Enzymological determination of free carnitine concentrations in rat tissues

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SUMMARY Purified carnitine acetyltransferase was employed to catalyze the reaction:

Carnitine + acetyl CoA ⇌ acetylcarnitine + CoA.

In the presence of a known excess of acetyl CoA, free CoA released was determined simultaneously in a coupled spectrophotometric assay. CoA formed was shown to be directly related to carnitine initially present. Rat tissue extracts were analyzed using this method, and μmoles of (−)-carnitine per gram dry weight were found to be 4.87 for heart; 3.14 for brown adipose tissue; 2.68 for skeletal muscle; 1.74 for testes; 1.26 for kidney; 0.55 for brain; and 0.051 for serum. The specificity and accuracy of the determinations are described.

Concentrations of carnitine (β-hydroxy, γ-trimethyl ammonium butyrate) in various tissues have been previously determined by: (*a*) bioassay procedures dependent upon the growth requirement of *Tenebrio molitor* for vitamin B_t (1, 2); (*b*) a bioassay dependent upon the response of frog rectus to carnitine derivatives (3); (*c*) methods based upon the complexing of quaternary compounds with bromophenol blue (4, 5), and (*d*) chromatographic separation, with subsequent conversion of the compound to its periodide derivative (6). In bioassay procedure (*a*), it has been implicitly assumed that vitamin B_t consists exclusively of carnitine. Even if this assumption is granted, it is apparent from values reported (1, 2) that the precision is no better than ±50%. Bioassay procedure (*b*) also lacks specificity and precision (3). In method (*c*), all reactive quaternary compounds other than carnitine must either be removed prior to colorimetric determination or be corrected for by various procedures. Further, it is essential to obtain complete esterification of carnitine for full color development (4). Although the reproducibility for the bromophenol-complex method is adequate with pure compounds, it becomes less so when crude tissue extracts are used (4, 5). The necessity to convert carnitine to its ethyl ester, the need for preliminary separation of carnitine from choline and betaine by using ion exchange resins (4, 5) or electrophoresis (7), and the ill-defined variables controlling color development make this method a rather laborious one. The fourth method cited has not yet been applied to tissues other than corn (6). Christianson et al. indicate, however, that carnitine recovery is low because of the high solubility of its periodide derivative.

We have recently developed an enzymatic assay employing purified carnitine acetyltransferase (8), which may be utilized to determine carnitine concentrations in tissues. The essential reactions in this coupled system are:

$$\text{Acetyl CoA} + \text{carnitine} \leftrightarrows \text{acetylcarnitine} + \text{CoASH} \quad \text{(I)}$$

$$\text{CoASH} + \text{5,5′-dithio}\textit{bis}\text{-2-nitrobenzoic acid} \rightleftarrows \underset{\text{Chromogenic}}{\text{NO}_2\text{-C}_6\text{H}_3(\text{COO}^-)\text{-S}^-} + \text{CoA—S—S—}\text{C}_6\text{H}_3(\text{COO}^-)\text{-NO}_2 \quad \text{(II)}$$

The spectrophotometric assay is based upon the premise that carnitine present will permit the liberation of an equimolar amount of free CoA under conditions in which the initial concentrations of acetyl CoA, carnitine acetyltransferase, and 5,5′-dithio*bis*-2-nitrobenzoic acid (DTNB) are held constant. Ellman (9) has described the combination of DTNB with sulfhydryl groups to form the thiophenolate ion which absorbs at 412 mμ

(E_{max} = 13.6 × 10^3 M^{-1} cm^{-1}). In this report, we present concentrations of free carnitine obtained in several rat tissues with the carnitine acetyltransferase assay, and compare these data with those in the literature obtained with other methods.

METHODS

Pig heart carnitine acetyltransferase was prepared in essentially the same manner as previously described (8). Commerical preparations[1] of DTNB and CoA were used and acetyl CoA was synthesized by the method described by Ochoa (10). Carnitine, used as a standard, was generously supplied by International Minerals and Chemicals, Skokie, Ill., while norcarnitine (N,N-dimethylamino-β-hydroxybutyrate) and its derivatives were gifts from Riker Laboratories, Northridge, Calif. Choline and its derivatives were obtained from the same sources as those previously described (11). Other compounds tested were purchased from Calbiochem, Los Angeles, Calif. Absorbancy changes were followed with the aid of a Beckman DU spectrophotometer having a Gilford automatic multiple sample recorder attachment.

Tissue Extracts

Male rats of the Sprague-Dawley strain, weighing between 150 and 230 g, had been fed ad lib. on a Rockland rat diet. They were killed by decapitation with the aid of a guillotine. Tissues to be analyzed were immediately excised and frozen in liquid nitrogen. In the case of brain, the entire head was immersed in liquid nitrogen and the frozen brain was subsequently removed by scraping away the skin and bone from split halves of the skull. Tissues were then transferred to 5-ml conical Kontes glass homogenizing tubes containing 1–2 ml/g wet weight of 2.5% $HClO_4$. Tissues were thoroughly homogenized, after which the same tubes were centrifuged at approximately 5000 × *g* in 50-ml cups having suitable adapters. The supernatant fraction was removed, and the residue was washed twice with equal volumes of distilled water. The pooled supernatant fractions were neutralized with KOH, and the resulting $KClO_4$ which formed while tubes were maintained for 30 min at 0–4° was removed by centrifugation. Aliquots of the clear supernatant solution were analyzed directly if the carnitine concentrations were sufficiently high. Otherwise, it was necessary to concentrate the solution in vacuo. In all cases, the solutions were heated at 90° for 5 min at pH 8.5 to oxidize any sulfhydryl groups which might be in the $HClO_4$ extracts of tissues. This was done to minimize the "tissue blank" which resulted from interaction between sulfhydryl groups in tissue extracts and DTNB. Residual tissue fractions remaining after the last wash were heated at 65° overnight in the original tubes, and were weighed on an analytical balance accurate to ±0.1 mg. Dry weights obtained were 20 to 25% of the original wet weights of heart, brain, testis, skeletal muscle, and kidney.

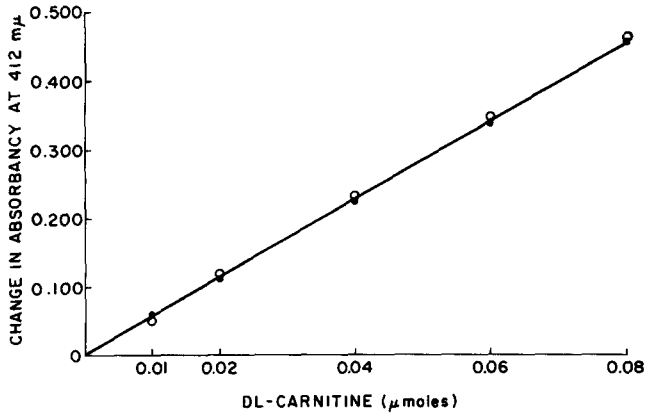

FIG. 1. Carnitine concentrations on the abcissa are plotted against absorbancy changes. Changes in absorbancy were recorded at 412 mμ on a Gilford automatic recorder attached to a Beckman DU spectrophotometer. Incubations were run for 10–15 min in 1 ml cuvettes containing 100 μmoles Tris–HCl buffer, pH 7.5 at 35°; 0.1 μmole DTNB (5,5′-dithio*bis*-2-nitrobenzoic acid); 0.05 μmole acetyl CoA and distilled H_2O to 1 ml final volume. The last component added was 0.035 mg purified carnitine acetyl transferase having a specific activity of 2.6 μmoles/mg protein. Final absorbancy changes plotted were obtained by subtracting absorbancy changes in reference cuvettes lacking carnitine from total absorbancy changes in complete cuvettes. The assay system for carnitine in tissues was the same, except that aliquots of tissue supernatant fractions prepared as indicated in Methods were used instead of known amounts of carnitine. Open and closed circles on the curve represent data obtained from experiments performed on separate days.

Enzymatic Assay

Reference cuvettes contained 100 μmoles Tris–HCl at pH 7.5 at 35°, 0.05 μmole acetyl CoA, 0.1 μmole DTNB (freshly prepared), aliquots of tissue extracts prepared as described above, and water to a final volume of 1.0 ml. Test cuvettes contained the same components plus carnitine acetyltransferase. It was necessary to determine in a separate cuvette the amount of absorbancy change which occurred when only DTNB and enzyme were added. This "blank," which was constant for a given amount of carnitine acetyltransferase and DTNB, was subtracted from all final readings. The reaction, which was usually complete within 15 min after addition of the last component (carnitine acetyltransferase), was allowed to proceed until no

[1] DTNB was purchased from Aldrich Chemical Co., Inc., Milwaukee 10, Wis., and CoA was obtained from either Calbiochem, Los Angeles 63, California, or Pabst Research Biochemicals, Milwaukee 5, Wis.

further absorbancy change occurred. In cases where tissue extracts did not interact with DTNB, it was unnecessary to add tissue extract aliquots to reference cuvettes.

Standard curves were obtained by determining absorbancy changes at 412 mμ after known amounts of carnitine were added to the system (Fig. 1). In each set of experiments, a carnitine standard was employed to insure that the enzyme and reagents were adequate. By comparison with the standard curve, it was possible to estimate the μmoles of carnitine present in aliquots of tissue extracts, and consequently the total amount per unit of initial tissue weight. Results are expressed both as μmoles (−)-carnitine per g dry weight and as micrograms/gram dry weight for the sake of comparison with data previously reported by other authors.

RESULTS

Enzymatic Method

As previously reported (8), a linear relationship exists between carnitine concentration and CoA released from acetyl CoA, as measured by changes in absorbancy at 412 mμ under conditions described in the legend to Fig. 1. For reasons previously cited (8), it is assumed that tissues contain only (−)-carnitine and that the transferase is specific for this isomer.[2] As previously shown, carnitine acetyltransferase was free of acetyl CoA hydrolase, and the reaction was completely dependent on added carnitine.

Recovery of added carnitine to either the whole tissue homogenate or to aliquots of the tissue supernatant fractions was essentially complete (Table 1). Duplicate aliquots of the same solution gave highly reproducible results with an error well within the range of carnitine concentrations found in comparable tissues obtained from different animals (Table 2). For example the mean ± the standard error was 5.55 ± 0.12 mμmoles (−)-carnitine in six 0.1 ml aliquots of perchloric acid extracts of rat testes. A linear relationship existed between aliquot size from a given tissue extract and absorbancy change.

The following compounds were shown not to interfere with the carnitine acetyltransferase assay when present in cuvettes at final concentrations up to 0.2 μmole/ml: deoxycarnitine, choline, acetylcholine, γ-aminobutyric acid, glycine, betaine, serine, histamine, D-glucosamine, and *p*-aminobenzoic acid. As previously described (8), norcarnitine at higher concentrations was able to serve as an acetyl acceptor from acetyl CoA. It remains to be established whether any of the carnitine reported in Table 2 is in fact norcarnitine. This possibility seems remote because equimolar amounts of norcarnitine gave about one-tenth the change in absorbancy which carnitine yielded under the conditions employed (8).

TABLE 1 RECOVERY OF ADDED CARNITINE*

Fractions Added					
Standard DL-Carnitine Solution (40 μmoles/0.1 ml)	$HClO_4$ Extract of Rat Heart	$HClO_4$ Residue of Rat Heart†	Total (—)-Carnitine	Recovery of Added (—)-Carnitine	Recovery of Added (—)-Carnitine
ml	*ml*	*ml*	*μmoles*	*μmoles*	%
0.1	0	0	0.20	0.20	100
0	0.1	0	0.255	—	—
0.1	0.1	0	0.45	0.195	97.5
0	0	0.1	0	—	—
0.2	0	0.1	0.40	0.40	100

* Results shown are averages of duplicate analyses from heart preparations, and are representative of 8 such experiments.

† Residue remaining after $HClO_4$ extraction and two water washes was hydrolyzed in HCl, and then dried in vacuo. The residue was subsequently homogenized in 1.0 ml water, and aliquots of the clear supernatant solution were analyzed as indicated in Methods.

Carnitine Concentrations in Tissue Extracts

In various rat tissues examined, highest carnitine concentrations were found in heart, and brown adipose tissue had the next largest amounts (Table 2). It will be of interest to determine whether carnitine plays

TABLE 2 FREE CARNITINE CONCENTRATIONS DETERMINED BY ENZYMATIC ASSAY

Rat Tissue	n*	Carnitine Concentration	
		μmoles/g dry weight	*μg/g dry weight*
Heart	8	4.87 ± 0.39†	793 ± 64
Brown Adipose Tissue (Interscapular)	4	3.14 ± 0.22	510 ± 36
Skeletal Muscle (Hind Leg)	4	2.68 ± 0.11	437 ± 18
Testes	8	1.74 ± 0.10	283 ± 17
Kidney	4	1.26 ± 0.046	204 ± 7.5
Brain	9	0.55 ± 0.038	90.0 ± 6.2
Serum	5	0.051 ± 0.0086	8.30 ± 0.14

* n represents the number of separate extracts made. Each extract was analyzed in duplicate.

† Values are expressed as the mean ± the standard deviation.

[2] *Note Added in Proof.* We have recently examined the specificity of carnitine acetyltransferase towards (—)-carnitine, kindly provided by Tanabe Seiyaku Co., Ltd., Osaka, Japan, and have observed that (—)-carnitine is twice as effective as DL-carnitine on a molar basis in accepting acetyl transfer from acetyl CoA. Thus, addition of 0.05 μmole (—)-carnitine to the enzymatic assay system resulted in an absorbancy change comparable to the addition of 0.1 μmole DL-carnitine. It therefore appears that the transferase is specific for the (—)-isomer.

TABLE 3 SUMMARY OF CARNITINE CONCENTRATIONS IN SELECTED TISSUES ESTIMATED BY PREVIOUS INVESTIGATORS

Species	Tissue	Reported Carnitine Concentration			
		Tenebrio Assay*	Frog Rectus Assay†	Colorimetric Analysis	
				Free	Bound
		μg/g dry weight			
Rat	Heart	—	250–1150	—	—
Rat	Brain	—	—	100‡	0‡
Rat	Kidney	—	200	—	—
Rat	Testes	—	—	575–775‡	—
Rat	Skeletal Muscle	560–1120	1550	600–900‡	—
Dog	Heart	560	—	—	—
Dog	Brain	87	—	<10§	310–340§

* Data are from Fraenkel (1).
† Data are from Strack et al. (3).
‡ Data are from Mehlman and Wolf (5).
§ Data are from Broekhuysen and Deltour (7).

a role in adipose tissue similar to that postulated for heart (see reference 12 for review). As expected from the findings of Fraenkel (1), skeletal muscle also was rich in carnitine, but it should be noted that its concentration was little more than half that found in heart. The lowest carnitine concentraton was found in serum, again a result not unanticipated because of the prior work of Fraenkel (1).

DISCUSSION

Results presented indicate that it is possible to analyze as little as 0.005 μmole (−)-carnitine in extracts of rat tissues in the presence of choline or other similar compounds. The enzymatically obtained carnitine analyses fell within a considerably narrower range of concentrations than those previously presented by other investigators (Table 3). Our results, which are in approximate agreement with those of Mehlman and Wolf for brain, are in sharp disagreement with claims by Hosein (13) and by Broekhuysen and Deltour (7), who maintain that almost all carnitine in brain is in "bound" form. As described in detail elsewhere (12), we feel that the methods employed by these latter investigators are open to question. Bender and Adams (14) have indicated that several claims made by Deltour and his colleagues regarding the presence of carnitine in various phospholipid moieties cannot be confirmed. More recently, Mehlman and Wolf (15) have demonstrated the occurrence of phosphatidyl carnitine in relatively large amounts in chick embryos, and in lesser amounts in extracts from livers of embryonic rats. With a more specific and precise means of determining carnitine concentrations, it is now possible to reexamine the possible existence of phosphatidyl carnitine in various tissues, and to investigate the distribution of free carnitine within cellular organelles. In addition, it will be possible to determine the distribution of carnitine in tissues of various species with a higher degree of precision than that previously available.

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Portions of this work will be incorporated into a thesis by Norman R. Marquis in partial fulfillment of his Ph.D. requirements at the University of Michigan.

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