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Effect of Deproteinization and Reagent Buffer on the Enzymatic Assay of *L*-Carnitine in Serum

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Summary: Tris and HEPES were systematically compared as buffers for the enzymatic assay of *L*-carnitine. The deproteinization methods preceding the assay were also compared. The following conclusions were drawn.

1. Both Tris and HEPES act on the catalytic site of the enzyme, acetylCoA: carnitine O-acetyltransferase (EC 2.3.1.7), which is used for the conversion of *L*-carnitine to acetylcarnitine. HEPES is a competitive inhibitor, and no acetylated product of HEPES is formed. In the presence of Tris a limited amount of acetylTris is formed, and an appropriate blank corrects for this effect.
2. The incubation time of the assay is strongly influenced by the preceding deproteinization method. The enzyme is influenced by inorganic salt, which acts as a competitive inhibitor.
3. If Tris is used in place of HEPES in end-point assays, optimal conditions and shorter assay times are achieved with less enzyme and less acetylCoA, provided more elaborate deproteinization methods are used.
4. The HEPES system is more costly, but preferable for the determination of both total and free *L*-carnitine in combination with a matched deproteinization method.

Introduction

L-Carnitine functions primarily in the transport of fatty acids across the mitochondrial membrane (1). It is present either free or esterified (1). Subnormal concentrations in serum may result from a primary cause (2), or can be secondary to pathological conditions such as kidney disease and dialysis (3, 4), parenteral nutrition (5) or inborn errors of metabolism (6). These conditions affect the metabolic processing of fatty acids. *L*-Carnitine is easily esterified in vitro, and this process can be exploited for measuring both free and total serum *L*-carnitine. Several methods have been described for the measurement of *L*-carnitine in serum or plasma. *Marquis & Fritz* developed an enzymatic assay (7): acetylCoA: carnitine O-acetyltransferase (EC 2.3.1.7)[1]) catalyses the formation of acetylcarnitine and CoASH[2]) from *L*-carnitine and acetylCoA. CoASH[2]) reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) to form 5-thio-2-nitrobenzoate, which can be measured at 412 nm. Deproteinization is necessary to avoid non-specific reduction of 5,5'-dithiobis-(2-nitrobenzoic acid). The procedure has been variously modified with respect to deproteinization, hydrolysis of esterified *L*-carnitine, and the nature of the buffer (8–10).

We compared different assay conditions, and we studied the influence of deproteinization, hydrolysis, and buffer on the performance of the assay.

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[1]) Enzyme
AcetylCoA : carnitine O-acetyltransferase (EC 2.3.1.7)

[2]) Abbreviations

CoASH	coenzyme A
EDTA	ethylenediaminetetra-acetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid
MOPS	3-(N-morpholino)propanesulphonic acid
Tris	Tris(hydroxymethyl)aminomethane

Materials and Methods

Materials

AcetylCoA : carnitine O-acetyltransferase (EC 2.3.1.7), *DL*-acetylcarnitine and acetylCoA were purchased from Boehringer, Mannheim (FRG); *L*-carnitine and *5,5'-dithiobis-(2-nitrobenzoic acid*) were from Sigma, St. Louis, MO 63178 (USA); other chemicals of analytical grade were obtained from Merck, Darmstadt (FRG).

Methods

For *deproteinization of serum without subsequent hydrolysis of L-carnitine esters* the following methods were used:

1: the perchloric acid method
Serum (2.0 ml) was mixed with 0.5 ml (140 g/l) of cold $HClO_4$. After 30 min at 4 °C the mixture was centrifuged for 5 min at 10 000 *g*. To 1.0 ml of supernatant cold 0.5 mol/l MOPS[2]) in 2.0 mol/l KOH or solid $KHCO_3$ was added until the pH was between 7 and 8. After 30 min at 4 °C the mixture was centrifuged for 5 min at 10 000 *g*. The clear supernatant was used for the assay.

2: the trichloroacetic acid method
Serum (2.0 ml) was mixed with 0.5 ml (200 g/l) of cold trichloroacetic acid. After 30 min at 4 °C the mixture was centrifuged for 5 min at 10 000 *g*. To the supernatant 2.0 mol/l NaOH was added until the pH was between 7 and 8, or the supernatant was extracted with 7.5 ml of diethyl ether to remove trichloroacetic acid. This was repeated twice until the pH was between 6 and 7. Remaining traces of diethyl ether were removed by a gentle nitrogen stream.

3: the $ZnSO_4$-$Ba(OH)_2$ method
This method was performed according to *Seccombe* et al. (8).

4: the heat denaturation and freeze-thawing method
This method was performed according to *Rodriguez-Segade* et al. (9).

In all methods a Cryofuge 5000 (Heraeus Christ, Osterode/Harz, FRG) was used for centrifugation.

Deproteinization with subsequent hydrolysis of L-carnitine esters

Serum (2.0 ml) was mixed with 0.5 ml (140 g/l) of cold $HClO_4$. After 30 min at 4 °C and subsequent centrifugation for 5 min at 10 000 *g*, 0.2 ml of KOH (5 mol/l) was added to 1.5 ml of the supernatant. After 10 min at 4 °C, the sample was centrifuged for 5 min at 10 000 *g* and the supernatant was incubated at 80 °C for 60 min. Cold $HClO_4$ was added until the pH was <7. After centrifugation for 10 min at 10 000 *g*, solid $KHCO_3$ was added to the supernatant until the pH was between 7 and 8. After centrifugation, the clear supernatant was used for the assay.

Assays

For the enzymatic assay of *L*-carnitine two methods were compared. The *first method* was according to the procedure as described by *Wieland* et al. (11). To 1.0 ml of deproteinized serum or standard solution, 0.9 ml reagent was added. The reaction was started with 0.1 ml of acetylCoA : carnitine O-acetyltransferase (0.25 g/l, 80 kU/g). Final reagent concentrations are summarized in table 1.

The *second method* was as described by *Pearson* et al. (12). To 1.0 ml of deproteinized serum or standard solution, 1.0 ml of reagent was added. The reaction was started by the addition of 10 µl of acetylCoA : carnitine O-acetyltransferase (1 g/l, 80 kU/g). Final reagent concentrations are summarized in table 1.

Tab. 1. Final reagent concentrations in commonly used enzymatic *L*-carnitine assays.

Reagent	Tris-buffered assay (*Pearson* et al.)	HEPES-buffered assay (*Wieland* et al.)
HEPES KOH pH 7.5	–	36 mmol/l
Tris-HCl pH 7.8	100 mmol/l	–
5,5'-Dithiobis-(2-nitrobenzoic acid	0.125 mmol/l	0.200 mmol/l
EDTA	1.25 mmol/l	0.72 mmol/l
AcetylCoA	0.20 mmol/l	0.55 mmol/l
AcetylCoA : carnitine-O-acetyltransferase	0.8 U	2 U

Standards were made by dilution of a *L*-carnitine stock solution (1 mmol/l) with isotonic saline. To investigate the effect of salt, the stock solution was diluted with solutions of NaCl, K_2SO_4 or sucrose to the desired concentration and conductivity c. q. osmolality. For the reagent blank, iso-osmotic saline solutions were used.

Absorbances (A_{412nm}) of the reaction mixtures were measured continuously at 25 °C for 5 to 15 min on a Perkin Elmer 554 spectrophotometer (Perkin Elmer Corp., Norwalk CT 06856, USA). The molar lineic absorbance of 5-thio-2-nitrobenzoate is 1360 m^2/mol (13). Osmolalities were measured on a Vapor Pressure Osmometer (Wescor Inc., Logan, Utah 84321, USA). Conductivities were measured on a Radiometer DCM 83 Conductivity Meter (Radiometer, Copenhagen, Denmark).

Results

The time required for complete conversion of *L*-carnitine to acetylcarnitine is dependent upon the conditions of the assay. In figure 1, lines a, b, g and h show the effect of deproteinization methods on the reaction times in the assay system according to *Pearson* et al. Similar results were obtained with the HEPES-buffered assay according to *Wieland* et al. (lines c, d, f). The shortest reaction times were obtained with a Tris[2])-buffered assay.

The effect of the reagent buffer

The ability of acetylCoA : carnitine O-acetyltransferase[1]) to acetylate Tris is shown in figure 2. Assay conditions were according to *Pearson* et al., but *L*-carnitine was omitted and the concentration of Tris in the assay was varied. NaCl was added to obtain the same conductivity in all assays. Tris is acetylated by the enzyme, the K_m of the reaction being 175 mmol/l and the V_{max} 1.3 nmol/min. No acetylation of HEPES[2]) was found. Nevertheless, figure 3 shows a competitive inhibitory effect of HEPES on the acetylation of *L*-carnitine in the Tris-buffered system according to *Pearson* et al. HEPES influences the acetylation of Tris in a similar way (not shown).

Similar effects are obtained when another *Good*'s buffer, MOPS[2]), is used.

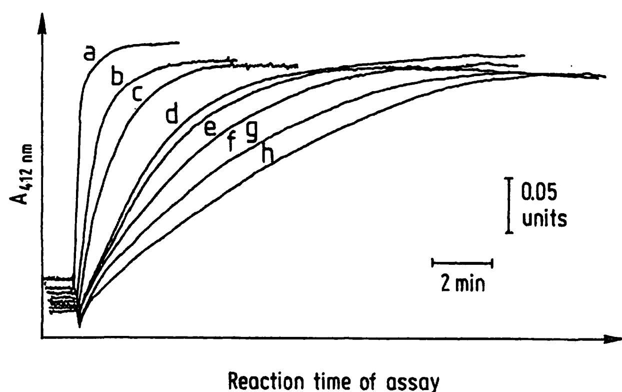

Fig. 1. Reaction times of *L*-carnitine assays. Different assay conditions (buffer type, enzyme and acetylCoA concentration) and different deproteinization methods were used.
line a: Tris-buffered assay according to *Pearson* et al. (12); aqueous *L*-carnitine standard (no deproteinization).
line b: Tris-buffered assay according to *Pearson* et al. (12); trichloroacetic acid deproteinization with ether extraction neutralization.
line c: HEPES-buffered assay according to *Wieland* et al. (11); $HClO_4$ deproteinization with $KHCO_3$ neutralization.
line d: HEPES-buffered assay according to *Wieland* et al. (11); trichloroacetic acid deproteinization with ether extraction neutralization.
line e: Tris-buffered assay according to *Pearson* et al. (12), but enzyme and acetylCoA concentration as in the HEPES-buffered assay according to *Wieland* et al. (11); $HClO_4$ deproteinization with $KHCO_3$ neutralization.
line f: HEPES-buffered assay according to *Wieland* et al. (11); aqueous *L*-carnitine standard (no deproteinization).
line g: Tris-buffered assay according to *Pearson* et al. (12); $HClO_4$ deproteinization with $KHCO_3$ neutralization.
line h: Tris-buffered assay according to *Pearson* et al. (12); $HClO_4$ deproteinization with MOPS-KOH neutralization.

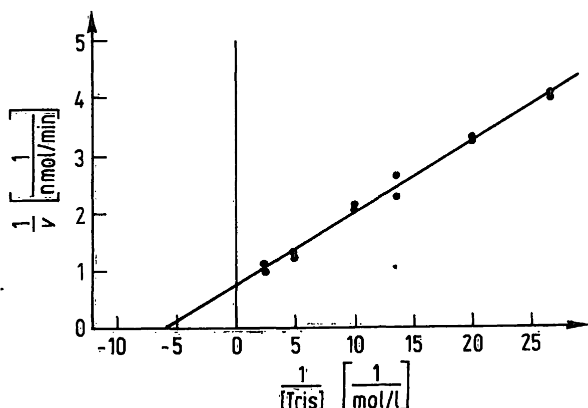

Fig. 2. *Lineweaver-Burk* plot of acetylCoA : carnitine O-acetyltransferase.
The concentration of Tris was varied; the osmolality of the assay was kept constant at 254 mosmol/kg with NaCl. Further conditions were as described in Materials and Methods.

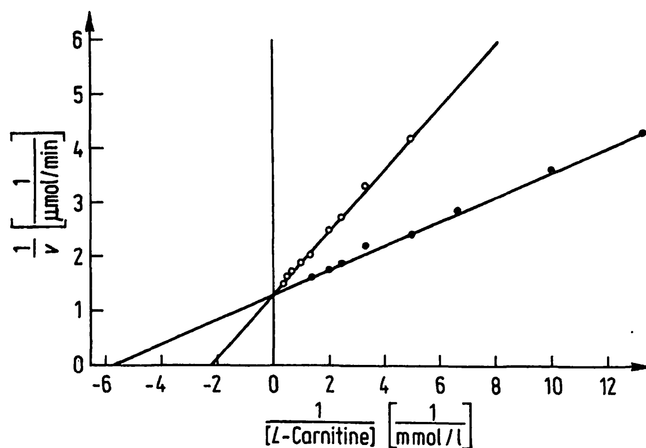

Fig. 3. The effect of HEPES on the kinetics of acetylCoA : carnitine O-acetyltransferase.
HEPES was added to the Tris-buffered assay with variable *L*-carnitine concentrations: ●—●: no addition; o—o: addition 100 mmol/l. Conditions were as described in Materials and Methods.

The effect of deproteinization

The effect of salt on the kinetics of acetylCoA: carnitine O-acetyltransferase is shown in figure 4.

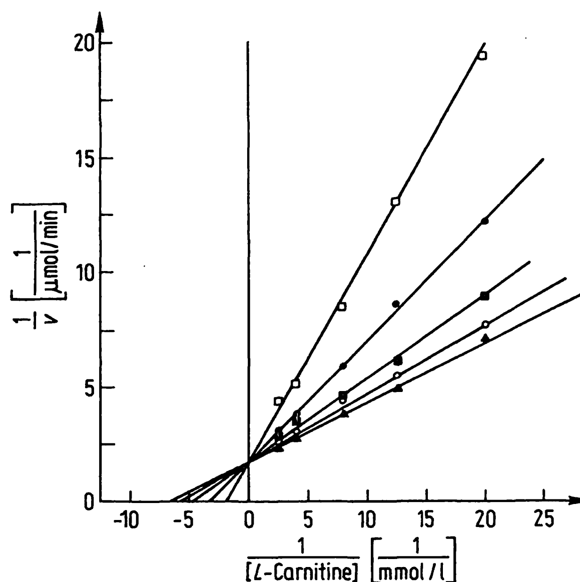

Fig. 4. The effect of sodium chloride on the kinetics of acetylCoA : carnitine O-acetyltransferase.
Different concentrations of NaCl were added to the Tris-buffered assay. Conditions were as described in Materials and Methods, exept for o—o: here, 50 mmol/l Tris was present in the assay instead of 100 mmol/l. After complete conversion of *L*-carnitine to acetylcarnitine, osmolalities and conductivities were measured.

Line	NaCl in assay mmol/l	Osmolality mosmol/kg	Conductivity mS/cm
o—o	—	159	3.9
△—△	—	198	6.9
■—■	100	295	11.0
●—●	200	373	17.4
□—□	300	513	23.7

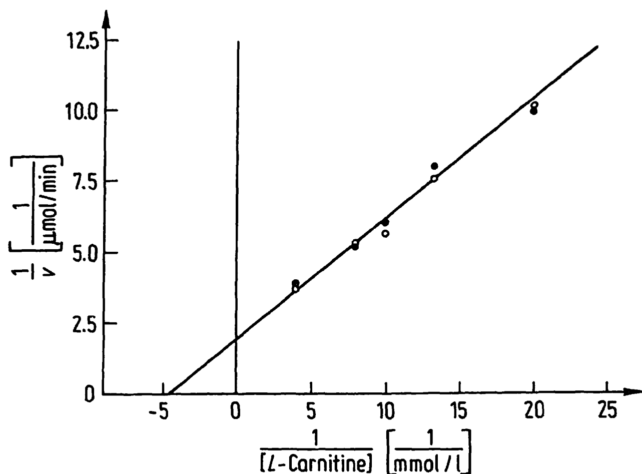

Fig. 5. The effect of potassium sulphate and sodium chloride on the kinetics of acetylCoA:carnitine O-acetyltransferase.
The Tris-buffered assay was used. Conditions were as described in Materials and Methods.
o—o K_2SO_4 140 mmol/l; ●—● NaCl 200 mmol/l.
After complete conversion of *L*-carnitine to acetylcarnitine, osmolalities and conductivities were: (o—o) 355 mosmol/kg and 16.4 mS/cm; (●—●) 360 mosmol/kg and 15.8 mS/cm.

Tab. 2. Osmolality and conductivity in the assay mixtures after deproteinization according to different methods.

Method	Osmolality mosmol/kg	Conductivity mS/cm
1: Trichloroacetic acid; ether extraction	330	11.4
2: Trichloroacetic acid; NaOH neutralization	430	14.6
3: $HClO_4$; MOPS-KOH neutralization	375	13.7
4: $HClO_4$; $KHCO_3$ neutralization	415	14.1
5: $ZnSO_4$ — $Ba(OH)_2$	210	7.4
6: Heat-Freeze-Thaw	300	11.0
7: Non-deproteinized serum	300	11.1
8: Water	200	7.0

1—6: serum was deproteinized as described in Materials and Methods, and 1 ml of supernatant was added to 1 ml of assay mixture. Final concentrations were as described in Materials and Methods, according to the method of *Pearson* et al. After complete conversion of *L*-carnitine to acetylcarnitine, osmolalities and conductivities were measured.
7—8: as a control, 1 ml of non-deproteinized serum (7) or 1 ml of water (8) was added to the assay reagent instead of deproteinized serum.

Using aqueous *L*-carnitine standards, *Lineweaver-Burk* plots were made at different NaCl concentrations. The lowest K_m is seen under the conditions described by *Pearson* et al. Increasing the concentration of NaCl to 300 mmol/l results in an increase of the K_m to 435 µmol/l. No effect on the V_{max} was observed. To exclude an effect of NaCl itself on the enzyme, K_2SO_4 (140 mmol/l) was added instead of NaCl (200 mmol/l). At identical osmolalities and conductivities no significant differences were observed (fig. 5) suggesting that the enzyme was influenced by the ionic strength in the assay. This was confirmed by the observation (not shown) that no increase in K_m was observed when sucrose was added to the assay, i. e. when the osmolality but not the ionic strength was increased.

Similar results were obtained with the HEPES-buffered assay according to *Wieland* et al. (not shown).

Deproteinization methods affect the conductivities and osmolalities of the Tris-buffered assays according to *Pearson* et al. (tab. 2). This is reflected in the time for the complete enzymatic conversion of *L*-carnitine to acetylcarnitine (fig. 1, line a, b, g, h). Similar effects are obtained with the HEPES-buffered assay according to *Wieland* et al., in which the osmolality is 106 mosmol/kg after the addition of an aqueous *L*-carnitine standard (fig. 1, line f). After the addition of a serum that had been deproteinized with $HClO_4$ and neutralized with $KHCO_3$, the osmolality was 331 mosmol/kg (fig. 1, line c).

The combination of reagent buffer and deproteinization

A comparison of results obtained with the HEPES-buffered assay of *Wieland* et al. and with the Tris-buffered assay of *Pearson* et al. is shown in table 3. Omission of a reagent blank in the latter assay results in a minor increase of the measured value. In table 3 the trichloroacetic acid method with subsequent ether extraction is used for deproteinization. In table 3b the

Tab. 3. Intra-assay variation of *L*-carnitine in two pool sera, as measured with enzymatic assays containing HEPES or Tris buffer.

	Pool serum 1 mean µmol/l	Pool serum 1 CV %	Pool serum 2 mean µmol/l	Pool serum 2 CV %
a: Trichloroacetic acid; ether extraction				
1: HEPES-buffered assay:	35.8	5.1	52.1	4.7
2: Tris-buffered assay,				
including blank:	35.0	3.1	51.5	2.1
without blank:	36.2	3.0	52.7	2.2
b: $HClO_4$; $KHCO_3$				
1: HEPES-buffered assay:	35.1	3.9	51.9	2.8
2: Tris-buffered assay,				
including blank:	36.1	10.2	53.0	7.6
without blank:	38.0	9.9	54.1	8.1

Assays were as described in Materials and Methods (n = 15). Sera were deproteinized, either by the trichloroacetic acid method with subsequent neutralization by ether extraction (a), or by the perchloric acid method with subsequent $KHCO_3$ neutralization (b). For the reagent blank, NaCl was added instead of serum to obtain the same assay osmolality.

perchloric acid method with subsequent $KHCO_3$ neutralization is used. By using this method of deproteinization, carnitine esters are hydrolysed and the recoveries of added *L*-carnitine and acetylcarnitine are between 89% and 103%. Moreover, dilution is minimal and no extra salt is introduced, compared with the non-hydrolysed sample (osmolalities: 428 ± 38 mosmol/l vs. 431 ± 32 mosmol/l; n = 5).

Discussion

Several enzymatic methods, both manual and automated, for the determination of *L*-carnitine in serum or plasma have been described (7–10, 14, 15). Most of them are colorimetric assays. Their principal differences are in the buffer and in the deproteinization method that precedes the assay.

The effect of reagent buffer

Christiansen & Bremer claim that Tris functions as an acetyl-group receptor (16). HEPES is not acetylated and is therefore recommended (16). These results are often referred to by other HEPES-using authors, and they are even used to explain grossly differing results (17).

We studied the acetylation of Tris in the assay system of *Pearson* et al. and quantitated the amount of acetylated product by the formation of 5-thio-2-nitrobenzoate. When the concentration of Tris in the assay was 100 mmol/l, about 2 µmol/l of acetylTris was formed in a reaction time of 5 min, whereas reference values of free *L*-carnitine range from 30 to 70 µmol/l. Longer incubation times lead to an increase in the formation of acetylTris, but a reagent blank corrects for this effect (tab. 3). Although the amount of acetylTris that is formed depends upon the assay, the formation of this compound should not be held responsible for the wide variation of results, as proposed by *Fürst & Glöggler* (17).

It is stated that HEPES is not acetylated and that it therefore does not interfere with the assay (16). This is only partially true. We have shown that HEPES functions as a competitive inhibitor of the enzyme. The same effect is observed with another *Good*'s buffer, MOPS. The catalytic centre of the enzyme must have affinity for these buffers, despite the fact that there is no detectable formation of an acetylated product. This could explain why higher concentrations of acetylCoA : carnitine O-acetyltransferase and acetylCoA are needed in the HEPES-buffered system according to *Wieland* et al. to obtain acceptable reaction times.

The effect of deproteinization

The efficiency of the assay is not only dependent upon the choice of the buffer. It should also be recognized that the efficiency is influenced by the deproteinization method preceding the assay. It is clear that these methods introduce a variable amount of salt (tab. 2). Even serum electrolytes affect the ionic strength. The $ZnSO_4$-$Ba(OH)_2$ method (8) seems more appropriate for keeping the ionic strength low, but this is due to dilution, thus decreasing the sensitivity of the assay. Amicon filter cones (8, 10), or the heat denaturation of freeze-thawing method (9) and the trichloroacetic acid method with subsequent ether extraction (18) have the least effect, but they are either expensive (Amicon) or elaborate.

The negative effect of salt on the properties of acetylCoA : carnitine O-acetyltransferase is clearly demonstrated in figure 4. The effect is independent of the nature of the salt (fig. 5) and solely due to the ionic strength of the assay. Addition of sucrose has no effect at all. Salts act as if they were competitive inhibitors. This could be due to masking of charges on molecules (*Debye-Hückel* effect). Increased amounts of acetylCoA : carnitine O-acetyltransferase and acetylCoA are required in the assay to obtain acceptable reaction times (fig. 1, line e, g). Thus the choice of the buffer should be combined with the choice of the deproteinization method.

The combination of buffer and deproteinization

One can use the Tris-buffered assay according to *Pearson* et al. A reagent blank is necessary, as well as a deproteinization method that introduced a minimum amount of salt, e. g. heat denaturation and freeze thawing or trichloroacetic acid with subsequent ether extraction. With the latter method, the overall performance is similar to the HEPES-buffered assay (tab. 3). If a deproteinization method is used that introduces more salt, then more acetylCoA and more acetylCoA : carnitine O-acetyltransferase are required in the assay for acceptable results, thereby depriving the Tris-buffered method of its only advantage (fig. 1, line e, f). A disadvantage of ether extraction is that long chain acylcarnitines may also be extracted, so that the method is not suitable for the determination of total *L*-carnitine.

The second possibility is the use of the HEPES-buffered assay according to *Wieland* et al. in combination with perchloric acid deproteinization with subsequent $KHCO_3$ neutralization (fig. 1, line c). Due to the unfavourably low osmolality of the HEPES-buffered

assay (± 100 mosmol/kg), the reaction with an aqueous *L*-carnitine standard is very slow (fig. 1, line f). The addition of salt increases the osmolality of the assay towards optimal values and decreases the reaction time (fig. 1, line c, d). This is in contrast to the Tris-buffered assay. With an aqueous *L*-carnitine standard the osmolality is already in the optimal range, so that additional salt increases the osmolality away from optimal values, and the reaction time increases (fig. 1, line a, b, g). Also, concentrations of acetylCoA and acetylCoA : carnitine O-acetyltransferase are higher in the HEPES-buffered assay. The results from table 3 show that the preferred method is the HEPES-buffered assay in combination with the perchloric acid deproteinization with subsequent $KHCO_3$ neutralization. An advantage of this method is the possibility of determining both free and acylated *L*-carnitine. By using the proposed method for the hydrolysis of esterified carnitine esters, the amount of extra salt can be kept within the same limits as those in the unhydrolysed sample.

Thus we prefer methods that employ HEPES rather than Tris, because the deproteinization method is more convenient, and because it is possible to determine both total and free *L*-carnitine. Moreover, the additional problem of the acetylation of Tris is avoided.

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References

1. Siliprandi, N., Sartorelli, L., Ciman, M. & DiLisa, F. (1989) Carnitine: metabolism and clinical chemistry. Clin. Chim. Acta *183*, 3–12.
2. Chapoy, P. R., Angelini, C., Brown, J., Stiff, J. E., Shug, A. L. & Cederbaum, S. D. (1980) Systemic carnitine deficiency A treatable inherited lipid-storage disease presenting as Reye's syndrome. N. Eng. J. Med. *24*, 1389–1394.
3. Vacha, G. M., Giorcelli, G., Siliprandi, N. & Corsi, M. (1983) Favorable effects of L-carnitine treatment on hypertriglyceridemia in hemodialysis patients: decisive role of low levels of high density lipoprotein-cholesterol. Am. J. Clin. Nutr. *38*, 532–540.
4. Leschke, M., Rumpf, K. W., Eisenhauer, Th., Becker, K., Bock, U. & Scheler, F. (1984) Serumspiegel und Urinausscheidung von L-Carnitin bei Patienten mit normaler und eingeschränkter Nierenfunktion. Klin. Wochenschr. *62*, 274–277.
5. Worthley, L. I. G., Fishlock, R. C. & Snoswell, A. M. (1983) Carnitine deficiency with hyperbilirubinemia, generalized skeletal muscle weakness and reactive hypoglycemia in a patient on long-term total parenteral nutrition: treatment with intravenous L-carnitine. J. Parent. Enter. Nutr. *7*, 176–180.
6. DiDonato, S., Rimoldi, M., Garavaglia, B. & Uziel, G. (1984) Propionylcarnitine excretion in propionic and methylmalonic acidurias: a cause of carnitine deficiency. Clin. Chim. Acta *139*, 13–21.
7. Marquis, N. R. & Fritz, I. B. (1964) Enzymological determination of free carnitine concentrations in rat tissues. J. Lip. Res. *5*, 184–187.
8. Seccombe, D. W., Dodek, P., Frohlich, J., Hahn, P., Skala, J. P. & Campbell, D. J. (1976) Automated method for L-carnitine determination. Clin. Chem. *22*, 1589–1592.
9. Rodriguez-Segade, S., Dela Pena, C. A., Paz, J. M. & Del Rio, R. (1985) Determination of L-carnitine in serum, and implementation on the ABA-100 and Centrifichem 600. Clin. Chem. *31*, 754–757.
10. Cederblad, G., Harper, P. & Lindgren, K. (1986) Spectrophotometry of carnitine in biological fluids and tissue with a Cobas Bio centrifugal analyzer. Clin. Chem. *32*, 342–346.
11. Wieland, O. H., Deufel, T. & Paetzke-Brunner, I. (1985) Free and esterified carnitines: Colorimetric method. In: *Methods of Enzymatic Analysis*. Third edit. (Bergmeyer, H. U., ed.) Vol. VIII, pp. 481–488, VCH Verlags-Gesellschaft.
12. Pearson, D. J., Tubbs, P. K. & Chase, J. F. A. (1974) Carnitine and acylcarnitines. In: *Methods of Enzymatic Analysis*. Second edit. (Bergmeyer, H. U., ed.) pp. 1758–1771, Weinheim Verlag Chemie.
13. Ellman, G. L. (1960) Tissue sulfhydryl groups. Arch. Biochem. Biophys. *82*, 70–77.
14. Deufel, T. & Wieland, O. H. (1983) Sensitive assay of carnitine palmitoyl transferase in tissue homogenates with a modified spectrophotometric method for enzymatic carnitine determination. Clin. Chim. Acta *135*, 247–251.
15. Rössle, C., Kohse, K. P., Franz, H. E. & Fürst, P. (1985) An improved method for the determination of free and esterified carnitine. Clin. Chim. Acta *149*, 263–268.
16. Christiansen, R. Z. & Bremer, J. (1978) Acetylation of Tris-(hydroxymethyl)aminomethane (Tris) and Tris derivatives by carnitine acetyltransferase. FEBS Letters *86*, 99–102.
17. Fürst, P. & Glöggler, A. (1987) Reappraisal of carnitine concentrations in blood. Clin. Chem. *33*, 1956–1957.
18. Chauhan, M. S. & Dakshinamurti, K. (1981) Fluorimetric assay of B_6 vitamers in biological material. Clin. Chim. Acta *109*, 159–167.

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