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Note

Use of anion-exchange resin in F^- form in sample processing for determination of carnitine

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Ion-exchange resins of Dowex 1 type are commonly used for processing biological samples in order to isolate carnitine(s) together with butyrobetaine. The purification steps are as follows: Dowex 50W in the H^+ form binds carnitine but in the NH_4^+ form it does not: Dowex 1 and 2 resins exclude carnitine(s) but they bind acidic compounds. The combination of Dowex 50 and Dowex 1 resins was successfully used to process small biological samples for analytical purposes [1–3]. Usually, the resins were contained in small columns (Pasteur pipettes). The procedure using this 'double' column results in a preparation containing carnitine, butyrobetaine and strongly basic amino acids, together with polyamines.

Dowex 1 resin in the OH^- form is very effective in removing anionic compounds, because the OH^- ion has the lowest affinity (relative value 1) for the resin. However, in this form the resin hydrolyses carnitine esters; therefore it can be used only for the isolation of total (free plus ester) carnitine. When it is necessary to measure acylcarnitines, such as in the radioactive carnitine assay, Dowex 1 or 2 is used in the Cl^- form [4,5]. However, Dowex 1 with a Cl^- counter-ion is insufficiently effective in sample purification because Cl^- has a relatively high affinity (relative value 22) for the resin; therefore it can hardly be replaced by weak acids.

The use of Dowex 1 X8 resin in the F^- form is promising for the following reasons: (a) F^- has a very low affinity for the resin (value 1.6); (b) in combination with Dowex 50W X8 resin in the NH_4^+ form, the resulting salt in the

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sample is NH_4F, which mostly sublimates during lyophilization when sample concentration is necessary.

EXPERIMENTAL

Materials

Carnitine acetyltransferase (CAT), coenzyme A (CoA) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Boehringer (Mannheim, F.R.G.). Dowex 50W X8 (200–400 mesh) and Dowex 1 X8 (200–400 mesh) ion-exchange resins were from Serva (Heidelberg, F.R.G.). 1-Heptanesulphonic acid was from Aldrich (Milwaukee, WI, U.S.A.). [1-^{14}C]Acetyl-CoA was from Amersham (Little Chalfont, U.K.). L-Carnitine and acetyl-L-carnitine were gifts from Sigma Tau (Rome, Italy). Acetyl-CoA and propionyl-CoA were prepared with the corresponding acid anhydride as described in ref. 6. L-[Methyl-^{3}H]carnitine was prepared as described in ref. 7. Acetyl-L-[^{3}H]carnitine and propionyl-L-[^{3}H]carnitine were prepared from the corresponding CoA esters and [^{3}H]L-carnitine with CAT (in the presence of N-ethylmaleimide) under conditions of the radioactive carnitine assay [5]. All other chemicals were of analytical grade.

Resins

The resins were converted into the desired form essentially as recommended by a recent Bio-Rad catalogue. Since Dowex 50W X8 resin was in the NH_4^+ form, it was washed with distilled water until it reached pH 7.2–7.4, as checked by a pH meter (indicator papers may be misleading). Dowex 1-X8 resin with Cl^- counter-ion was converted into the F^- form by washing the column with 1 *M* NaF until the effluent was free of Cl^-. Then the resin was taken out of the column, washed twice with 10 volumes of water and once with 10 volumes of dilute HF solution (50 μl of 30% HF in 1 l of water). After this treatment the resin was washed with water until it reached pH 4.0–4.5.

Carnitine determinations

Tissue extraction and photometric determination were carried out as described previously [8]. Alkaline hydrolysis of the samples in order to obtain total carnitine was performed as follows: to a 500-μl sample were added 30 μl of 25% NH_4OH in an Eppendorf tube; the tube was capped and kept at 37°C for 1 h. After the hydrolysis, the sample was evaporated to dryness under a stream of air and then reconstituted with water to the original 500-μl volume. The radioactive carnitine assay was done basically as originally described [4].

Combined use of Dowex 50W NH_4^+ and Dowex 1 F^- resins

The volume of both resins was 4.0 cm × 0.5 cm in Pasteur pipettes. The pipette containing Dowex 50W NH_4^+ was stacked over the another pipette con-

TABLE I

EFFECTIVENESS OF ION-EXCHANGE RESINS IN COMBINATION TO REMOVE SUBSTANCES FROM BIOLOGICAL SAMPLES AND BUFFER

A 1.0-ml neutralized perchloric acid extract of rat liver represented 200 mg of original tissue and contained 1041 nmol of -SH, determined by the DTNB test. The arrangement of columns and elution of samples were as specified in Experimental. Values are means ± S.D. for triplicate measurements. KHB, Krebs-Henseleit bicarbonate buffer.

	Upper column (Dowex 50W X8)/lower column (Dowex 1 X8)					
	NH_4^+/F^-	NH_4^+/F^-	NH_4^+/Cl^-	NH_4^+/Cl^-	NH_4^+/Cl^-	NH_4^+/F^-
Applied to the column:						
Liver extract, 1.0 ml	+	+	+	+		
DTNB, 500 nmol, 100 μl		+		+		
KHB, 1.0 ml, 35 μg P_i					+	+
Recovered in the effluent:						
-SH group (nmol)	No	No	204 ± 19.0	132 ± 8.8	–	–
P_i (μg)					11.7 ± 1.9	No
Cl^- (qualitative)	No	No	+	+	+	No
Free carnitine (nmol		42.1 ± 1.9[a]				
		43.2 ± 3.6[b]				
Total carnitine (nmol)		51.6 ± 2.6[a]				
		50.5 ± 5.5[b]				

[a]Assayed by the photometric test [8].
[b]Assayed by the radioactivity test [4].

taining Dowex 1 X8 F^-. The sample was applied to the upper resin followed by water to elute the sample, and the effluent was collected from the lower pipette.

High-performance liquid chromatography (HPLC)

The separation was carried out by reversed-phase ion-pair liquid chromatography. The HPLC system (Labor MIM, Budapest, Hungary) consisted of a pump (Liqoupump 312/1) and a Chromosil C_{18} (250 mm×4.6 mm I.D., 10 μm) column. The eluent, with 1-heptanesulphonic acid as ion-pairing reagent, was prepared as previously described [1]. The flow-rate was 1.0 ml/min, and 1.0-ml fractions were collected directly in scintillation vials. The methanol step-gradient was as follows: the solution was started from a reservoir containing 70 ml of eluent with 7.5% (v/v) methanol, and at the 20th min 10 ml of methanol were added in 3 min to the remainder of the eluent under constant stirring.

RESULTS AND DISCUSSION

In the first experiment the efficiencies of Dowex 1 resins in the Cl^- and F^- forms were compared in order to confirm the prediction that the F^- resin is more effective in binding anionic compounds. Dowex 1 resins (Cl^- or F^-) were employed in the lower column under the Dowex 1 X8 NH_4^+ resin (Table I). Table I shows that of the 35 μg of inorganic phosphate (P_i) (applied with 1 ml of Krebs–Henseleit buffer) the Cl^- resin let through 11.7 μg, whereas in the effluent from the F^- resin no P_i was detectable. No Cl^- was found in the effluent from the F^- resin either, indicating that it exchanged for F^- ions.

For carnitine determination two main types of enzymatic analysis, the photometric assay using DTNB [8] and the radioactive assay [4,5], are generally used. The former is much simpler but the latter is considered to be more reliable. The photometric assay suffers from the difficulty that thiol compounds in biological samples react with DTNB to give blank values. Also 'invisible' disulphide compounds (e.g. in urine) may cause falsely low readings. The removal of thiol compounds from rat liver extract was attempted (Table I) by combined anion- and cation-exchange resins. Prior addition of DTNB reduces the remaining thiols in the effluent from 204 to 132 nmol. (This effect was confirmed in a model experiment with cysteine on Dowex 1-X8 resin. The resin let cysteine pass through quantitatively. However, with precolumn addition of DTNB to the cysteine sample no thiol group was detected in the effluent.) It is important that DTNB and its derivatives bind quantitatively to the resins, so that they do not interfere with any assay in the effluent. Table I also shows that when the F^- resin is used as second resin a completely thiol-free effluent is obtained. The liver sample purified in this way can be analysed by the photometric assay [8]. The obtained values of carnitine in the effluent were the

TABLE II

RECOVERY OF CARNITINE AND ACETYLCARNITINE FROM URINE SAMPLES PURIFIED ON RESINS

Urine was treated with 2.5 M K_2CO_3 to adjust the pH to 7.0–7.2. To 1.0 ml of urine, 50 μl or DTNB solution (250 nmol) were added and the whole sample was applied to the resin columns. The arrangement of columns and elution was as detailed in Experimental. Carnitines were assayed in the eluent by the photometric method [8] and confirmed by the radioactive method [4]. Quantitative values are means ± S.D. for triplicate measurements.

	Columns, NH_4^+/F^-		
	I	II	III
Applied to the column:			
Urine (ml)	1.0	1.0	1.0
Carnitine (nmol)	-	100	-
Acetylcarnitine (nmol)	-	-	96[a]
Recovered in effluent (nmol):			
Free carnitine	132 ± 17	230 ± 15	134 ± 19
Total carnitine	166 ± 16	268 ± 26	263 ± 20
Acetylcarnitine[b]	34	38	129

[a]100 nmol was planned, preparation contained some free carnitine.
[b]Obtained by subtracting free from total.

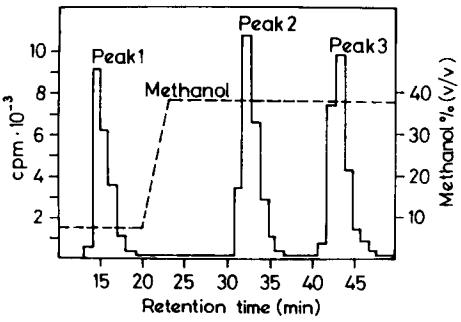

Fig. 1. Resolution of radioactive carnitine and carnitine esters by HPLC. A mixture of [^{3}H]L-carnitine, acetyl-[^{3}H]L-carnitine and propionyl-[^{3}H]L-carnitine (25 000 cpm each) was added to 1.0 ml of rat plasma. Sample processing and HPLC analysis are as specified in Experimental. Peaks: 1 = carnitine; 2 = acetylcarnitine; 3 = propionylcarnitine. In the three peaks 65 000 cpm of radioactivity was recovered.

same when determined by the photometric or the radioactive method (Table I). Before passage through the columns the sample from rat liver contained 52.1 nmol of total carnitine as determined by the radioactive assay [4] (not shown), so the recovery in the effluent (Table I) was 96.9%.

For the clinical laboratory the simpler photometric carnitine assay of human

TABLE III

BACKGROUND IN RADIOACTIVE CARNITINE ASSAY AS EFFECTED BY USE OF DIFFERENT ION-EXCHANGE RESINS

The radioactive carnitine assay was briefly as follows: to the solution (sample plus N-2-hydroxyethylpiperazin-N′-ethanesulphonic acid buffer containing N-ethylmaleinimide) of 200 μl were added 20 μl of a mixture to initiate the reaction. The 'starting' mixture contained 20 nmol of acetyl-CoA, 25 000 cpm of [1-^{14}C]acetyl-CoA and 2 μl (8 U) of carnitine acetyltransferase. Following 30 min incubation at 37 °C, 200 μl of the 220 μl of incubation solution were applied to a 8 cm × 0.5 cm Dowex 1 X8 column contained in a Pasteur pipette. The column was eluted with 1.0 ml of water into a vial for scintillation counting. Values are cpm recorded in the eluent.

[1-^{14}C]Acetyl-CoA preparation	Resin form		Carnitine in the sample (nmol)			
	Dowex 1 X8	Dowex 50W X8	0	1.9	4.75	9.0
			cpm obtained			
Untreated[a]	Cl^-	-	906	2780	5395	9430
Untreated[a]	F^-	-	895	-	-	-
Untreated[a]	Cl^-[b]	H^+[b]	45	-	-	-
Untreated[a]	Cl^-[b]	NH_4^+[b]	620	2376	4720	8950
Lyophilized	Cl^-	-	810	-	-	-
Purified on Dowex 50 H^+	Cl^-	-	55	1860	4530	8960

[a]Two-year-old Amersham preparation dissolved in water and kept at −20 °C for one more year.
[b]For these samples the pipette contained 2.0 cm of Dowex 50W X8 resin in the NH_4^+ or H^+ form (lower layer) and 6.0 cm of Dowex 1 X8 resin in the Cl^- form (upper layer).

urine may be of interest. Based on findings in Table I we employed the best combination: Dowex 1 X8 F^- resin in the lower column and prior addition of DTNB (Table II). It is important to neutralize the urine sample before applying it to the column. Table II shows the results of carnitine determinations by the photometric assay [8] in purified human urine samples, and also a complete recovery of added carnitine and acetylcarnitine. Data, as in Table I, were confirmed by the radioactive assay (not shown).

For the separation of sample components by chromatography, it is often necessary to concentrate the sample as well as desalt it. The combination of cation exchanger in the NH_4^+ form and anion exchanger in the F^- form offers a special advantage: the salt content of the sample exchanges for NH_4F, which mostly evaporates during lyophylization. This procedure was tested as shown in Fig. 1. The mixture of L-[^{3}H]carnitine, acetyl-L-[^{3}H]carnitine and propionyl-L-[^{3}H]carnitine (25 000 cpm each) was added to 1.0 ml of rat plasma, and a neutralized perchloric acidic extract was prepared as describing previously [8]. The extract was applied to the Dowex 50W NH_4^+ and Dowex 1 F^- double column as described above, with the exception that both columns were 8 cm long. The columns were eluted with 2.0 ml of water. The effluent was

collected in a conical glass tube and acidified with 20 μl of 95% acetic acid and 10 μl of 1 *M* HCl. (Acidification was necessary because some carnitine in the betaine form may evaporate during subsequent lyophilization.) After lyophilization the residue was extracted twice with 500 μl of cold (−20°C) methanol, the methanol extract was evaporated under a stream of air, and the residue was reconstituted in 200 μl of the HPLC eluent. The resolution of carnitine and its esters is shown in Fig. 1. The percentages of the radioactivity recovered in carnitine, acetylcarnitine and propionylcarnitine were 30.1, 33.8 and 36.1%, respectively. In a parallel experiment the same mixture of isotopic carnitines was directly applied to the HPLC column, and we obtained 29.4, 35.6 and 35.0% distribution of radioactivity in the above order of compounds. This, together with the observations given in Tables I and II, proves that no hydrolysis of carnitine esters occurred during sample processing on the resins or subsequently.

In current radioactive assays [4,5], at termination of incubation the sample is applied to a Dowex 2 (or 1) Cl^- resin to bind the excess of [1-^{14}C]acetyl-CoA and possibly hydrolysed [1-^{14}C]acetate, while the radioactivity in [1-^{14}C]acetylcarnitine passes through into a scintillation vial. The blank value is radioactivity that passes through the column from the sample without carnitine. Therefore, a lower blank value was expected with Dowex F^- than with the Cl^- resin. However, it was not lower (Table III). Instead, cation exchanger Dowex 50W in the H^+ or NH_4^+ form markedly reduced the background. (However, this resin cannot be used in the H^+ form for the carnitine assay, because it would not let the acetylcarnitine pass through.) Thus, Dowex 50W resin in the H^+ form can be recommended for pre-purification of radioactive acetyl-CoA preparations, if necessary. The component of some aged [1-^{14}C]acetyl-CoA preparations responsible for high blank value might be [^{14}C]acetylcysteamine, but its identification was not within the scope of this work.

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