ISOLATION OF CARCINOLIPIN BY COMBINED LIQUID–SOLID AND LIQUID–LIQUID CHROMATOGRAPHY

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A substance stimulating protein synthesis in cell-free systems of rat liver[1] has been isolated in our laboratory. Because of its lipid nature and carcinogenic activity the designation "Carcinolipin" was proposed for this factor[2]. Methods used originally for the isolation of this compound[3] did not allow us to prepare it in a sufficiently pure form to elucidate its chemical nature. However, its low polarity and positive colour reactions for sterols[3] indicated that it might be a sterol ester. This paper describes methods which were developed for further purification of the active substance and which allowed us to reach a more precise conclusion as to its chemical constitution.

MATERIALS AND METHODS

Animals and biological materials

Wistar rats of both sexes, weighing 120–200 g, bred in this Institute and kept on a mixed diet were used as the source of liver tissue for both the isolation of cholesterol esters and preparation of subcellular fractions. Fresh beef and pig liver was obtained from the slaughter house and used within 1–2 h after delivery.

Chemicals

Silicic acid for chromatography (Silica Gel CH, 150/300 mesh), used for both column and thin-layer chromatography, was a product of Spolana, Velvary, Czechoslovakia. Before use for liquid–solid chromatography it was activated at 120° for at least 48 h. All solvents were redistilled before use. Petroleum ether was the fraction boiling between 30 and 50°. ATP (sodium salt) was obtained from Reanal, Budapest, Hungary and purified as described by COHN AND CARTER[4]. Radioactive (U- ^{14}C)-labelled algal protein hydrolysate (769 mC/g) was purchased from the Institute for Research, Production, and Utilisation of Radioisotopes, Prague.

Lyophilisation and extraction of liver tissue

Beef, pig, or rat liver was chopped in a meat grinder and the resulting slurry homogenized in a blendor. Homogenate was poured into large Petri dishes, frozen at —30° and freeze-dried (Deutsche Vakuumapparate, Sangerhausen, Germany). The resulting material was ground to a fine powder and stored in well-stoppered bottles at 0°. Small quantities of dried liver tissue (up to 100 g) were extracted in a Soxhlet apparatus, for large-scale extractions (2–3 kg) Jena L-12-624 extractors

(Schott und Gen., Jena, Germany) were used. Extracts were evaporated to dryness using vacuum rotary evaporators.

Separation of cholesterol esters by liquid–solid chromatography

For the separation of cholesterol esters from other lipids silicic acid columns were used and esters eluted by diethyl ether–petroleum ether mixtures[5]. Activated silicic acid was suspended in petroleum ether and the resulting slurry poured in several smaller portions into glass columns (diameter 40 mm, length 1000 mm) containing sintered glass discs. About 500 ml of a petroleum ether extract (from 1 kg of freeze-dried liver tissue) was carefully added to the column. After this material had soaked into the column, cholesterol esters were eluted with a 2 % solution of diethyl ether in petroleum ether. Time-operated fraction collectors (Mikrotechna, Prague) were used and the rate of flow of the columns was adjusted to give 10–15 ml fractions over 20 min. For further analyses the solvent from individual test tubes was evaporated on a water bath. Cholesterol esters of saturated fatty acids were separated from those of unsaturated ones by liquid–solid chromatography using carbon tetrachloride for elution. Columns were prepared and elution performed as described previously.

Separation of cholesterol esters by liquid–liquid chromatography

For further fractionation of individual cholesterol esters a reversed-phase liquid–liquid chromatography system was developed. Apiezon M grease on Celite 545, 80/100 mesh, served as stationary phase and acetone–methanol mixtures were used as mobile phases. For impregnation of the supporting material an amount of Apiezon M grease necessary for a 10 % coating was dissolved in chloroform and the calculated quantity of Celite added. The resulting suspension was left at room temperature with occassional shaking for 3–4 h. Solvent was then evaporated in a rotary evaporator and the impregnated Celite dried in a thin layer at room temperature overnight. All solvents used for chromatography were saturated with Apiezon M grease. Since saturation of solvents with the grease at room temperature was found to be insufficient, solvents or their mixtures were refluxed with an excess of Apiezon M grease for 2–3 h. After cooling to room temperature the clear supernatant was decanted and used as mobile phase for chromatography. Columns 40 mm in diameter and 2000 mm long were prepared from slurries of impregnated Celite in the initial solvent mixture in the same way as described above. Because of the low solubility of cholesterol esters in solvents used as mobile phases the usual mode of sample application could not be used . Cholesterol esters (usually 200 mg for one column) were therefore dissolved in 20–30 ml of petroleum ether and 1–2 g of Apiezon M-coated Celite was added. This suspension was left 1 h at room temperature with occasional mixing and the solvent then evaporated in a rotary evaporator. The resulting loose fine powder was carefully placed on the top of the column above which a solvent layer 0.5–1 cm thick has been left. The stopcock at the end of the column was then opened and the solvent above the top of the column left to soak into the column. Three to five further small volumes of the solvent were then carefully added until the solution above the column remained clear and uncoloured. This mode of sample application resulted in the formation of a narrow and sharp initial zone of esters. After this the elution was started. Constant flow-rate of the column was maintained by a laboratory micropump (Mikrotechna, Prague) by which the mobile phase

was delivered on to the top of the column. The rate of flow was adjusted to 0.4–0.5 ml/min. When a gradient elution was used the micropump was connected to a flask equipped with a magnetic stirrer from which the initial solvent mixture (2000 ml) was admitted. This was connected to another reservoir from which the second solution was delivered. Teflon tubing was used for all connections. 10 ml fractions were collected in fraction collectors operated by micropumps. When necessary, several fractions were pooled and separated from Apiezon M grease by thin-layer chromatography on silicic acid as described below.

Thin-layer chromatography of cholesterol esters

This method was used for both analytical and micropreparative separations of cholesterol esters from other lipids or impurities of Apiezon M. Thin layers (unbound) of activated silicic acid were prepared as described by MOTTIER AND POTTERAT[6]. Plates were developed in a 5 % solution of diethyl ether in petroleum ether. Spots of cholesterol esters were detected by their bluish-violet fluorescence in the light of a mercury high pressure lamp (Phillips Philora HPW 125). Under these conditions cholesterol esters had an R_F of 0.4–0.5 and were completely separated from other lipids. Portions of silicic acid containing cholesterol esters were sucked-off into a flask and esters eluted with diethyl ether when necessary.

Paper chromatography of cholesterol esters

Two chromatographic procedures were developed for the rapid checking of the composition of individual fractions obtained by column chromatography. The first, using silicic acid-impregnated paper allowed the separation of the group of cholesterol esters of saturated fatty acids from cholesterol oleate and palmitoleate, linolate, and linolenate. Whatman No. 3 paper was impregnated as described by MICHALEC *et al.*[7] and developed by the descending technique in carbon tetrachloride–*n*-heptane (1:1). Before the development papers were activated at 100° for 2 h. The development was usually completed within 2 h. For the detection of cholesterol esters the papers were sprayed with a 10% solution of phosphomolybdic acid in ethanol and heated at 100° for 2 min in a drying cabinet[8]. For the separation of individual cholesterol esters reversed-phase partition chromatography was used. Sheets of Whatman No. 2 paper, 34 cm long and 26 cm wide were dipped into a 0.75 % solution of paraffin oil

TABLE I

RELATIVE MOBILITIES OF SOME CHOLESTEROL ESTERS ON SILICIC ACID-IMPREGNATED PAPER ELUTED WITH CARBON TETRACHLORIDE–*n*-HEPTANE (SYSTEM A) AND PARAFFIN OIL-IMPREGNATED PAPER ELUTED WITH ACETONE–METHANOL–WATER MIXTURES (SYSTEM B)

Details of both experimental procedures are given in the section Materials and methods. All mobilities are referred to cholesterol palmitate ($R_{CP} = 1.00$).

Compound	R_{CP} *in system*	
	A	*B*
Cholesterol myristate	1.00	1.32
Cholesterol palmitate	1.00	1.00
Cholesterol stearate	1.00	0.65
Cholesterol oleate	0.77	1.17
Cholesterol linolate	0.61	—

in ether and dried at room temperature for 10–15 min. Samples (0.02 ml) containing 10–50 μg of cholesterol esters in chloroform were then applied and the chromatograms were placed in a Chropa developing tank No. 002 (Glasswerke Ilmenau, Germany). The lower ends of the papers were immersed in 600 ml of acetone saturated with paraffin oil in a rectangular dish placed on the bottom of the tank and equipped with a magnetic stirrer. A mixture of methanol–water (8:2) saturated with paraffin oil was added (8 ml/h) to the initial mobile phase by a laboratory micropump. Chromatograms were developed for 14 h. They were then dried at 100° in a drying cabinet and cholesterol esters detected by phosphomolybdic acid as described above. The gradient-elution system used allowed a good separation of individual esters of cholesterol with saturated fatty acids and even a partial resolution of some "critical pairs" (cholesterol palmitate and oleate) as given in Table I.

Preparation of samples for gas–liquid chromatography

Solvents from all fractions to be tested were evaporated on a water bath. 4 ml of ethanol and 1 ml of a 33 % solution of potassium hydroxide in water was added and the mixture was heated at 60° for 20 min. After cooling, 3 ml of petroleum ether were added and after shaking the upper phase was discarded. This procedure was repeated twice more. After that the lower phase was acidified by hydrochloric acid to acid reaction (indicator paper) and extracted three times with petroleum ether as before. Extracts were pooled, an ethereal solution of diazomethane was added and solvents evaporated in a stream of nitrogen. The residues were dissolved in a small volume of diethyl ether and injected into the gas chromatograph.

Gas–liquid chromatography

Capillary columns 0.1 mm × 50 m coated with Apiezon L grease were used for the separation of methyl esters of fatty acids present in cholesterol esters. A Chrom II Gas Chromatograph (Laboratorní přístroje, Prague) equipped with a flame ionisation detector was used. The temperature of the column was 201°, carrier gas nitrogen, inlet pressure 3.0 atm., hydrogen 25 ml/min, and air 600 ml/min. Nitrogen bypass ratio was 1:50 and sensitivity of the instrument 1:1. Calibration was done using an artificial mixture of odd-numbered saturated fatty acids C_{12}–C_{18}. Carbon numbers of individual methyl esters were determined as described by WOODFORD AND VAN GENT[9].

Quantitative determination of cholesterol

This was done using the method of PEARSON *et al.*[10]. Admixture of Apiezon M grease in samples from chromatographic columns did not interfere with this determination. All fractions of column chromatography were evaporated to dryness prior this determination.

Assay of biological activity

Effect of individual fractions on the incorporation of labelled algal protein hydrolysate into s-RNA* of rat liver *in vitro* was adopted as a standard procedure for this purpose. For this test, cholesterol esters were dissolved in polyethylene

* s-RNA = soluble or transfer RNA.

glycol, mol.wt. 600 as described elsewhere[11]. Isolation of rat liver pH 5 enzymes, composition of incubation mixtures, washing procedures, and radioactivity assay were also described earlier[11]. All fractions were tested at least in duplicate. Active fractions showed a stimulation corresponding to 130–150% of the energy-dependent incorporation in the control samples for dilutions corresponding to a concentration of 0.1 μg of cholesterol per ml[1]. Pooled fractions were tested in doses 0.1 mg–0.001 μg of cholesterol per incubation mixture.

RESULTS

Starting material and extraction

Egg-yolks which served as the starting material for the isolation of Carcinolipin in our original studies[3] were not suitable for the large-scale isolation of cholesterol esters. This material contains a high quantity of lipids and, in particular, some pigments which seriously interfere with the separation of cholesterol esters. Therefore liver tissue from various sources was investigated quantitatively for the presence of these substances in order to find the most suitable material for the isolation.

As seen in Table II, rat liver tissue contains the highest quantity of cholesterol esters in all tissues tested. However, since this material is not readily available in quantities sufficient for large-scale preparations, beef liver was chosen for this purpose.

TABLE II

CONTENTS OF CHOLESTEROL ESTERS IN THE LIVER TISSUE OF VARIOUS ANIMALS

Lipids were extracted from freeze-dried liver tissue by petroleum ether in a Soxhlet extractor. Cholesterols esters were separated by thin-layer chromatography of the extract and determined after elution quantitatively as described in the section Materials and methods. All values are milligrams of cholesterol esters per gram of freeze-dried tissue.

Source of liver	*Cholesterol esters*
Rat	84.2
Beef	77.5
Pig	21.3

Freeze-dried beef liver was extracted with various solvents to find the most suitable one. Cyclohexane extracted the highest quantity of cholesterol esters from this material (see Table III). However, since this solvent is relatively expensive, petroleum ether which also gave good yields of cholesterol esters was chosen instead.

Separation of cholesterol esters by liquid–solid chromatography

In preliminary experiments total cholesterol esters of beef liver were eluted by 2% ether in petroleum ether from silicic acid columns. This preparation had a stimulating effect on the amino acid incorporation in the subcellular system used. GLC of acids present in this fraction showed that it is composed of several higher fatty acid as seen in Fig. 1. Methyl palmitate was the most prominent peak visible in these chromatograms, all other saturated fatty acids, even- and odd-numbered, were also present, however. Rather high quantities of unsaturated fatty acids, in

TABLE III

QUANTITY OF CHOLESTEROL ESTERS EXTRACTED FROM FREEZE-DRIED RAT LIVER TISSUE BY DIFFERENT SOLVENTS

Cholesterol esters were extracted and determined as described in Table II. All values are milligrams of cholesterol esters per gram of freeze-dried rat liver.

Solvent	*Cholesterol esters*
Petroleum ether	84.2
Chloroform	43.7
Diethyl ether	66.5
Isooctane	25.3
Toluene	78.0
Cyclohexane	92.0

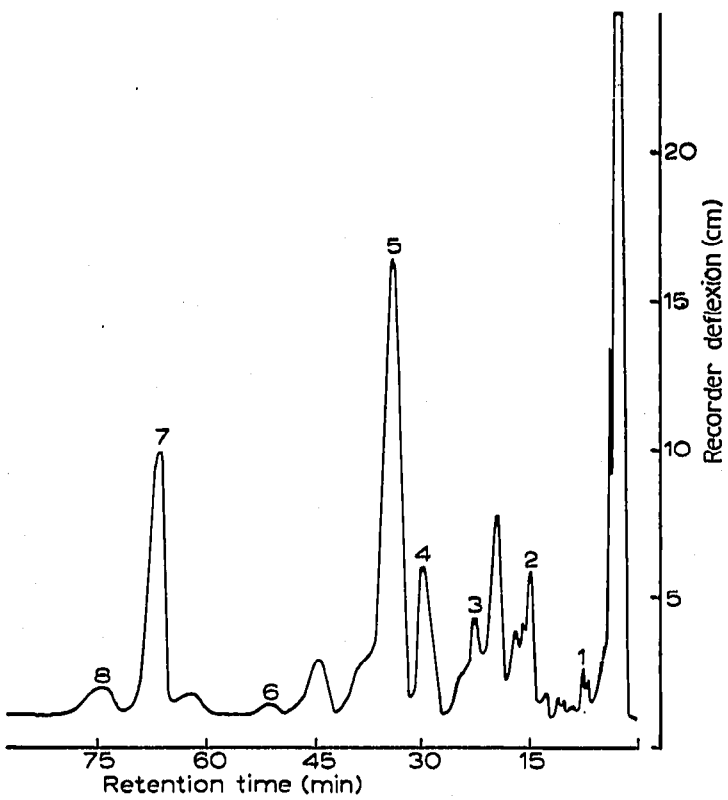

Fig. 1. GLC pattern of fatty acids present in cholesterol esters of beef liver. Petroleum ether extract of 1 kg freeze-dried liver tissue was separated on a column (diameter 40 mm, length 1 m) of activated silicic acid and cholesterol esters eluted by a 2% solution of diethyl ether in petroleum ether. Esters were hydrolysed and subjected to GLC as described in the section Materials and methods. Fatty acid methyl esters of individual peaks are the following: 1 = laurate; 2 = myristate; 3 = pentadecanoate; 4 = palmitoleate; 5 = palmitate; 6 = margarate; 7 = oleate; 8 = stearate.

particular oleate and palmitoleate, were present, too. However, only traces of polyunsaturated fatty acids were found. Most of them were probably destroyed during the isolation procedure.

Elution of cholesterol esters from silicic acid columns with carbon tetrachloride instead of petroleum ether resulted in the separation of esters with saturated fatty

acids from cholesterol oleate and palmitoleate. No definite peaks, however, could be demonstrated if the cholesterol contents in individual fractions were plotted against the effluent volume. Instead, more or less the same quantity of cholesterol esters was found in most of the fractions examined. The typical course of such a chromatogram is given in Table IV. Although some overlapping of cholesterol esters of saturated fatty acids with cholesterol oleate occured, most initial fractions were not contaminated with this latter ester. GLC revealed that some separation may be achieved even within the group of saturated acid esters. The first fractions contained relatively more of the lower acids, in particular cholesterol myristate, while in terminal fractions relatively more stearate was found. Cholesterol palmitate, however, represented the major portion of each fraction of cholesterol esters with saturated fatty acids. Protein synthesis-stimulating acitivity was also present in most fractions of saturated acid esters except for a few initial fractions. However, fractions containing cholesterol oleate and palmitoleate were free of this activity. In addition, pooled fractions of saturated esters whose GLC composition is given in Fig. 2 were active. It may be noted that only traces of unsaturated acids, oleic and palmitoleic, were present when compared with the composition of total cholesterol esters from beef liver (Fig. 1). Also most of the unsaturated acids in the C_{13}–C_{15} region were removed by this procedure. Yields of the saturated ester fraction were usually 0.5–0.8 g/kg of freeze-dried liver tissue.

TABLE IV

ELUTION PATTERN OF CHOLESTEROL ESTERS FROM A SILICIC ACID COLUMN ELUTED WITH CARBON TETRACHLORIDE

Petroleum ether extract of 1 kg of freeze-dried beef liver was separated on a column of activated silicic acid (diameter 40 mm, length 1 m) and cholesterol esters eluted with carbon tetrachloride. Fractions of 12 ml each were collected. The cholesterol content was determined in every fifth fraction. Qualitative assay by paper chromatography (silicic acid-impregnated paper developed with carbon tetrachloride–*n*-heptane) was performed on every fifth fraction, GLC of fatty acids in every twentieth fraction. For assay of the protein synthesis-stimulating activity content, every tenth tube was diluted to give a cholesterol concentration of 0.01 μg/0.1 ml of polyethylene glycol, mol.wt. 600. The activity was tested in the cell-free system of rat liver as described in the section Materials and methods. The total yield of saturated fatty acid esters (fraction 216–510) was 0.56 g.

Fraction No.	*Fatty acid composition*		*Activity*
	Paper chromatography (R_{CP})	*GLC* (*carbon number*)	
1–215	—	—	—
216–240	1.00	12.0, 13.0, 14.0, 15.0, 16.0, 14.7	No
240–510	1.00	12.0, 13.0, 14.0, 15.0, 16.0, 18.0, 14.7, 16.7	Yes
511–585	0.77, 1.00	14.0, 15.0, 16.0, 18.0, 14.7, 16.7, 17.6	Yes
586–1380	0.77	17.6	No

Separation of cholesterol esters by liquid–liquid chromatography

Esters of cholesterol with saturated fatty acids, isolated by the method described above, were subjected to further fractionation using partition chromatography. A 40% solution of acetone in methanol was used as mobile phase. The elution pattern of cholesterol esters from such an experiment is given in Fig. 3.

This procedure gave a fairly good separation of individual esters, however,

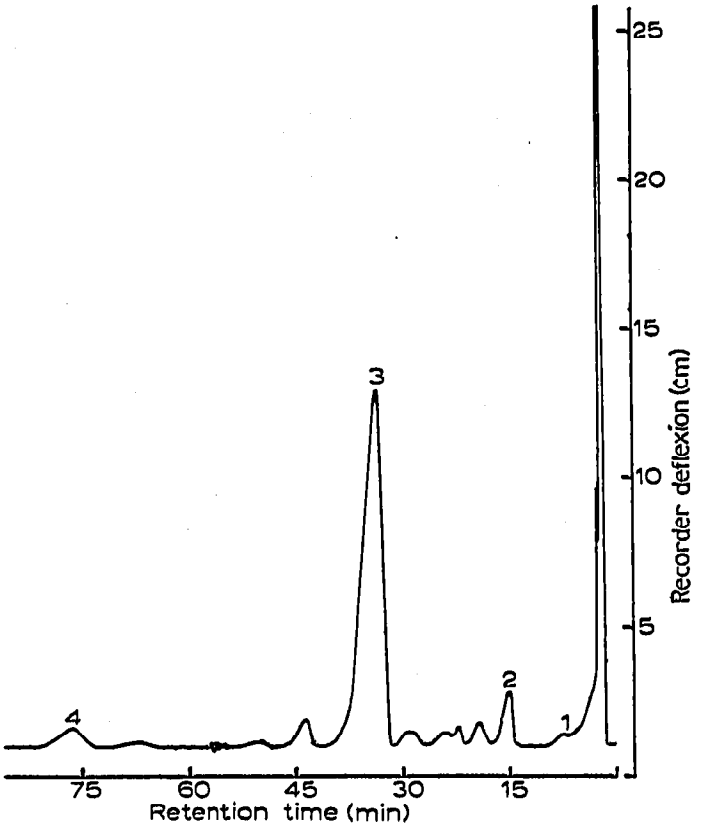

Fig. 2. GLC pattern of fatty acids present in the fraction of cholesterol esters with saturated fatty acids. This fraction was separated as described in Table IV, esters hydrolysed and subjected to GLC as given in the section Materials and methods. The composition of individual peaks is as follows: 1 = laurate; 2 = myristate; 3 = palmitate; 4 = stearate.

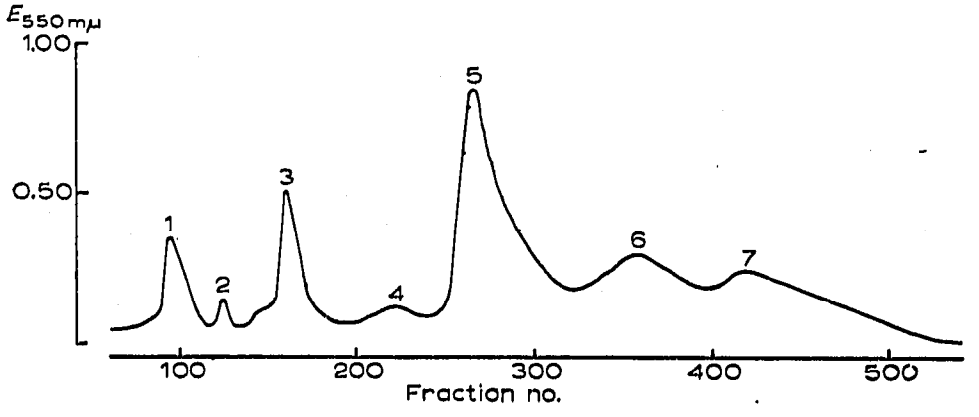

Fig. 3. Separation of cholesterol esters with saturated fatty acids by liquid–liquid chromatography. 200 mg of the saturated ester fraction isolated as described in Table IV was separated on a column (diameter 40 mm, length 2 m) of 10% Apiezon M grease on Celite 545 and eluted with a 40% solution of acetone in methanol. Details of this procedure are described in the section Materials and methods. The carbon numbers of predominant fatty acids in individual peaks are as follows: 1 = 12.0; 2 = 13.0; 3 = 14.0; 4 = 17.6; 5 = 16.0; 6 = 16.7; 7 = 18.0.

considerable tailing occurred particularly in the last fractions. Stimulating activity on protein synthesis was found to be associated with fractions from the three last peaks. They contained, according to GLC, fatty acids of carbon number 16.0, 16.7, and 18.0 as the main components. Smaller quantities of acids with carbon numbers 14.0, 15.0, and 17.0 were also present.

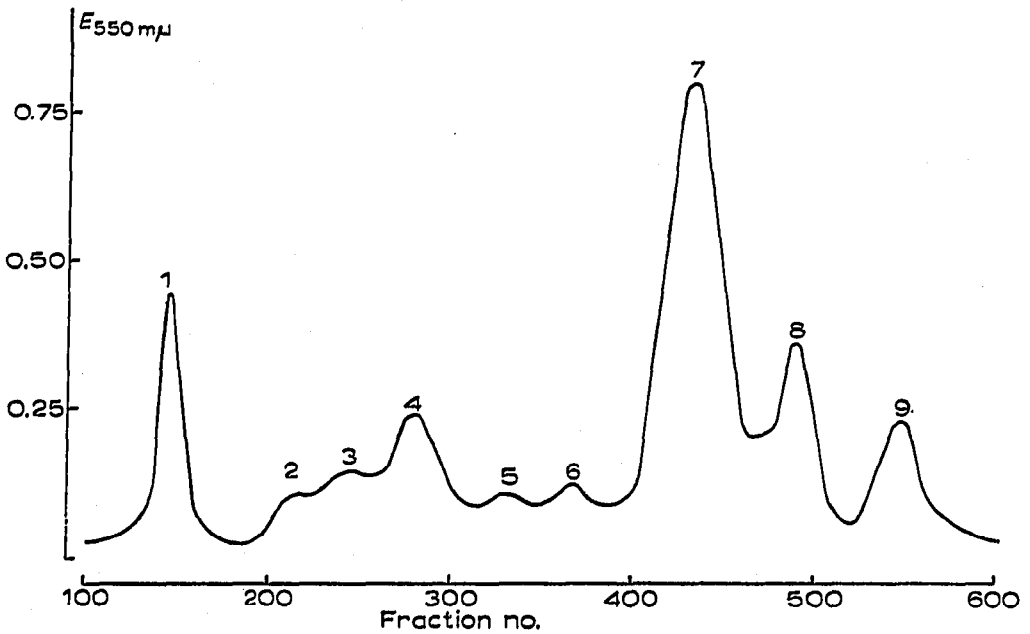

Fig. 4. Separation of cholesterol esters with saturated fatty acids by liquid–liquid chromatography using gradient elution. The starting material and column were the same as described in the legend for Fig. 3. Elution was started with 2000 ml of a 20% solution of acetone in methanol and acetone was gradually added as described in the section Materials and methods. The composition of fractions and their protein synthesis-stimulating activity is given in Table V.

TABLE V

FATTY ACID COMPOSITION AND PROTEIN SYNTHESIS-STIMULATING ACTIVITY OF FRACTIONS FROM THE LIQUID–LIQUID CHROMATOGRAM GIVEN IN FIG. 4

Conditions used for chromatography are indicated in the legend for Fig. 3. Paper chromatography was done on paraffin oil-impregnated papers developed by an ascending gradient technique with methanol–water in acetone as described in the section Materials and methods. All mobilities are relative to cholesterol palmitate. In GLC results, the carbon number of the predominant fatty acid present is given first. Fatty acids present only in traces are not given. Fractions of individual peaks were pooled as indicated.

Peak No.	*Fraction No.*	*Fatty acid composition by*		*Activity*
		Paper chromatography (R_{CP})	*GLC* (*carbon number*)	
1	130–170	—	12.0, 12	—
2	200–220	—	13.6, 12.0, 13.0	—
3	230–260	1.40	13.0, 12.0, 13.6, 14.0,	—
4	270–300	1.32	14.0, 13.0, 13.6, 14.7, 15.6	—
5	315–345	1.25	14.7, 14.0, 15.0, 15.6	—
6	350–385	1.17	17.6, 14.0, 14.7, 15.0, 15.6	—
7	410–470	1.00	16.0, 14.0, 14.7, 15.0, 16.7	450–470 +
8	475–520	0.88	16.7, 16.0, 17.0, 18.0	+
9	525–600	0.65	18.0, 16.0, 16.7, 17.0	525–540 +

Gradient elution of cholesterol esters was tested in the next experiment to reduce tailing and to obtain a better separation. The elution pattern of such a column is given in Fig. 4, where experimental conditions are also indicated. The fatty acid composition of individual peaks is seen in Table V.

Far better resolution of individual cholesterol esters was achieved on using the gradient-elution technique than by the use of mobile phases with constant composition. The protein synthesis-stimulating acitivity was associated only with fractions contained in the peak No. 8 with some small overlap into the neighboring peaks, No. 7 and 9. These fractions contained the fatty acid with carbon number 16.7 as the main component but also significant quantities of cholesterol palmitate and stearate as well as traces of cholesterol myristate and margarate as seen in Fig. 5.

Yields of this active fraction (after separation from impurities of Apiezon M grease as described in Materials and methods) were usually 10–15 mg from a column on which material corresponding to 250–500 g of freeze-dried beef liver tissue had been separated.

These results indicated that ester of cholesterol with fatty acid of carbon number 16.7 may be responsible for the biological activity, since both cholesterol palmitate and stearate present as main components in the neighboring peaks were free of protein synthesis-stimulating activity. However, attempts to purify this cholesterol ester were not successful. Even repeated chromatography of the active fractions in the system described was not able to remove traces of cholesterol pal-

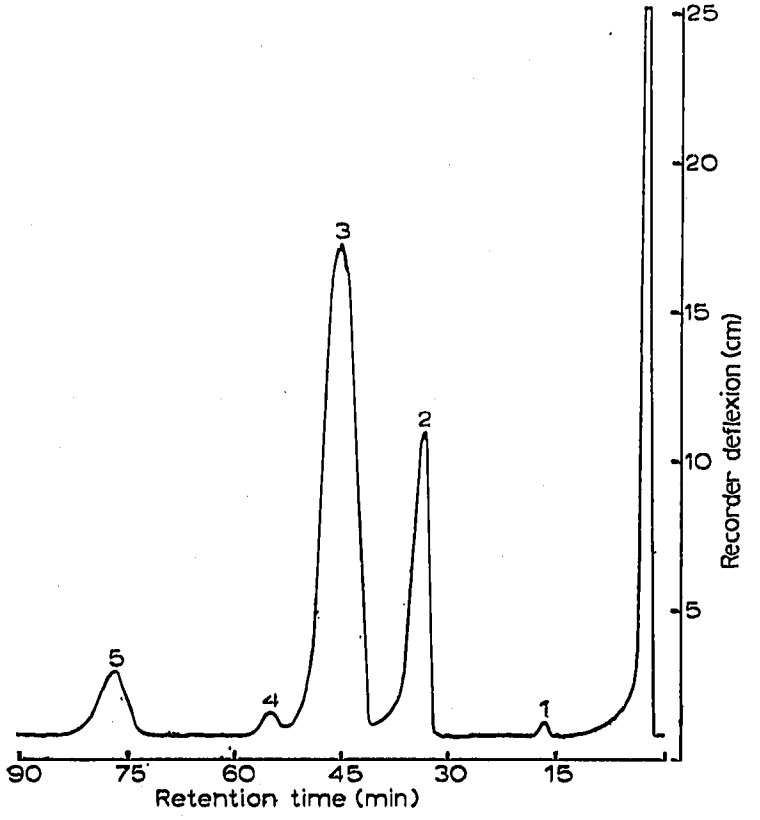

Fig. 5. GLC pattern of fatty acids present in fraction No. 8 (see Fig. 4 and Table V) separated by liquid–liquid chromatography which showed a protein synthesis-stimulating activity. The carbon numbers of individual components are as follows: 1 = 14.0; 2 = 16.0; 3 = 16.7; 4 = 17.0; 5 = 18.0.

mitate in order to obtain the active ester in a pure form which is necessary for the elucidation of the chemical constitution of the fatty acid present.

In addition, attempts to purify the active fraction by crystallisation were not successful. Although crystalline material could be readily obtained from methanol–ether mixtures, the melting point remained unchanged at 77–78°, and the original fatty acid composition of this material remained unaltered even after seven recrystallisations.

DISCUSSION

Saponification of the starting material was used in our original method for the isolation of Carcinolipin[3]. The majority of sterol esters were undoubtedly hydrolysed by this treatment and this could explain the higher yields of active fractions obtained in the present experiments.

No completely satisfactory method so far exists for the preparative separation of individual cholesterol esters. However, several analytical procedures have been described, giving a good resolution of these compounds, which could be used as a basis for the development of preparative techniques described in this paper.

Since some cholesterol esters are known to form "critical pairs" in partition chromatography[12], a preliminary separation of esters with saturated fatty acids from those with unsaturated fatty acids seemed desirable. ZÖLLNER AND WOLFRAM[13] demonstrated that good resolution of both these classes may be obtained on thin layers of silicic acid using carbon tetrachloride as eluting agent. This method has been adapted successfully to a preparative procedure in our experiments. However, the original method was not suitable as a routine test for the composition of fractions from preparative columns because it takes 2–3 days to obtain good resolution. For this purpose our method using silicic acid-impregnated paper was found more advantageous. This method is similar to that used by MULDREY *et al.*[14].

Partition chromatography seemed to be the method of choice for the separation of individual esters with saturated fatty acids. Even GLC does not give a completely satisfactory resolution of these compounds[15]. Some attempts were made in our laboratory to fractionate these substances by gel filtration on Sephadex LH-20 using chloroform or chloroform–methanol mixtures for elution. However, cholesterol esters were eluted as a single peak with no resolution of individual substances. Probably the relatively high molecular weight of these compounds and slight differences in this respect between members of the homologous series were responsible for this failure.

KAUFMAN *et al.*[16] obtained an excellent resolution of the individual cholesterol esters by a two-dimensional combination of adsorption and reversed-phase partition chromatography on thin layers of silicic acid. However, such a method could hardly be adapted to a preparative procedure.

MICHALEC reported a satisfactory resolution of cholesterol esters with some saturated fatty acids by both paper[17] and thin-layer chromatography[18] using paraffin oil as the stationary phase and acetic acid–chloroform mixtures as the mobile phase. Experiments with columns of paraffin oil-impregnated Whatman cellulose powder or Celite and similar solvent mixtures did not yield satisfactory separations in our hands, however. Replacement of paraffin oil with silicone oils did not improve the

resolution significantly. Apiezon M grease, a stationary phase frequently used for GLC, showed the best selectivity for cholesterol esters of all the phases tested. Impregnation of Celite with less than 10% Apiezon M gave worse separations and considerable tailing, while concentrations over 10% yielded much longer elution times with no better resolution.

The favourable effect of impregnation with Apiezon M was further increased by the use of acetone–methanol mixtures for elution. Although cholesterol esters are not very soluble in either of these solvents, rather high differences in solubility exist for individual esters in these mixtures[19]. These solvents were also very suitable for gradient elution paper chromatography which gave a good resolution not only of esters with odd-numbered fatty acids but also with intermediate compounds, as for instance the cholesterol ester of an acid with carbon number 16.7. For these reasons this method was more suitable for rapid checking of the composition of column chromatography fractions than that of MICHALEC AND STRAŠEK[17]. The considerable tailing when using this procedure frequently made it impossible to obtain complete resolution of two neighbouring spots.

In our previous experiments[1], no increase in specific activity of s-RNA resulted from the addition of Carcinolipin into incubation mixtures containing some labelled amino acids. A more rapid labelling of s-RNA was only induced by the presence of this substance. In the present experiments, however, active fractions enhanced the labelling of s-RNA with algal protein hydrolysate. Different results may be obviously obtained in this respect when different amino acids are used.

Results of our present experiments show clearly that Carcinolipin belongs to the group of sterol (most probably cholesterol) esters with higher saturated fatty acids. A more exact definition of this acid is not possible as yet although the carbon number of the acid present in active fractions indicates that a branched-chain fatty acid might be involved. Our results also indicate that it was not possible to isolate the active substance in a pure form by the present chromatographic techniques. The rather high molecular weight of these compounds and, in particular, very small chemical differences between individual cholesterol esters are not in favour of using such procedures. Subsequent attempts have therefore been made to purify the fatty acid alone, present in the active ester. Results of these experiments will be reported elsewhere.

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SUMMARY

The low polarity of Carcinolipin and its positive reaction to colour tests for sterols indicated that this substance might be a sterol ester. Cholesterol esters separated from beef liver lipids by silicic acid chromatography showed a stimulating effect on the incorporation of labelled algal protein hydrolysate into soluble RNA of rat liver *in vitro*. A group of cholesterol esters with saturated fatty acids was sepa-

rated from cholesterol oleate and palmitoleate by elution with carbon tetrachloride from silicic acid columns. Only the saturated fraction had a protein synthesis-stimulating effect. A reversed-phase liquid–liquid chromatographic technique was developed for the fractionation of individual esters with saturated fatty acids. 10% Apiezon M grease on Celite 545 was used as stationary phase and the esters were eluted with acetone in methanol by an ascending technique. Only one fraction, containing a fatty acid with carbon number 16.7 as the main component, was active. It seems that Carcinolipin is an ester of cholesterol with a branched-chain fatty acid.

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