Synthesis of unsaturated carnitine esters with N-acyl imidazoles

J. Červenka and H. Osmundsen[1]

Institute of Medical Biochemistry, University of Oslo, Blindern, Oslo 3, Norway

Summary A novel procedure is described for the synthesis of acylcarnitines. This entails the synthesis of an N-acyl imidazole intermediate, which subsequently is reacted with L-carnitine perchlorate to form the acylcarnitine. The procedure is rapid and simple, and does not require a large excess of N-acyl imidazole to achieve a reaction yield of 50–70%. A procedure, based on reverse-phase Sep-Pak cartridges for rapid isolation of acylcarnitines from these reaction mixtures is also described. These procedures have, in particular, been applied in the synthesis of polyunsaturated acylcarnitines, including docosahexaenoylcarnitine.—**J. Červenka and H. Osmundsen.** Synthesis of unsaturated carnitine esters with N-acyl imidazoles. *J. Lipid Res.* 1982. **23:** 1243–1246.

Supplementary key words polyunsaturated fatty acids • N,N′-carbonyldiimidazole

Synthesis of polyunsaturated acylcarnitines is usually achieved by using fatty acid chlorides as an intermediate. These are highly reactive compounds, and conditions must be carefully controlled (1). Also, the purification of the resulting acylcarnitines has been difficult and time-consuming, because these acylcarnitines, unlike saturated esters, do not readily crystallize (1).

N-acyl imidazoles have been used as a derivatization reagent for gas–liquid chromatography (2), for synthesis of triglycerides possessing a spin-labeled fatty acid (3), and, more recently, for synthesis of fatty acyl-CoA esters (4). Fatty acyl imidazoles are readily synthesized, and appear much less reactive than fatty acid chlorides. We here report a novel route for the synthesis of polyunsaturated acylcarnitine esters using N-acyl imidazoles as an intermediate. A procedure for subsequent isolation and purification of the acylcarnitines, employing reverse-phase Sep-Pak cartridges, is also presented.

MATERIALS AND METHODS

Materials

Linoleic acid, linolenic acid, γ-linolenic acid, linoelaidic acid, vaccenic acid, *trans*-vaccenic acid, arachidonic acid, and docosahexaenoic acid were purchased from Nu-Chek Prep Inc., Elysian MN. Oleic acid, N,N′-carbonyldiimidazole, acetonitrile, triethylamine, and dimethylsulfoxide were purchased from Fluka AG, Buchs, Switzerland. Silver perchlorate (anhydrous) was obtained from BDH Ltd., Poole, Dorset, United Kingdom. Reverse-phase (C_{18}) Sep-Pak cartridges were purchased from Waters Assoc., Milford, MA; L-carnitine was kindly donated by Sigma-Tau S.p.A., Rome, Italy; [^{3}H]carnitine was a personal gift from Professor J. Bremer, Institute of Medical Biochemistry, University of Oslo. Butylated hydroxytoluene was purchased from Sigma Chemical Co., St. Louis, MO.

Procedure

Measurement of acylcarnitine yields. The synthesis was always carried out with L-carnitine to which had been added [^{3}H]carnitine to give a specific activity of about 10^6 dpm/μmol. This faciliated measurements of reaction yields by a simple butanol extraction (1 volume of butanol to 2 volumes of aqueous phase), followed by measurement of radioactivity in the butanol phase and water phase. This entailed removal of a sample (50 μl) of reaction mixture which was added to 2 ml of distilled water. After extraction with 1 ml of butanol, 100 μl of each of the two phases was removed for measurement of radioactivity. This procedure leaves unreacted carnitine in the aqueous phase, while acylcarnitines are extracted into the butanol phase (1).

Analysis of acylcarnitines. Purity of acylcarnitine solutions was checked by thin-layer chromatography as previously described (1).

The fatty acid content of synthesized acylcarnitines was analyzed by gas–liquid chromatography using a column of 10% SP-2340 on 100/120 Chromosorb W AW (Supelco Inc., Bellefonte, PA). Free fatty acid content was measured as the amount of fatty acid extracted from acidified solutions. Concentrations of acylcarnitines were measured as fatty acid extracted after hydrolysis with 2 N KOH at 50°C for 15 min. Prior to analysis, fatty acids were methylated using Methyl-8 (Pierce Chemical Co., Rockford, IL). Butylated hydroxytoluene (0.005%, w/v) was included in all solvents. Absolute amounts of fatty acids were measured using pentadecanoic acid as an internal standard.

RESULTS AND DISCUSSION

Synthesis of acyl imidazoles

Fatty acid (0.2 to 0.8 mmol) was dissolved in 2 ml of benzene (that had been dried over metallic sodium) con-

[1] Present address: Institute of Biochemistry, Veterinary College of Norway, Box 8146, Dep, Oslo 1, Norway.

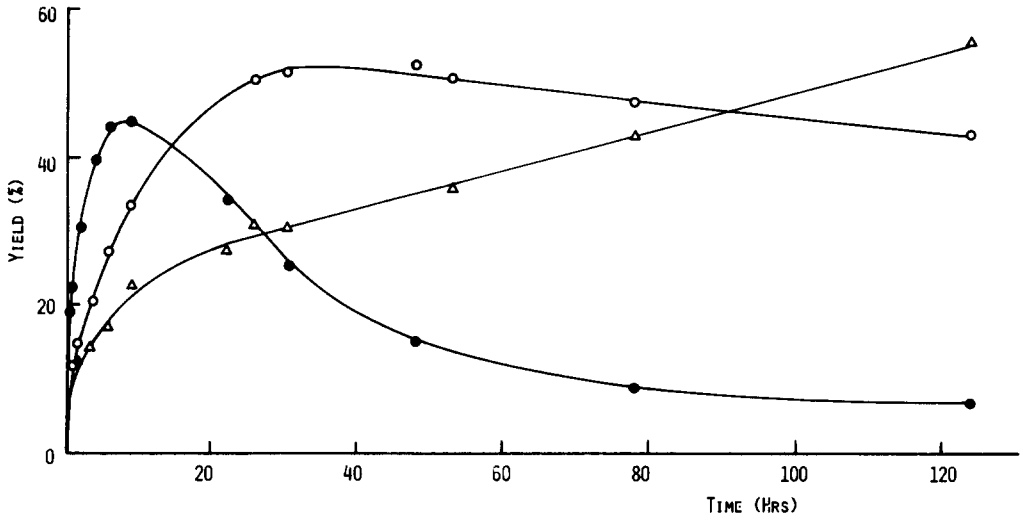

Fig. 1. Effects of reaction time on yields of oleoylcarnitine. Reaction mixtures containing [^{3}H]L-carnitine:deoylimidazole in molar ratios of 1:2 were incubated at 45°C (△), 65°C (○), or at 80°C (●) in the presence of 0.5 mM triethylamine. Samples of the reaction mixtures were removed at the times indicated in the figure and analyzed for reaction yields of oleoylcarnitine as described in the Methods section.

taining 0.005% (w/v) of butylated hydroxytoluene as antioxidant. A 10% molar excess of solid N,N′-carbonyldiimidazole was added to the fatty acid solution contained in a glass-stoppered reaction vessel. The mixture was stirred during this addition. Stirring was continued until effervesence had ceased completely (about 30 min) indicating completion of the reaction between fatty acid and N,N′-carbonyldiimidazole. Any insoluble residue from N,N′-carbonyldiimidazole was removed by brief centrifugation and decantation of supernatant into a clean test tube. Benzene was subsequently removed on a rotary evaporator and the N-acyl imidazole was redissolved in 1–2 ml of acetonitrile (dried over anhydrous $MgSO_4$) containing 0.005% (w/v) of butylated hydroxytoluene.

TABLE 1. Yields of various acylcarnitines

Acylgroup	Reaction Temperature	Reaction Time	Yield
	°C	hr	%
Oleoyl-	40	140	69 ± 14 (n = 5)
trans-Vaccenoyl-	50	63	48
Vaccenoyl-	50	86	43
Linoyl-	40	144	68, 62
Linolenoyl-	45	162	56, 40
Linoelaidoyl-	52	69	62
γ-Linolenoyl-	50	63	47
Arachindonoyl-	50	63	50, 38
Decosahexaenoyl-	50	86	28, 26

The tabulated yields represent individual reaction yields, except for oleoylcarnitine, which shows a mean value with S.D. indicated. The final yields (after purification on a Sep-Pak cartridge) were 50–75% of the reaction yields. This was, in part, due to deliberate losses in order to ensure the highest possible purity of the resulting acylcarnitine. Yields have been expressed as the percentage of total radioactivity (from [^{3}H]L-carnitine), which was extracted into the butanol phase, as described in the Methods section. These yields were obtained using a molar ratio of L-carnitine to N-acyl imidazole of 1:2.

Reaction of N-acyl imidazole with L-carnitine perchlorate

Carnitine perchlorate, which is readily soluble in acetonitrile, was prepared from silver perchlorate and L-carnitine chloride as described by Christophersen and Bremer (1). Carnitine perchlorate solutions in acetonitrile (0.4 mmol/ml) were kept dry over a molecular sieve (type 4A). Carnitine perchlorate solution was added to the N-acyl imidazole solution, usually to give a twofold molar excess of N-acyl imidazole. To this mixture was then added trimethylamine to a concentration of 0.5 M (higher concentrations did not increase yields; results not shown). Although acetonitrile was routinely used as solvent, the reaction gave very similar yields when carried out in dimethylsulfoxide (data not shown).

It is important to recognize that yields can decrease with increasing times of reaction, as is shown in **Fig. 1.** At reaction temperatures of 65°C and 80°C, yields of oleoylcarnitine showed an intermittent peak. At 45°C, however, the yield increased steadily with increasing times of reaction. A temperature of 50°C was, however, often used without apparent loss of yield (see **Table 1**). Usually reaction times of 60–160 hr were used. In Table 1 are presented yields for various acylcarnitines, together with reaction times and temperatures.

Isolation and purification of acylcarnitine esters from reaction mixtures

At the end of reaction time, 2 ml of methanol was added to the reaction mixture (typically about 2 ml con-

taining 0.2 mmol L-carnitine and 0.3–0.4 mmol of N-acyl imidazole). After about 10 min further standing at the reaction temperature, solvents were removed by rotary evaporation. The remaining oily liquid was extracted once with 15 ml of hexane. The hexane phase was discarded, and the remaining oily liquid was dissolved in about 1 ml of methanol. To this solution was added 10–15 ml of hexane, with continuous shaking. This procedure precipitates carnitine esters as oily liquids. The hexane phase was removed, and the procedure was repeated. In this way most of the contaminating free fatty acid and N-acyl imidazole were removed. Remaining solvents were removed by rotary evaporation, and the remaining oily liquid was dissolved in about 1 ml of distilled water.

This aqueous solution of carnitine and acylcarnitine (together with imidazole) was then passed through a reverse-phase Sep-Pak cartridge. To the eluate was added acetic acid to a final concentration of 5–10% (v/v) (this was found to enhance acylcarnitine binding). The acidified eluate was recycled through the cartridge once, to ensure complete binding of acylcarnitine. The cartridge was subsequently washed with 5–7 ml of distilled water, which also eluted unreacted carnitine (see **Fig. 2**). The acylcarnitine was then eluted in a step-wise fashion with 5–10 ml of aliquots of methanol–water mixtures, usually starting with 50% (v/v) of methanol. The concentration of methanol was usually increased by 10% in each step, to a final value of 100%. The concentration of methanol required to give a sharp peak of elution differed somewhat with different acylcarnitines, but all acylcarnitines were eluted between 70 and 85% (v/v) of methanol. Fractions of 0.3–0.5 ml were usually collected at the concentration of methanol known to elute an acylcarnitine, as this facilitated separation of acylcarnitine from colored reaction products, which usually were eluted sharply off the cartridge immediately prior to the acylcarnitine. Elution of carnitine and acylcarnitine were monitored by measuring radioactivity in the eluate, and by thin-layer chromatography of eluted fractions. In Fig. 2 is shown a typical elution diagram.

Polyunsaturated acylcarnitines were stored at −20°C as methanol solutions, also containing 0.005% (w/v) of butylated hydroxytoluene. Prior to use a measured volume was withdrawn, methanol was removed by rotary evaporation, and the acylcarnitine was redissolved in a known volume of distilled water. Monounsaturated acylcarnitines were stored as solids at −20°C.

Characteristics of the purified reaction product

Purified acylcarnitines ranged from semi-solids to viscous oils, depending on the degree of unsaturation,

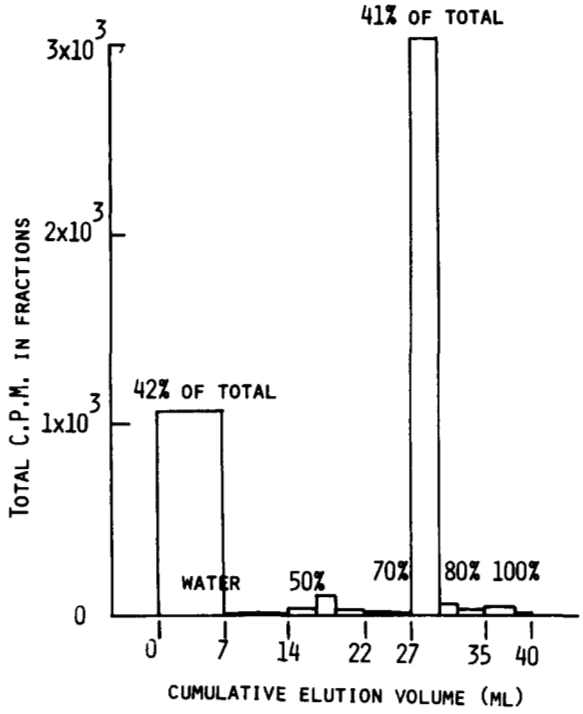

Fig. 2. Elution diagram from a reverse-phase Sep-Pak cartridge of a reaction mixture containing [^{3}H]L-arachidonoylcarnitine. The reaction mixture, which had contained 0.2 mmol of [^{3}H]L-carnitine and 0.4 mmol of arachidonoylimidazole, was incubated at 50°C for 120 hr. The reaction mixture was treated as described in the Methods section, and cartridge elution was carried out as shown in the figure. The two small fractions collected immediately prior to the arachidonoylcarnitine peak (the major peak of radioactivity was eluted with 70% of methanol) here contained practically all of the colored reaction products. Remaining imidazole was eluted together with carnitine, as judged by thin-layer chromatographic analysis of eluted fractions.

as described elsewhere (1). They all gave a positive hydroxamate reaction (1). The preparations were devoid of colored reaction products.

Analysis by thin-layer chromatography showed one single component, which also contained all detectable radioactivity. Unsaturated acylcarnitines gave an R_f of about 0.50, which was slightly higher that that of palmitoylcarnitine (0.47), also in agreement with previous findings (1). Oleoylcarnitine, synthesized as described here, was chromatographically indistinguishable from oleoylcarnitine synthesized via the acid chloride (1). The infrared spectra of these two compounds were not significantly different.

Analysis by gas–liquid chromatography showed that purified acylcarnitines always contained one single fatty acid, indistinguishable from the starting fatty acid, as judged by retention times. The free fatty acid content of these preparations was about 0.5% of the total amount of fatty acid in the preparations.

Using these acylcarnitines as substrates for β-oxidation by isolated rat liver mitochondria (5), the amounts

of O_2 consumed during oxidation of a limiting amount of acylcarnitine corresponded well with the theoretical amount calculated from acylcarnitine concentration (95–110% of theoretical). This concentration was based on measurement of fatty acid content by gas–liquid chromatography. Therefore, this shows that, within limits of experimental error, all esterified fatty acid was available for β-oxidation. Rates of oxidation of polyunsaturated acylcarnitines were similar to those previously reported (1). These preparations are currently being used in studies of mitochondrial metabolism of polyunsaturated fatty acids.

This work was supported by the Royal Norwegian Council for Scientific and Industrial Research.

Manuscript received 21 December 1981 and in revised form 17 May 1982.

REFERENCES

1. Christophersen, B. O., and J. Bremer. 1972. Carnitine esters of unsaturated fatty acids. Preparation and some aspects of their metabolism. *Biochim. Biophys. Acta.* **260:** 515–526.
2. Horning, M. G., A. M. Moss, E. A. Boucher, and E. C. Horning. 1968. The GLC separation of hydroxyl-substituted amines. Preparation of derivatives for electron capture detection. *Anal. Lett.* **1:** 311–321.
3. Boss, W. F., C. J. Kelly, and F. R. Landsberger. 1975. A novel synthesis of spin label derivatives of phosphatidylcholine. *Anal. Biochem.* **64:** 289–292.
4. Kawaguchi, A., T. Yoshimura, and S. Okuda. 1981. A new method for the preparation of acyl-CoA thioesters. *J. Biochem. (Tokyo).* **89:** 337–339.
5. Borrebaek, B., H. Osmundsen, and J. Bremer. 1980. In vivo induction of 4-enoyl-CoA reductase in liver mitochondria, and its effect on pent-4-enoate metabolism. *Biochem. Biophys. Res. Commun.* **93:** 1173–1180.