PURIFICATION OF THE FATTY ACID PRESENT IN CARCINOLIPIN

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Isolation of Carcinolipin was described in our previous paper and evidence presented that this substance is probably an ester of cholesterol with a branched chain fatty acid[1]. Methods developed for the isolation of Carcinolipin did not allow it to be prepared in a pure form. It was also felt that it would be hardly possible to isolate it pure by chromatographic techniques in its natural form, *i.e.* as a cholesterol ester[1].

Another approach was therefore chosen in further experiments. Attempts were made to purify only the fatty acid present in Carcinolipin, to synthesize the cholesterol ester of this acid and to test the activity of this semisynthetic preparation. These attempts were successful and resulted in the preparation of this particular fatty acid in a pure form as described in this paper.

MATERIALS AND METHODS

Chemicals

All solvents were redistilled before use. Petroleum ether was the fraction boiling between 35 and 50°. Cholesterol was a commercial preparation purified through the dibromide[2]. It had a m.p. 149–150°. Cholesterol esters were isolated from a petroleum ether extract of freeze-dried beef liver and separated from other lipids by liquid–solid chromatography on silicic acid columns using petroleum ether–ether mixtures for elution as described earlier[1].

Hydrolysis of cholesterol esters

This was performed as described elsewhere[1]. Free fatty acids were extracted from the acidified hydrolysate with petroleum ether and the solvent evaporated in a vacuum rotary evaporator. The same method was also used for the hydrolysis of fatty acid methyl esters. Free fatty acids were crystallized from methanol–water mixtures.

Esterification of fatty acids

In large scale preparations fatty acids liberated from cholesterol esters were suspended in two volumes of absolute methanol and 6 drops of concentrated sulphuric acid added per 100 ml of this mixture. The mixture was protected from the air and then refluxed for 5 h. Solvent was evaporated using a rotary evaporator, a small volume of petroleum ether added and the lower phase containing sulphuric acid discarded. This method of esterification gave a nearly 100 % yield of fatty acid methyl esters. For the esterification of smaller quantities of fatty acids diazomethane was used[1].

Synthesis of cholesterol esters

Two parts of fatty acids were added to one part of cholesterol and this mixture heated at 200° for 3 h under a stream of CO_2[3]. After cooling the yellowish reaction product was dissolved in a small volume of chloroform and applied on to thin layers of activated silicic acid. Preparative thin layer chromatography for the separation of cholesterol esters has been described in detail elsewhere[1]. When sufficient quantities were available cholesterol esters eluted from thin layers were crystallized from methanol–ether mixtures. The yield of a purified cholesterol ester obtained by this procedure was 30–35 % of the theory. The method described was successfully used for quantities of the starting material as low as 1–2 mg of fatty acid.

Fractionation of fatty acids with urea

The methods of GRIMMER AND JACOB[4] and of LINSTEAD AND WHALLEY[5] were used for the precipitation of straight chain fatty acids from solutions of fatty acid methyl esters.

Clathrate chromatography of fatty acid methyl esters

For paper chromatography, sheets of Whatman No. 3 paper were dipped with a saturated solution of urea in water, left at room temperature for 2–3 h and finally dried at 80° for 2 h in a drying cabinet. After sample application the chromatograms were left for 24 h at room temperature in an atmosphere of methanol in well-stoppered tanks containing dishes of this solvent. This treatment resulted in the formation of an easily visible white zone of clathrate on the starting line. After that, chromatograms were dried for 10 min at room temperature to remove excess methanol and then developed with petroleum ether by the descending technique. Dishes containing methanol were placed in the developing tanks to ensure sufficient methanol in the atmosphere of the tanks during the development. After development the papers were dried at room temperature and the methyl esters of the fatty acids were detected by their fluorescence in the ultraviolet light of a Philips Philora HPW 125 high pressure mercury vapour lamp. They were then eluted from the paper with petroleum ether when necessary.

For micropreparative separation of fatty acid methyl esters a chromatographic procedure using thin layers of urea was developed. Three parts of urea were mixed with one part of Celite 545 (120 mesh) suspended in urea-saturated methanol and homogenized in a blendor. The resulting thick paste was quickly poured on to glass plates (12 × 20 cm) on to which a plastic frame 3 mm thick was placed to allow free edges to be left on the plate. Excess of urea suspension was scraped off with a plastic rod and the layer left to dry at room temperature for about one hour. After this the frame was removed and layers dried at room temperature overnight. The procedure described resulted in the formation of uniform homogeneous layers with excellent mechanical stability. Because of their high urea content these layers allowed a good separation of mixtures of fatty acid methyl esters up to about 500 mg. After sample application layers were left in an atmosphere of methanol overnight and developed with petroleum ether. Detection was performed in the same way as in the case of paper chromatograms. Portions of the layer containing fatty acid methyl esters were scraped off and suspended in water. This suspension was shaken 3 times with an equal volume of ether, the extracts were pooled, washed with water, dried over sodium

sulphate overnight and evaporated to dryness in a vacuum rotary evaporator to yield fatty acid methyl esters. Thin layers of thiourea were prepared in a similar way.

For large-scale separation of branched chain fatty acids from those with a straight chain, chromatography on urea columns was used. Equal parts of urea and Celite 545 (80/100 mesh) were suspended in a 2 % solution of methanol in petroleum ether (saturated with urea) and homogenized in a blendor. From the resulting homogeneous fine suspension columns 40 × 1000 mm were prepared in the usual way[1]. Methyl esters of fatty acids were dissolved in 2 % methanol in petroleum ether saturated with urea and columns eluted with the same solvent mixture. Clathrate formation could be observed following the progress of the sample front through the column. Elution was carried on until the clathrate front descended to 8–10 cm from the lower end of the column. The eluate was washed with water, dried over anhydrous sodium sulphate overnight and the solvent evaporated in a vacuum rotary evaporator. The procedure described allowed the separation of fatty acid methyl esters up to about 100 g. Although the separation was not as clear as when using paper or thin layer chromatography, the eluate after column chromatography contained only 1–2 % of the higher straight chain fatty acids present in the original mixture.

Gas–liquid chromatography

A LPCH-1 Gas Chromatograph (Vývojové dílny ČSAV, Prague) equipped with a thermal conductivity detector was used for preparative GLC. The instrument was slightly modified for this purpose[6]. Fatty acid methyl esters were separated using a 6 mm × 2 m column of 15 % Apiezon L on Chromosorb W (80/100 mesh) at 215°. Carrier gas was helium, inlet pressure 1.0 atm. Fractions were collected into hot chlorobenzene. 100–120 mg of methyl esters were injected for one run. Recoveries were about 85 % of the calculated yield. Isolated methyl esters were freed from split-products of Apiezon bled from the column by thin layer chromatography on silicic acid when necessary.

Analytical GLC was performed in the same way as described earlier[1].

Assay of biological activity

The effect of cholesterol esters resynthesized from individual fatty acid fractions on the incorporation of labelled algal protein hydrolysate into s-RNA* of rat liver *in vitro* was used for testing the activity of fatty acids isolated. Details of these procedures were described elsewhere[1,7].

RESULTS

Fractionation of fatty acids with urea

The mixture of fatty acids obtained by hydrolysis of beef liver cholesterol esters (for composition see ref. 1) was treated with urea as described by GRIMMER AND JACOB[4]. Although 10 precipitations with urea were carried out, no clear separation of branched chain fatty acids from those with straight chain could be obtained. Although precipitates contained considerably less unsaturated and branched chain fatty acids than the original mixture, as revealed by GLC, large quantities of palmitic and even traces of stearic acid were present in filtrates. In conformity with these

* Abbreviations: s-RNA = soluble or transfer RNA.

results both the cholesterol esters prepared from the fatty acids present in the final precipitates as well as the filtrate were active.

Better separations were obtained when fatty acid methyl esters were precipitated with urea three times as described by LINSTEAD AND WHALLEY[5]. The final precipitate did not contain detectable quantities of unsaturated and branched chain fatty acids. Cholesterol esters synthesized from fatty acids present in the clathrate were inactive. The final filtrate contained in addition to unsaturated and branched chain fatty acids considerable quantities of methyl palmitate and traces of stearate. Cholesterol esters prepared from these fatty acids showed a stimulating effect on protein synthesis.

These results supported our original opinion that Carcinolipin is an ester of cholesterol with a branched chain fatty acid. However, treatment of fatty acids with urea in solution was not really capable of separating branched chain fatty acids from those with a straight chain.

Clathrate chromatography of fatty acids

Both paper and thin layer chromatography on urea resulted in the separation of fatty acids into three groups. A clearly visible white zone of clathrate remaining on the starting line of chromatograms was composed, according to GLC, of straight chain saturated fatty acids. Methyl palmitate was the most prominent peak present

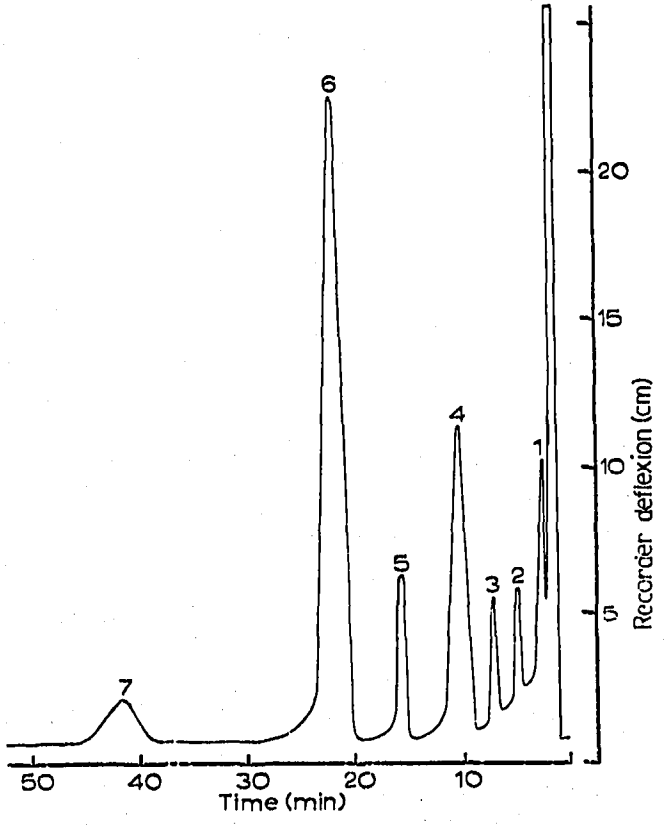

Fig. 1. GLC pattern of fatty acid methyl esters from the clathrate zone on a thin layer chromatogram. Methyl esters of fatty acids (430 mg) from beef liver cholesterol esters were separated on a thin layer of urea mixed with Celite as described in the section Materials and methods. Carbon numbers [12] of fatty acids in individual peaks are as follows: (1) 11.0; (2) 12.0; (3) 13.0; (4) 14.0; (5) 15.0; (6) 16.0; (7) 18.0.

in these chromatograms, a number of other higher fatty acids, in particular methyl myristate, being present as well, as seen in Fig. 1.

A yellowish zone with a bluish-violet fluorescence in ultraviolet light having an R_F 0.4–0.5 was evidently composed mainly of branched chain fatty acids, a fatty acid with carbon number 16.7 being the predominant constituent. Some lower straight chain fatty acids, in particular methyl laurate and myristate, were also present. However, this fraction contained only traces of methyl palmitate, as shown in Fig. 2.

Another yellowish fraction near the front of the chromatogram was composed mainly of unsaturated fatty acids, oleic and palmitoleic acid being the predominant components.

Cholesterol esters were synthesized from all three fractions. Only the intermediate fraction containing branched chain fatty acids was active.

When thiourea was used as material for thin layer chromatography, separation of the fatty acids into two fractions was obtained. Branched chain acids remained on the starting line and both straight chain saturated and unsaturated fatty acids moved near the top of the layer. However, the fraction of branched chain fatty acids was rather heavily contaminated with straight chain compounds.

For large-scale separation of branched chain fatty acids clathrate column chromatography was used. This method did not yield such a good separation as paper or thin layer chromatography. Although methyl stearate was completely removed by this treatment, methyl palmitate was found as a contaminant of the column eluate

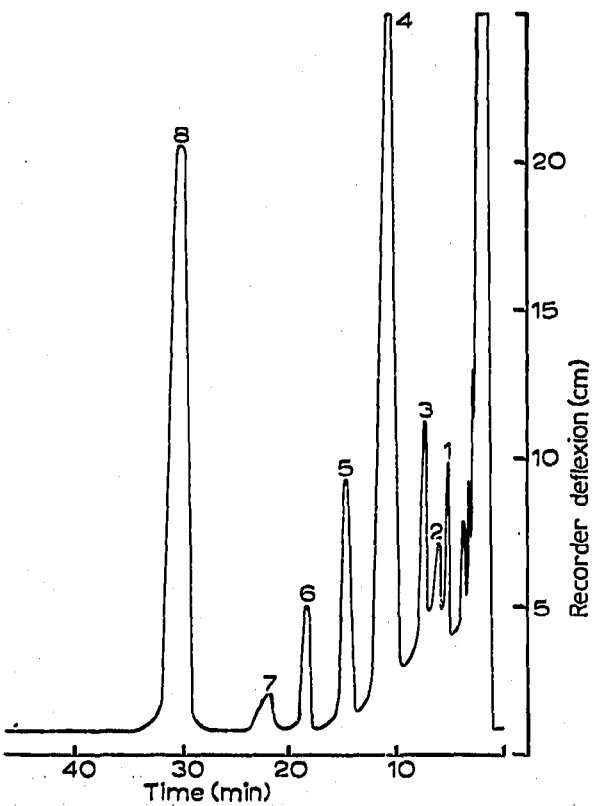

Fig. 2. GLC pattern of fatty acid methyl esters in the intermediate zone of a thin layer clathrate chromatogram. Carbon numbers of fatty acids in individual peaks are as follows: (1) 12.0; (2) 12.7; (3) 13.0; (4) 14.0; (5) 14.7; (6) 15.7; (7) 16.0; (8) 16.7.

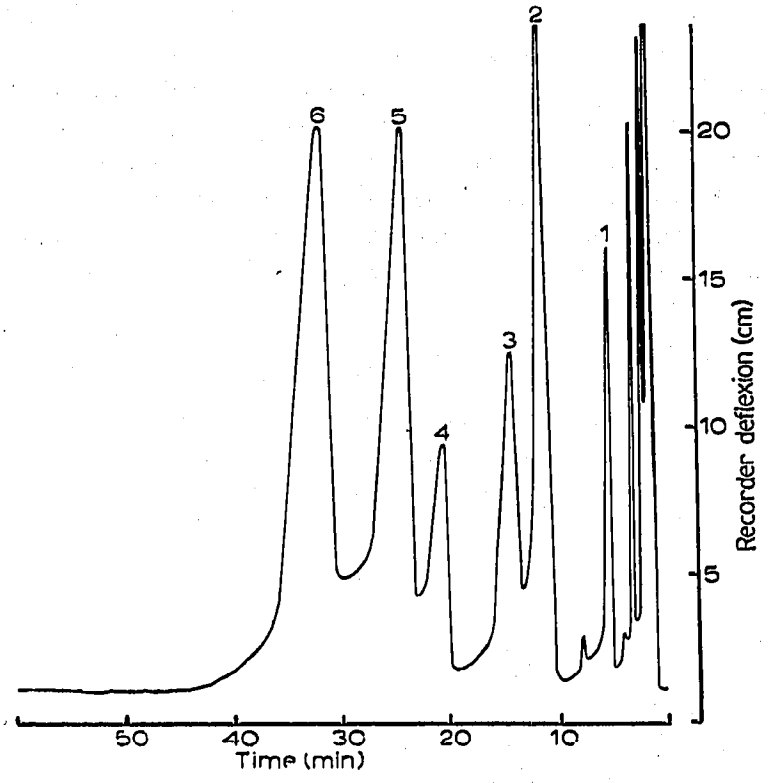

Fig. 3. GLC pattern of fatty acid methyl esters from the eluate of a urea–Celite column. Methyl esters of fatty acids (16.3 g) from beef liver cholesterol esters were separated on a column of urea and Celite (1 : 1) and eluted as described in the section Materials and methods. Carbon numbers of individual fatty acids are as follows: (1) 11.0; (2) 14.0; (3) 14.7; (4) 15.7; (5) 16.0; (6) 16.7.

(see Fig. 3). However, the fraction of branched chain fatty acids obtained in this way was found to be a satisfactory starting material for preparative GLC.

Preparative GLC of the branched chain fatty acid fraction

The fraction of branched chain fatty acids prepared by clathrate chromatography (for composition see Fig. 3) was subjected to preparative GLC. Six fractions representing individual fatty acids or their simpler mixtures were isolated in this way. The composition of fractions isolated by preparative GLC corresponded to the individual peaks seen in Fig. 3, fraction No. 1 containing all the lower acids up to and including hendecanoic acid. Only one of the cholesterol esters prepared from each of these fractions showed a stimulating effect on protein synthesis. This was the ester of the fatty acid with carbon number 16.7 and this was in good agreement with our previous results[1].

This fatty acid methyl ester was purified by further preparative GLC. In this way the active acid was obtained in a purity 98–99 % as checked by analytical GLC (see Fig. 4). After hydrolysis of the methyl ester and three crystallisations from methanol–water a white, crystalline substance was obtained whose m.p. 39.5–40.0° remained unaltered by further recrystallisations.

DISCUSSION

Methods were described in our previous paper[1] for the preparation of relatively pure Carcinolipin from beef liver. However, these procedures are time-consuming and yields of the active substance rather low. For these reasons it did not seem feasible to use Carcinolipin isolated by these techniques as a starting material for the final purification of the fatty acid present in this cholesterol ester. Instead, fractionation of total fatty acids present in beef liver cholesterol esters was found more advantageous for these purposes.

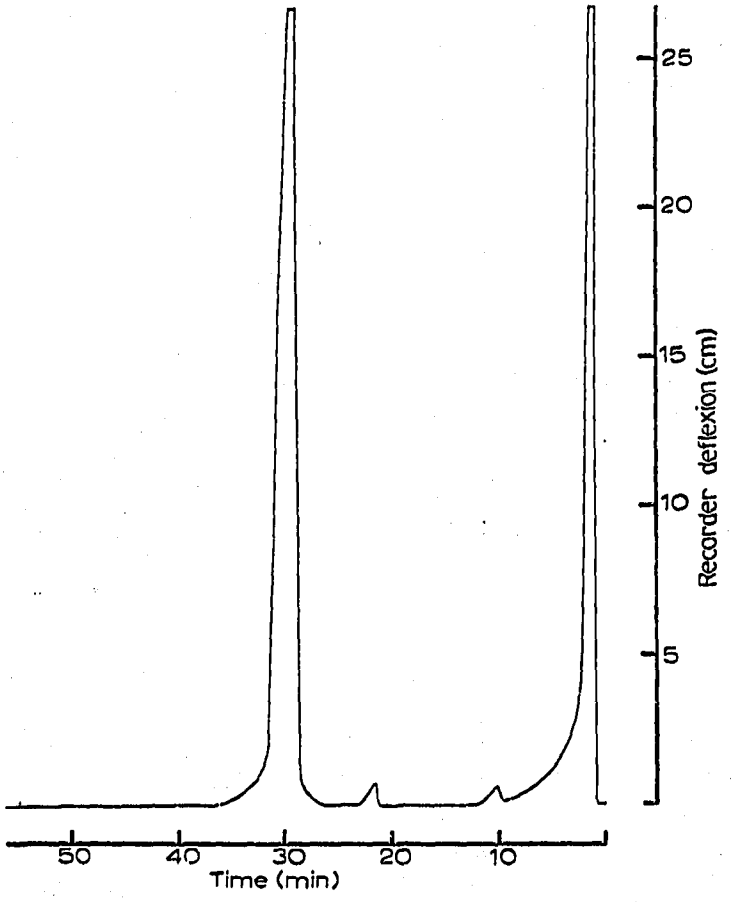

Fig. 4. Gas–liquid chromatogram of the final preparation of C-16.7 fatty acid methyl ester. The acid was purified by repeated preparative GLC and, after hydrolysis, by three crystallisations from methanol–water.

Preparative GLC is at the present time without doubt the most suitable method for the purification of individual fatty acids if they have different retention times as was so in this case. However, if only small amounts of the active substance are present in addition to large quantities of other compounds this procedure becomes time-consuming and loses most of its advantages. This was true in this case where only a few percent of the active acid were present in the original mixture. Moreover, methyl palmitate whose retention time is very similar to that of this acid was the most abundant substance in these mixtures. This fact made the purification of the acid with carbon number 16.7 very difficult and a preliminary separation of palmitic acid seemed desirable.

Relatively few branched chain fatty acids have so far been isolated from biological materials and consequently only a small number of techniques have been described for this purpose (see ABRAHAMSSON *et al.*[8] for a recent review). In preliminary experiments we have tried to separate the lead salts of branched chain fatty acids from those with a straight chain on the basis of their different solubilities in ether[9] but without much success. Fractionation of fatty acids with urea, a procedure often used for the purification of branched chain fatty acids[8], seemed to be the method of choice.

Techniques using precipitation of straight chain fatty acids from their solutions[4,5] seem to suffer from a common disadvantage that filtrates are contaminated by components present in the precipitate. Chromatographic methods guaranteeing a better and more prolonged contact of the fatty acids with urea seemed more promising. Urea column chromatography was successfully used by CASON *et al.*[10] for the separation of branched chain from straight chain fatty acids.

Two points seem to be of a critical importance for good separations on urea-impregnated carriers: sufficiently high urea contents incorporated in the carrier material, and, more important, the necessity of securing a sufficiently prolonged contact of the fatty acid methyl esters with urea since the clathrate formation proceeds relatively slowly. BHATNAGAR AND LIBERTI[11] described paper and thin layer chromatography on urea-containing materials but the relatively low content of urea in their carriers only allows the separation of minute quantities of fatty acids as was found when testing their method. Sufficient time for clathrate formation may easily be secured by simply placing papers or thin layers in an atmosphere supporting the complex formation (as do methanol vapours in our method). This effect can hardly be achieved when using column chromatography. We suppose that this is in fact the main reason why worse separations are obtained when using column chromatography instead of thin layers. High urea contents in the column material are, however, of primary importance here, too. Although it is possible to prepare columns of pure urea only, this procedure seems to suffer from severe disadvantages. Due to the clathrate formation proceeding within the column the elution becomes very slow and in some cases the flow of the column is practically stopped. Use of Celite as an inert support allows the preparation of columns with satisfactory and stable elution properties.

The use of thiourea[11] for the separation of branched chain fatty acids seems to offer no advantage over urea. In this case complexes of branched chain fatty acids remain on the starting line and their contamination with straight chain acids becomes more likely than in the case of urea clathrate formation.

Methods of clathrate chromatography described in this paper seem to be suitable for a preliminary fractionation of complex fatty acid mixtures on both analytical and preparative levels. Their use in combination with preparative GLC represents one possible way in which very pure branched chain fatty acids may be isolated from biological materials.

SUMMARY

Methods were developed for the chromatography of fatty acid methyl esters on urea-impregnated papers, thin layers and columns of urea and Celite. Fatty acids of cholesterol esters from beef liver were separated by clathrate chromatography into

fractions of unsaturated, branched chain saturated and straight chain saturated fatty acids. Cholesterol esters were synthesized from these fractions and commercial cholesterol and their effect was tested on the incorporation of labelled amino acids into s-RNA of rat liver *in vitro*. Only esters of the fraction of branched chain fatty acids were active. This fraction was further separated into 6 components by preparative GLC. The cholesterol ester of only one of these components, a saturated fatty acid with carbon number 16.7, showed a stimulating effect on protein synthesis. This fatty acid was purified to 98–99 % by repeated preparative GLC and obtained in crystalline form. It is concluded that the combination of clathrate chromatography and preparative GLC is very suitable for the isolation of pure branched chain fatty acids from biological materials.

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