.4

The assays for free and total carnitine were carried out as described under Experimental Procedure.

volume plus 0.13 ml of water present in the slurry) the maximum possible count rate per 0.5 ml of fluid can be calculated to be 22,457 cpm. Considering that in this particular experiment the blank value increased from approximately 700 to 1300 cpm per 0.5 ml during the 5 to 60 min interval, it is clear that, as was the case in the experiments of Figs. 1B and D, a 1:1 stoichiometry existed between the amounts of carnitine added and acetylcarnitine formed.

In light of the findings outlined above, the standard assay procedure employed in all future determinations utilized 1 unit of carnitine acetyltransferase and an incubation period of 30 min. Although 25 nmole and 0.025 μCi of [1-^{14}C]acetyl-CoA were routinely employed, the sensitivity of the method can, of course, be enhanced simply by increasing the specific activity of the labeled acetyl-CoA.

Application of the method to the determination of carnitine in plasma. Before the standard assay was applied to the measurement of plasma carnitine the following points were established. Prior deproteinization of the samples was unnecessary, as evidenced by the identical values for carnitine concentration obtained for the same plasma sample both before and after extraction with perchloric acid (data not shown). Secondly, the method reliably measured the amounts of free and esterified carnitine when various combinations of untreated plasma, carnitine, and acetylcarnitine were tested (**Table 1**). Thirdly, the response of the assay was linear over a sample range of 50 to 150 μl (data not shown). Finally, choline, a compound structurally related to carnitine, did not interfere with the method (data not shown).

On the basis of the above experiments we have elected to run standards of 2.5, 5.0, and 10.0 nmole of carnitine with 50 μl of sample for all routine assays of plasma carnitine. The resultant standard curves have been reproducibly linear, and replicate analyses carried out simultaneously on a given sample of human plasma gave values of 55.7 ± 1.99 and 65.7 ± 2.55 μmole per liter for free and total carnitine, respectively (mean ± SD; n = 10), results which are in close agreement with those reported by others (3, 5, 11).

It is important to recognize that the assay for carnitine in untreated plasma as reported here is feasible only if it has been established that the sample does not contain significant amounts of acetyl-CoA or carnitine acetyltransferase. We have found no evidence for the presence of either in any of the plasma samples we have analyzed. In contrast to plasma, perchloric acid extracts of other tissues will undoubtedly contain some acetyl-CoA which might conceivably interfere with the assay for free carnitine by causing variable dilution of the specific activity of the [1-^{14}C]acetyl-CoA added. Should this be suspected, the problem could be minimized by raising the concentration of the labeled acetyl-CoA. This potential source of interference would, however, be eliminated in the assay for total carnitine since any acetyl-CoA present in the sample would be hydrolyzed during the preliminary treatment with alkali.

In summary, the advantages of the procedure described here over the original radioisotopic assay for carnitine (3) and the modification of Bøhmer et al (5) are: (*i*) a linear response over a wide range of carnitine concentrations; (*ii*) the lack of interference by short (and presumably long) chain acylcarnitines; (*iii*) the ability to measure free and total carnitine in small volumes of plasma without extraction or deproteinization; (*iv*) increased rapidity and simplicity of the assay through avoidance of column preparation; (*v*) utilization of a CoASH oxidizing reagent, tetrathionate, which is without inhibitory effects on carnitine acetyltransferase.

We believe that the modified assay should have wide applicability for the measurement of carnitine in mammalian tissues and promises to be useful in the largely unexplored area of the biosynthesis and turnover of carnitine in the animal body.

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