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IMPROVED METHOD FOR MEASURING CONCENTRATIONS OF CARNITINE AND I--ETC(U)
JAN 77 J A PACE, R W WANNEMACHER, H A NEUFELD

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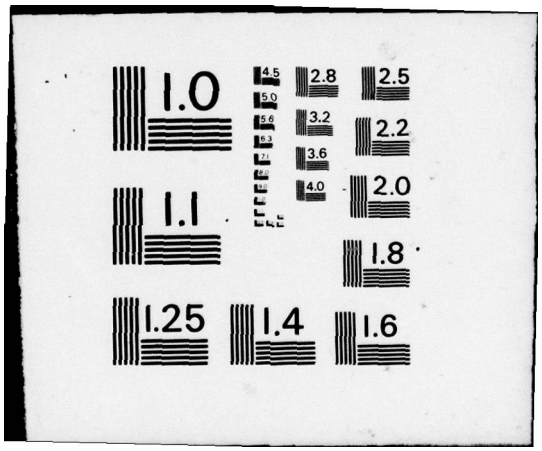
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Improved Method for Measuring Concentrations of Carnitine and Its Derivatives in Tissue Extracts

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Running Head: METHOD FOR MEASURING CARNITINE

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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ABSTRACT

A method was developed to measure carnitine and short-chain and long-chain acylcarnitines in perchloric acid extracts of liver and muscle. The radioisotopic assay employing, [1-^{14}C]acetyl-CoA and the measurement of labled acetylcarnitine, resulted in a sensitivity range of < 1 to 50 nmol L-carnitine/50-µl sample. Samples frozen at -20°C revealed no losses in carnitine concentrations for several months. Recovery of the carnitine added to perchloric acid extracts of liver was 103 $\pm$ 2%.

Many techniques for measuring carnitine have been reported in the literature (1-6). Of these, the methods which utilize the enzyme carnitine acetyltransferase (ACT) are the most sensitive and specific. The radioisotopic method introduced by Cederblad et al. (4) and modified by McGarry et al. (6) is designed to measure picomole quantities of carnitine in plasma. Basically, the reaction is:

$$\text{L-carnitine} + [1\text{-}^{14}\text{C}]\text{acetyl-CoA} \overset{\text{ACT}}{\rightleftharpoons} \text{acetyl-L-carnitine} + \text{CoASH}.$$

The CoASH formed is removed from the system by sodium tetrathionate which drives the reaction in one direction for the formation of acetyl-L-carnitine. The labeled acetyl-L-carnitine formed is separated from the labeled acetyl-CoA by passing the mixture through a Dowex anion exchange resin and determining the isotope content of the effluent fluid.

Carnitine, by the participation of mitochondrial carnitine acyltransferases, is known to facilitate the entry of fatty acids through the mitochondrial inner membrane. Carnitine's role in fatty acid transport (9) and ketogenesis (6) makes measuring of liver carnitine essential to the understanding of metabolic changes that might occur under different nutritional and hormonal stimuli.

When confronted with the problem of measuring the concentration of carnitine in liver, it appeared that the above method could be adapted readily to liver and muscle extracts.

In perchloric acid extracts of tissues endogenous acetyl-CoA may cause some interference. By increasing the concentration of labeled acetyl-CoA 4-fold over that used for the plasma assay, this interference

could be eliminated. This modification has been used to develop a procedure for the analysis of free, short-chain, and long-chain carnitine content of liver and muscle tissue.

MATERIALS

Chemicals. Tris, hydrochloride [tris (Hydroxymethyl) aminomethane and hydrochloride], DL-O-acetylcarnitine hydrochloride, and acetyl-coenzyme A (trilithium-trihydrate), were purchased from Calbiochem, San Diego, Calif. Sodium tetrathionate was obtained from ICN Pharmaceuticals, Inc., Plainview, NY. Dowex 2X-8 anion exchange resin in the Cl^- form (100-200 mesh) was from BioRad Laboratories, Richmond, CA. L-carnitine HCl (GIBCO) was a gift from J. D. McGarry, The University of Texas Health Science Center at Dallas, Dallas, Tex. Carnitine acetyltransferase (ACT) (EC 2.3.1.7) from pigeon breast muscle was from Sigma Chemical Co., St. Louis, MO. [1-^{14}C]acetyl-CoA (specific activity 55 mC/mol) was from New England Nuclear, Boston, MA.

METHODS

Homogenization and centrifugation. All homogenization was done using a Brinkman Polytron. Centrifugation was carried out at 3,000 g for 10 min in a Sorvall RC2-B at 5°C.

Preparation of solutions. Tris buffer: a 0.24 M stock solution of Tris was prepared by dissolving 7.56 g of the buffer crystals in 200 ml of triple distilled water. This solution was brought to pH 7.3 and stored at 4°C. Sodium tetrathionate: 54 mg were dissolved in 25 ml of triple distilled water.

Carnitine standard. DL-carnitine (9.88 mg) was dissolved in 25 ml of triple distilled water. This primary stock solution was stable and could be stored at 4°C for several months.

Enzyme. Carnitine acetyltransferase (600 units/ml) was diluted 1:3 with 3.2 M $(NH_4)_2SO_4$ solution. The enzyme was prepared fresh daily.

Labeled acetyl-CoA (0.4 mM). One-half milliliter of stock [1-^{14}C]acetyl-CoA was added to 8.81 mg of cold acetyl-CoA and diluted to 25 ml with triple distilled water. This stock solution was stable for several days when stored at 4°C.

Animals. The animals used were male rats (Fisher-Dunning, F-344/Mai f, obtained from Microbiological Associates, Walkersville, MD) weighing 175 to 250 g. Rats were maintained on a commercial diet (Wayne Lab Blox, Allied, Inc., Chicago, Ill.) and housed in rooms maintained at 23 ± 1°C.

Collection and preparation of tissue. Rats were stunned by a blow to the head and killed by cervical dislocation. The liver was removed and crushed between liquid nitrogen cooled aluminum blocks within 10 sec according to the method of Wollenberger et al. (8). Blood which had collected in the chest cavity was removed as quickly as possible. Skeletal muscle was removed from the hind leg and freeze clamped within 2 min. The frozen tissue was either stored at -20°C or immediately homogenized in dilute perchloric acid. The extraction procedure was a modification of the method of Pearson and Tubbs (2).

The deproteinization of frozen tissue with dilute perchloric acid (3%) divides the total carnitine into two fractions, an acid-soluble and an acid-insoluble, which were separated by centrifugation (Fig. 1). Free carnitine and short-chain acylcarnitines (e.g., acetylcarnitine, proprionylcarnitine and acetoacetylcarnitine) remain in the soluble fraction;

long-chain acylcarnitines (e.g., palmitoyl carnitine) were precipitated. Free carnitine (FC) was measured directly on the neutralized perchloric acid (PCA) extract. Short-chain acylcarnitines (SC) were obtained by making an aliquot of the PCA extract alkaline with 3.5 M KOH and incubating at 40°C for 1 hr. After neutralizing with HCl (0.5 N) the extract was assayed for L-carnitine. This gave the "total acid-soluble carnitine" (TC). Total acid-soluble carnitine minus free carnitine gave the short-chain acylcarnitines(SC). The precipitate (containing long-chain acylcarnitines) obtained on extracting the frozen tissue with dilute perchloric acid was washed with water three times and suspended in KOH (3.5, pH 13). This mixture was incubated for 2 hr at 50°C, cooled, neutralized with perchloric acid and assayed for L-carnitine.

Carnitine assay. Liver extract (50 µl representing 30-50 mg tissue wet weight) was added to 0.5 ml of Tris (0.24 M), 0.25 ml of sodium tetrathionate (8 mM), and 0.25 ml of [1-^{14}C]acetyl-CoA (0.4 mM). The reaction was initiated by the addition of 5 µl (1 U) of carnitine acetyltransferase. After 30 min incubation at room temperature, 0.5 ml of the reaction mixture was chromatographed on a 5 x 65 mm Dowex 2X-8 (Cl^-) column. The column was washed twice with 0.5 ml of water; 200 µl of the effluent fluid was added to 5 ml of a scintillation fluid containing: 1 volume Scintisol-GP with 10 volumes of Scintolute Fluor-containing cocktail base. The isotope content was determined using a Searle Model Mark III liquid scintillation spectrometer.

RESULTS

Standard curve. It is assumed that tissue contains only L-carnitine (9) and that the transferase is specific for this isomer (10). To verify this

assumption standard curves were run using DL-carnitine (0-100 nmol) and L-carnitine (0-50 nmol). The curves for DL-carnitine and L-carnitine were respectively $y = 164 x + 6$, $y = 126 x + 37$. Figure 2 shows a typical standard curve obtained when the assay was performed with 0-50 nmol of carnitine in the incubation with 0.1 μmol of [1-^{14}C]acetyl-CoA. A series of standards were run with daily assays. Standard curves were nearly superimposable over several months. The mean slope for 12 runs using DL-carnitine standards was 164 ± 6 (mean ± SD) y intercept was 6 ± 35 indicating that only L-carnitine reacted.

Recovery of carnitine. Carnitine (5 nmol) was added to liver samples prior to perchloric acid extraction and to a series of carnitine standards assayed as described in Methods. Table 1 and 2 show that the recovery of added carnitine to standard solutions (99 ± 4%) or to whole tissue homogenates (103 ± 2%) was essentially complete.

Reproducibility of extraction procedure. Five rats were starved for 24 hr and killed as described in Methods. Livers were removed within 10 sec and frozen in liquid nitrogen. The livers were divided into three parts. All part I portions were assayed at the same time. Parts II and III were stored at -20°C and assayed 1-2 months later. Each part was extracted and assayed as described in the Methods Section. Table 3 shows the excellent reproducibility of the analytical procedure as well as the stability of the liver when frozen at -20°C. The precision of the method expressed as relative standard deviation of triplicate liver analyses was 1 to 5% for all livers except liver 2 which was 10%.

Effect of varying proportions of carnitine and acetylcarnitine in the assay. As reported by McGarry et al. (6) when tetrathionate is included in the assay the relationship between the recovered acetylcarnitine and the amount of carnitine present is linear. Short-chain acylcarnitines do not interfere with the determination of free carnitine. Table 4 shows that identical results are obtained whether or not unlabeled acetylcarnitine is present.

Carnitine measurements. Free carnitine, short-chain and long-chain acylcarnitines were measured in liver, muscle and plasma from rats that were fed a balanced laboratory diet. Table 5 shows the absolute carnitine levels and the relative distribution of carnitine and its derivatives in liver, muscle and plasma from the same rat.

DISCUSSION

The assay system described makes use of a radioisotope in determining free carnitine, short-chain and long-chain acylcarnitines in liver and muscle extracts. Table 5 shows the wide variation between liver, muscle and plasma carnitine levels as well as the distribution of carnitine within these tissues. In the fed rat approximately 50% of the liver and muscle carnitine is in the free state. Free carnitine constitutes about 80% of the total plasma carnitine pool. Short-chain acylcarnitines make up about 30% of the total hepatic carnitine, whereas muscle short-chain acylcarnitines and free carnitine are evenly distributed. This is in agreement with results reported by Bøhmer (3). The short-chain content of rat muscle was found to be about seven times higher than in rat liver. Rat muscle long-chain acylcarnitines are twice that found in liver, however, on a relative basis hepatic long-chain acylcarnitines make up about 17%

more of the total carnitine pool than in muscle. The hepatic long-chain acylcarnitines are significantly higher in our studies than values found in the literature (3). However, Bøhmer (3), also finds a 10-fold variation in the level of long-chain acylcarnitines. This difference may be explained by the length of time taken for sample collection and the method of sample preparation. The 10-sec quick-kill (8) seems to have its greatest preservation effect on long-chain acylation states. Only about 2% of the acylcarnitines present in plasma is long-chain derivatives, therefore, short- and long-chain are reported as one and analyzed for acid-soluble carnitine as described in Methods.

The techniques reported in the literature (1-5) are less sensitive and more tedious than the one presented here. This modified ratioisotopic assay is one of the few available for measuring carnitine and its derivatives in liver and muscle extracts in addition to its use in plasma analyses.

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TABLE 1

RECOVERY OF ADDED CARNITINE IN ASSAY

Carnitine (nmol)		Recovered	
Initially Present	+ Internal Standard	nmol	% of total
50.0	55.0	57.7	105
25.0	30.0	29.6	99
10.0	15.0	15.5	103
2.5	7.5	6.5	87
Mean ± SEM			99 ± 4

TABLE 2

RECOVERY OF ADDED CARNITINE IN EXTRACTION

Carnitine (nmol)		Recovered	
Sample[a]	+ Internal Standard	nmol	% of total
5.9	10.9	11.1	102
5.6	10.6	10.9	103
4.4	9.4	9.6	102
5.4	10.4	11.0	106
Mean ± SEM			103 ± 2

[a] Four livers.

TABLE 3

REPRODUCIBILITY OF LIVER EXTRACTION PROCEDURE[a]

Rat Liver	Free carnitine (nmol/g)			
	Part I	Part II	Part III	Mean ± SD
1	202	195	215	205 ± 10
2	204	239	196	213 ± 23
3	194	197	197	196 ± 2.0
4	181	184	197	188 ± 8.6
5	181	184	181	183 ± 1.9

[a] Livers from 5 rats were removed within 10 sec, extracted and assayed as described in text. The precision of the method expressed a relative standard deviation of triplicate liver analyses was 1 to 5%.

TABLE 4

LACK OF INTERFERENCE FROM ACETYLCARNITINE[a]

Carnitine (nmol)	Amount Measured (nmol)
25.0	24.
10.0	10.1
5.0	5.1
2.5	2.5
Liver Extract containing:	
4 nmol carnitine	4.0
Extract + 1 nmol	4.9
Extract	4.0
Extract + 1 nmol	4.6

[a] Acetylcarnitine (4 nmol) was added to the carnitine standards and a liver extract containing 4 nmol of carnitine.

TABLE 5

CARNITINE, CARNITINE DERIVATIVES AND THEIR RELATIVE DISTRIBUTION IN LIVER, MUSCLE AND PLASMA OF FED RATS[a]

Carnitine	nmol/g (nmol/ml)[b]	% Total
	LIVER	
Free	171 ± 19	47 ± 5
Short-chain	114 ± 12	29 ± 3
Long-chain	122 ± 8	33 ± 2
Total Pool	369 ± 28	
	PLASMA	
Free	85 ± 8	78 ± 3
Short + Long-chain	22 ± 3	23 ± 3
Total Pool	100 ± 3	
	MUSCLE	
Free	738 ± 48	50 ± 7
Short-chain	753 ±196	35 ± 9
Long-chain	247 ± 20	16 ± 2
Total Pool	1616 ±169	

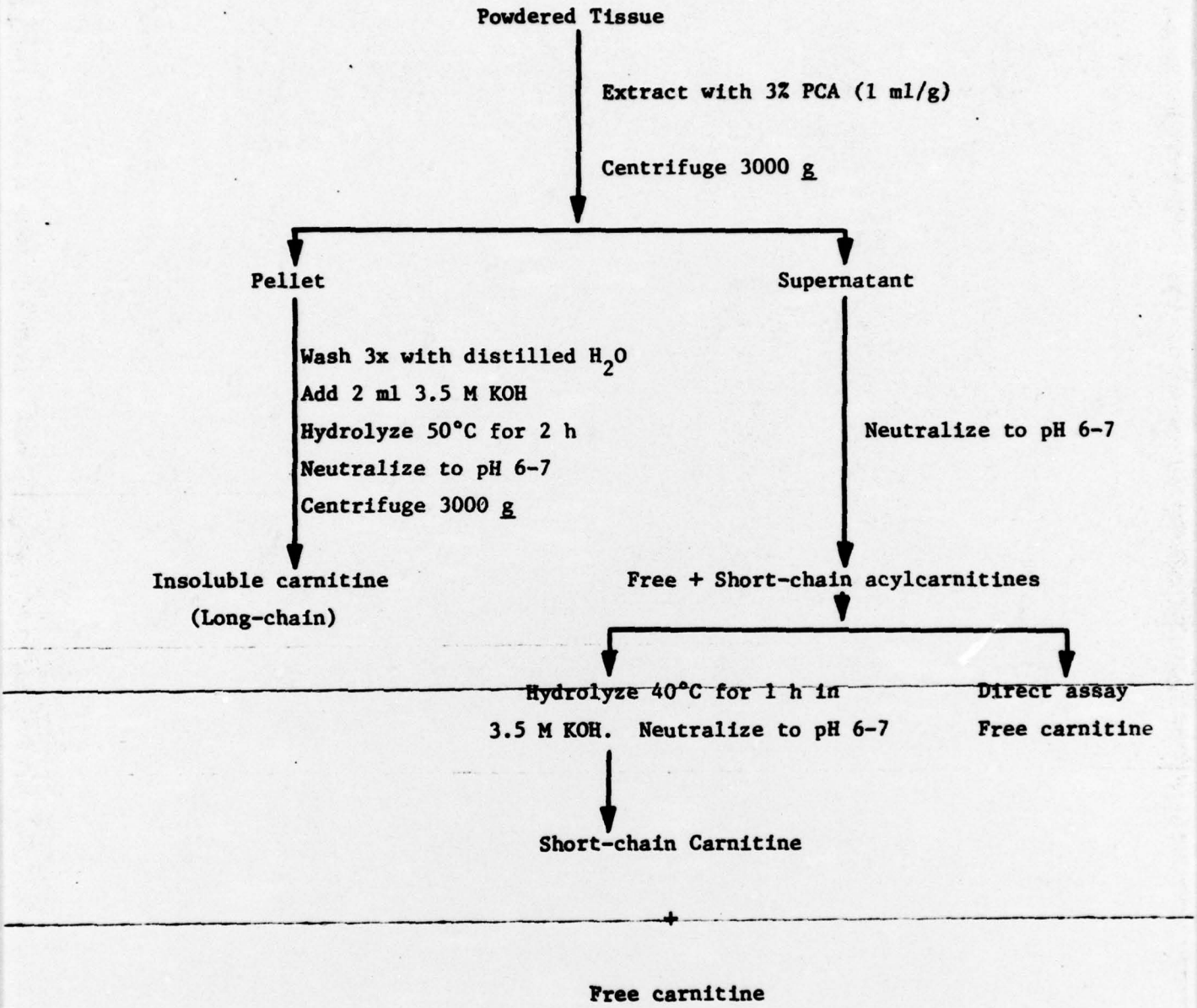
[a] Six rats

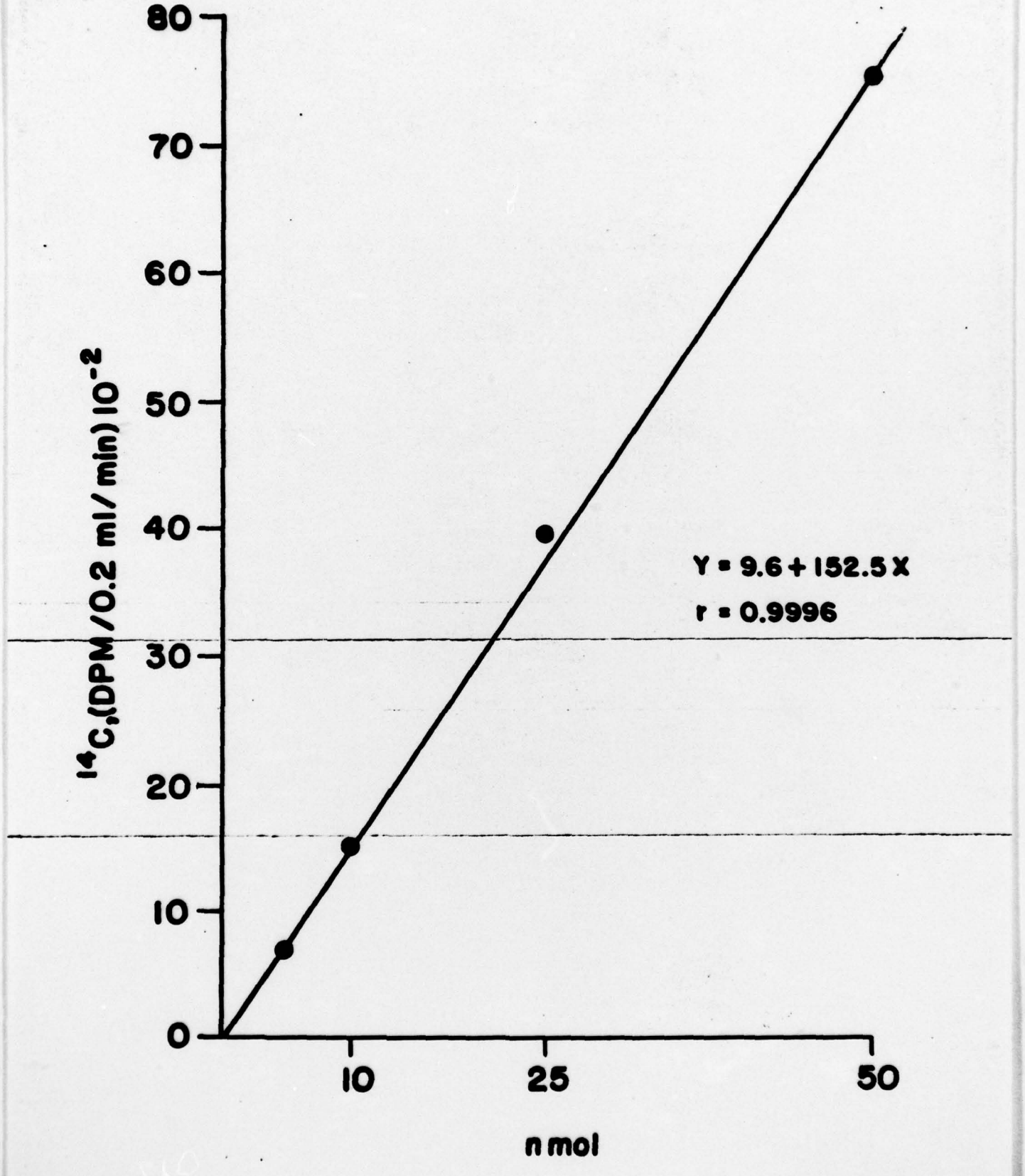
[b] Plasma values reported as nmol/ml

LEGENDS TO FIGURES

Figure 1. Tissue extraction procedures (7).

Figure 2. Typical curve for analysis of carnitine. D,L-carnitine standards containing 5, 10, 25, 50 nmol of L-carnitine were assayed for free carnitine as described under Methods.


Powdered Tissue
Extract with 3% PCA (1 ml/g)
Centrifuge 3000 g
Pellet
Supernatant
Wash 3x with distilled H_2O
Add 2 ml 3.5 M KOH
Hydrolyze 50°C for 2 h
Neutralize to pH 6-7
Centrifuge 3000 g
Neutralize to pH 6-7
Insoluble carnitine
(Long-chain)
Free + Short-chain acylcarnitines
Hydrolyze 40°C for 1 h in
3.5 M KOH. Neutralize to pH 6-7
Direct assay
Free carnitine
Short-chain Carnitine
+
Free carnitine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