ACETYLATION OF TRIS(HYDROXYMETHYL)AMINOMETHANE (TRIS) AND TRIS DERIVATIVES BY CARNITINE ACETYLTRANSFERASE

Renata Z. CHRISTIANSEN and Jon BREMER
Institute for Medical Biochemistry, University of Oslo, Karl Johans gt. 47, Oslo 3, Norway

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1. Introduction

The most sensitive method for determining carnitine is based on the separation of [^{14}C]acetylcarnitine from [1-^{14}C]acetyl-CoA on a small Dowex-2-Cl column after incubation of the carnitine with excess [1-^{14}C]acetyl-CoA and carnitineacetyl-transferase [1,2].

This method has been used for studies of carnitine uptake and release in isolated liver cells [3]. We observed erratic high carnitine values in the cells, especially when Tricine was used in the cell suspension medium, but also Tris, Tes and phosphate interfered in the carnitine analysis.

We have studied Tris and Tris derivatives in this assay more closely. It turns out that these buffers, especially Tricine, can act as acetyl acceptors for carnitine acetyltransferase, thus interfering with the assay of carnitine.

2. Materials and methods

2.1. *Chemicals*

Carnitine acetyltransferase was from Boehringer, Mannheim, FRG; [1-^{14}C]acetyl-CoA from Amersham Radiochemical Centre, England. (−)Carnitine was a gift from Otsuka Pharmaceutical Factory, Osaka, Japan. Tris(hydroxymethyl)aminomethane (Tris), (trishydroxymethyl)methylglycine (Tricine), tris-(hydroxymethyl)aminoethansulfonic acid (Tes), and hydroxyethylpiperazineethansulfonic acid (Hepes), were obtained from Sigma, OH.

O-Acetylated derivatives of Tris, Tricine and Tes were prepared from the corresponding free amines as follows: The amine was dissolved in trifluoroacetic acid (1 mequiv./ml) and acetylchloride (1–5 mequiv./equiv. amine) was added dropwise with shaking. After 1 h at room temperature several vol. diethyl ether were added. The sticky precipitate obtained was washed with ether. The reaction products were tested for ester bonds with alkaline hydroxylamine in water [4]. After acidification with perchloric acid a violet colour with ferric ions was obtained. With Tris some formation of the *N*-acetyl Tris could not be excluded, but quantitative measurements of ester bonds as acethydroxamic acid [4] showed that mainly *O*-acetylderivatives had been formed.

When chromatographed on thin-layer silica G plates with phenol–water (3:1, w/v) the acetylated products gave several spots when exposed to iodine vapour. Acetylated Tris gave at least 3 spots with higher R_F values than Tris itself. If more acetylchloride was used in the acetylation procedure, the spots with high R_F values increased in intensity, and with a huge excess of acetylchloride only one strong spot near the solvent front was obtained. The spots therefore evidently represented mono-, di- and triacetylated Tris.

2.2. *Radiochemical assay*

The standard incubation mixture contained [1-^{14}C]acetyl-CoA, 20 nmol; (2000 cpm/nmol) carnitine acetyltransferase, 0.6 units; and one of the buffers (pH 7.4) to be tested, 150 μmol in total vol. 0.325 ml at 37°C. After incubation the reaction mixture was passed through a column of Dowex-2-Cl (approx. 0.5 × 3 cm) to adsorb unreacted [1-^{14}C]-acetyl-CoA. Radioactivity in the eluate was measured in a Tricarb scintillation spectrometer.

2.3. *Spectrophotometric assay*

The acetylation of Tricine was followed by measuring the decrease in acetyl-CoA A_{233} in an Aminco dual-wavelength spectrophotometer. The reaction mixture contained approx. 150 nmol. acetyl-CoA, 2.5 units carnitine acetyltransferase, and varying concentrations of Tricine or carnitine, pH 7.4, 25°C and total vol. 2 ml. The acetylation of Tris and Tes was too slow to be measured with this method.

2.4. *Identification of reaction products*

The column eluate from the radiochemical assay was concentrated to a small volume under an air current and put on silica G thin-layer plates (0.25 mm) and developed with phenol–water (3:1). Spots of reference acetylated buffers were localized with iodine vapour. Distribution of radioactivity on the plates was determined after scraping bands (3–5 mm) of the silica layer into counting vials containing scintillation fluid.

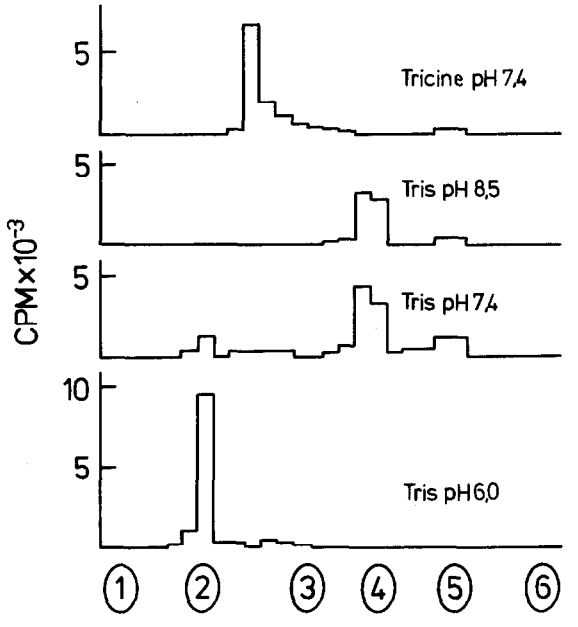

Fig.1. Thin-layer chromatography on silica G of acetylated products of Tris and Tricine with phenol–water (3:1). The peaks in the radiochromatograms show the position of radioactive products formed when [1-^{14}C]acetyl-CoA was incubated with carnitine acetyltransferase and Tricine or Tris at the pH values shown. The numbered spots at the bottom of the figure show the positions of reference compounds in the thin-layer chromatograms. (1) Acetyl-CoA (near the origin). (2) Radioactive spot when [1-^{14}C]acetyl-CoA and Tris were chromatographed together. (3) Tris. (4–6) Spots from Tris acetylated with acetylchloride in trifluoroacetic acid, presumably mono-, di- and triacetylated Tris.

Figure 1 shows that Tris gave one main radioactive spot with an R_F which coincided with that of monoacetyl-Tris. A smaller radioactive spot with a higher R_F could be diacetyl-Tris. The radioactive spot from Tricine similarly coincided with monoacetyl-Tricine (not shown). To test whether *N*- or *O*-acetylated Tris or Tricine had been formed samples from the column were added to a mixture of 0.05 ml 28% hydroxylamine hydrochloride and 0.25 ml 3.5 N NaOH. After neutralization with 3.5 N HCl the mixture was evaporated to dryness and the residues extracted with 0.1 ml methanol. A sample of the methanol was chromatographed on a thin-layer cellulose plate with butanol/acetic acid/H_2O (4:1:5, upper organic phase) [5]. Both from Tris and Tricine incubations we found only one radioactive spot with an R_F value which coincided exactly with that of synthetic acethydroxamic acid which was detected by spraying with 0.5% $FeCl_3$ in dilute $HClO_4$. This was taken to indicate that only *O*-acetylated Tris or Tricine had been formed.

Further identification of the reaction products by gas chromatography was attempted, but was not successful.

3. Results and discussion

Table 1 shows the apparent formation of radioactive products from [1-^{14}C]acetyl-CoA which were not retained on a column of Dowex-2-Cl after incubation of Tris, Tricine, Tes, Hepes and phosphate with and without carnitine acetyltransferase. Tricine was the better substrate, and the formation of its product was completely dependent on the presence of the enzyme. Tris and Tes gave less radioactive products, and Hepes was not a substrate at all.

With Tris and with phosphate, radioactive products seemed to be formed also in the absence of enzyme. The phosphate product was not investigated further. Presumably the buffer eluted part of the acetyl-CoA from the column.

The nonenzymic Tris product was chromatographed on silica G thin-layer plates and found to be partly acetyl-Tris and partly unchanged acetyl-CoA (see below).

Table 1
Acetylation of Tris and Tris derivatives by carnitine acetyltransferase

	Buffer	Radioactivity (%) not retained on Dowex 2			
		Without enzyme		With enzyme	
Exp. I	Tris			22[a]	
	Tricine	1.0		60	
	Tes			4.1	
	Hepes			1.2	
	Phosphate			14[b]	
Exp. II	Tris	6.5[a]	12[a]	27[a]	26[a]
	Tricine	1.1	2.1	61	61
	Tes	1.2	2.1	5.2	5.0
	Phosphate	12[b]	13[b]	13[b]	14[b]
	None	0.7	0.7	3.5[b]	3.6[b]

[a] Probably partly unchanged acetyl-CoA (see fig.1)
[b] The radioactive product(s) from the incubations with phosphate or water only were not investigated further

[1-^{14}C]acetyl-CoA, 20 nmol and buffer (pH 7.4) 150 μmol, were incubated with and without carnitine acetyltransferase, 0.6 units, total vol. 0.325 ml, at 37°C. Incubation time was 20 min (exp. I) or 40 min (exp. II)

There was little or no increase in the formation of radioactive products from 20–40 min. This was found to be due to a rapid inactivation of the enzyme, demonstrated by additions of carnitine after varying lengths of time in the spectrophotometric assay. When sufficient amounts of enzyme were used, nearly 100% of the acetyl-CoA was converted to acetyl-Tricine or to acetyl-Tris.

Figure 1 shows thin-layer chromatography in the phenol–water system of the products obtained from Tris incubated with enzyme at pH 6.0, 7.4 and 8.5. While Tricine and Tes (not shown) gave only one main radioactive product, Tris apparently gave two depending on the pH of the medium. The high R_F product obtained at pH 7.4–8.5 moved as synthetic acetyl-Tris. A small amount of this product was found in incubations without enzyme, showing that a spontaneous acetylation of Tris with acetyl-CoA can take place.

The lower R_F product from Tris, obtained mainly at pH 6, was most likely unchanged acetyl-CoA eluted by the Tris at this pH. We concluded this because (i) a high concentration of Tris prevented the binding of acetyl-CoA to the Dowex-2 column and (ii) the eluted radioactive product was able to react with carnitine in the presence of carnitine acetyltransferase. Also, when radioactive acetyl-CoA was chromatographed together with Tris in the phenol–water system, the radioactivity moved to the position of the low-R_F peak in fig.1. Acetyl- CoA alone stayed at the origin.

Tricine, the most reactive of the Tris derivatives, has a very high K_m for carnitine acetyltransferase – about 50 mM. Also its reaction rate is very slow, only 1–2% of that obtained wtih carnitine.

The results presented show that Tris, Tris derivatives and phosphate should not be used in the assay of carnitine with carnitine acetyltransferase. Their use probably explains the relatively high blank values observed by some workers [2]. The substrate properties of these commonly used buffers can also show up as false carnitine [3] depending on what kind of blank is used.

Hepes did not show any of these disadvantages.

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