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The Chemical Constitution of Carcinolipin

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1. The neutral portion of the molecule of carcinolipin was found to be cholesterol by comparison of mixed melting points with cholesterol, its dibromide and its acetate. 2. The fatty acid present in carcinolipin was subjected to oxidative degradation by chromic acid and permanganate. Butan-2-one was the main neutral degradation product resulting from both these procedures. A mixture of dibasic acids was obtained after the oxidation with chromic acid. Permanganate oxidation yielded a complete homologous series of branched-chain C_5–C_{17} fatty acids. 3. The mass spectrum of the acid was characteristic for a saturated C_{17} acid. The alcohol prepared by lithium aluminium hydride reduction of the original acid showed a mass spectrum typical for an anteiso compound. 4. Comparison of mixed melting points, gas–liquid-chromatographic behaviour and mass spectra of the fatty acid isolated from carcinolipin with an authentic sample of 14-methylhexadecanoic acid demonstrated the identity of these compounds. Cholesterol esters synthesized from authentic cholesterol and the fatty acid isolated from carcinolipin or synthetic 14-methylhexadecanoic acid showed an identical stimulating effect on the incorporation of labelled algal-protein hydrolysate into rat liver transfer RNA *in vitro*. 5. Mass spectra, results of oxidative degradations and comparisons with an authentic sample, as well as biological activity of the synthetic cholesterol 14-methylhexadecanoate, provided good evidence that carcinolipin is cholesterol (+)-14-methylhexadecanoate.

Carcinolipin, the carcinogenic lipid (Hradec & Kruml, 1960) that stimulates protein synthesis (Hradec, 1961), is a natural constituent of biological materials (Hradec & Štroufová, 1960). Improved procedures for its isolation have been devised and evidence has been presented that this substance is probably a sterol ester (Hradec & Sommerau, 1968). Some properties of the fatty acid present in this ester indicated that it might be a branched-chain fatty acid (Hradec & Menšík, 1968).

The present paper describes experiments that were performed to elucidate the exact chemical structure of carcinolipin and that gave the final evidence that this compound is cholesterol (+)-14-methylhexadecanoate.

MATERIALS AND METHODS

Materials. Carcinolipin was isolated from a freeze-dried ox liver homogenate. This material was extracted with light petroleum (b.p. 35–50°) and esters of cholesterol with saturated fatty acids were separated on silicic acid columns by using CCl_4 for elution. Further fractionation of these esters was performed on columns of Celite impregnated with 10% Apiezon M grease. The columns were eluted with an ascending gradient of $CHCl_3$ in methanol. Fractions exhibiting a stimulating effect on the incorporation of labelled algal-protein hydrolysate into transfer RNA of rat liver were pooled and rechromatographed in the same way. Carcinolipin obtained by these procedures was a white crystalline substance, m.p. 75°, containing traces of cholesterol palmitate and stearate. Methods used for the isolation of carcinolipin have been described by Hradec & Sommerau (1968). The acid present in carcinolipin was isolated from the fraction of esters of cholesterol with saturated fatty acids separated from ox liver lipids, by silicic acid chromatography as described above. Cholesterol esters were hydrolysed with boiling ethanolic KOH, and fatty acids were extracted from the hydrolysate with ether. The branched-chain fatty acid fraction was separated by chromatography on urea columns, and the acid present in carcinolipin was purified by subsequent preparative gas–liquid chromatography. The purity of the final preparation was about 98%. The isolation and purification of this fatty acid have been described in detail by Hradec & Menšík (1968). It was a white crystalline substance, m.p. 39·5–40·0°, $[\alpha]_D^{20}$ +3·3°, carbon number 16·7 on both polar and non-polar phases in gas–liquid chromatography. A sample

of synthetic 14-methylhexadecanoic acid was kindly supplied by Professor J. Cason, Department of Chemistry, University of California, Berkeley, Calif., U.S.A. Cholesterol was a commercial preparation purified as the dibromide (Schoenheimer, 1935).

Oxidative degradation of the acid. Oxidation with chromic acid was done as described by Cason, Fessenden & Agre (1959) with minor modifications. The reaction mixture was made alkaline and shaken three times with equal volumes of ether. Extracts were pooled and dried over anhydrous Na_2SO_4 overnight, and the ether was distilled off through a 30cm.-long column. The original reaction mixture was acidified and extracted with ether in the same way. The combined extracts were dried over Na_2SO_4 overnight and evaporated to dryness in a vacuum rotary evaporator. The acids were esterified with diazomethane as described by Hradec & Sommerau (1968). For permanganate oxidation the procedure of Murray (1959) was used.

Synthesis of cholesterol esters. This was done as described by Hradec & Menšík (1968).

Gas–liquid chromatography. A Chrom II Gas Chromatograph (Laboratorní přístroje, Prague, Czechoslovakia) was used for all gas–liquid-chromatographic analyses. Since this instrument had no facilities for temperature programming it was necessary to separate fatty acids in two steps. Lower fatty acids (up to C_{11}) were separated by using columns 0·6cm. × 240cm. filled with Chromosorb W (100–120 mesh) coated with 20% polyethylene glycol adipate. The column temperature was 150°, the carrier gas N_2, the inlet pressure 0·70atm., the H_2 flow rate 30ml./min., the air flow rate 600ml./min. and the sensitivity 1:200. The conditions used for the separation of higher fatty acids on capillary columns were as described by Hradec & Sommerau (1968). Dibasic acids were analysed on the same columns as lower fatty acids at column temperature 180°, N_2 inlet pressure 2·0atm., H_2 flow rate 25ml./min., air flow rate 600ml./min. and sensitivity 1:500. A mixture of the methyl esters of adipic acid, azelaic acid and sebacic acid was used for the calibration of the instrument. For the separation of ketones the same polyethylene glycol adipate columns were used at 90°, with N_2 inlet pressure 0·55atm., H_2 flow rate 25ml./min., air flow rate 600ml./min. and sensitivity 1:100. A mixture of acetone, butan-2-one and hexan-2-one (kindly prepared by Dr J. Sommerau) was used for the calibration of retention times.

Mass spectrometry. The mass spectra were measured with a MCH 1303 mass spectrometer (U.S.S.R.) by using a direct inlet system.

Assay of biological activity. These procedures were as described in detail elsewhere (Hradec, 1967; Hradec & Sommerau, 1968).

RESULTS

Identification of cholesterol. A white glossy crystalline substance, m.p. 149–150°, was obtained after crystallization from ethanol of residues from a neutral extract of carcinolipin hydrolysate. Its m.p. remained unchanged by admixture with an authentic sample of purified cholesterol. The dibromide of this compound had m.p. 124° and the acetate had m.p. 114°. Melting points of both these derivatives were unchanged by admixture with the same derivatives of authentic cholesterol.

The evidence that cholesterol forms the neutral part of the molecule of carcinolipin was further supported by the fact that esters prepared synthetically from some fatty acids and authentic cholesterol stimulated protein synthesis (Hradec & Menšík, 1968).

Oxidative degradation of the fatty acid. Oxidation of the acid with permanganate resulted in the formation of a complete homologous series of branched-chain fatty acids from carbon number 5·7 up to 15·7 (including a large quantity of the original unchanged acid of carbon number 16·7). Since methyl esters of branched-chain fatty acids have shorter retention times in gas–liquid chromatography than the corresponding straight-chain homologues, their carbon numbers are slightly smaller than whole numbers characteristic for straight-chain acids (Abrahamsson, Ställberg-Stenhagen & Stenhagen, 1963). Minute quantities of n-C_{16} and n-C_{14} acids were also present in the reaction mixture, apparently representing impurities contained in the original preparation. These results are shown in Figs. 1 and 2.

Butan-2-one was the main cleavage product

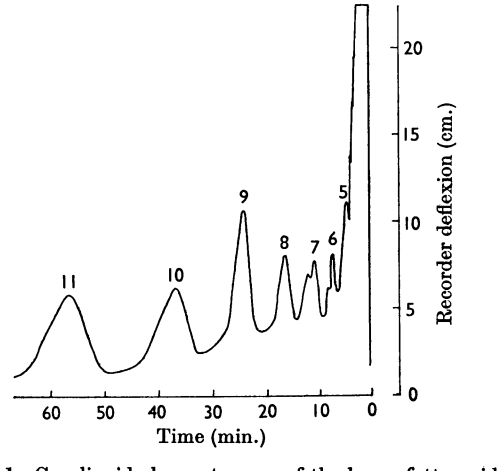

Fig. 1. Gas–liquid chromatogram of the lower fatty acids resulting from the permanganate oxidation of the fatty acid present in carcinolipin. The numbers of individual peaks indicate the numbers of carbon atoms in branched-chain fatty acids present. The fatty acid of carcinolipin (150mg.) was refluxed in 50ml. of acetone containing 1·5g. of potassium permanganate for 40hr. Neutral and acid portions of the reaction mixture were separated and prepared for gas–liquid chromatography as described by Murray (1959). Gas–liquid chromatography of fatty acid methyl esters was performed as described in the Materials and Methods section.

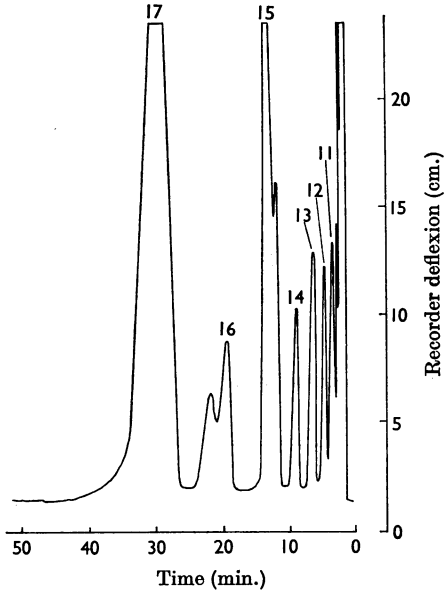

Fig. 2. Gas–liquid chromatogram of the higher fatty acids produced by the permanganate oxidation of the fatty acid present in carcinolipin. The numbers of individual peaks indicate the number of carbon atoms in single branched-chain fatty acids. The conditions of oxidation were as described for Fig. 1.

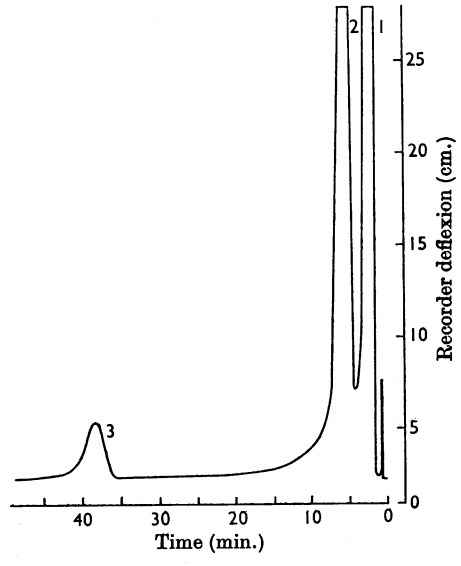

Fig. 3. Gas–liquid chromatogram of the neutral fraction of the reaction mixture resulting from the oxidation of the fatty acid present in carcinolipin with permanganate. Composition of individual peaks: 1, acetone; 2, butan-2-one; 3, unidentified. The conditions of oxidation were as described for Fig. 1. Acetone was used as the solvent (Murray, 1959).

present in the neutral fraction. In addition, another minor component was found in the ketone fraction. It could not be identified exactly, but it was apparently not a ketone. A chromatogram of the neutral fraction is given in Fig. 3.

A homologous series of higher dibasic acids was produced by the treatment of the fatty acid with chromic acid. In addition, the original fatty acid of carcinolipin was also present, together with *n*-hexadecanoic and an unidentified compound, both of these last-named substances apparently representing some impurities present in the original acid. However, no dominant degradation product could be identified, since the quantitative distribution of individual dibasic acid methyl esters was rather uniform, as shown in Fig. 4.

Butan-2-one was again the main product present in the neutral fraction. In addition, smaller

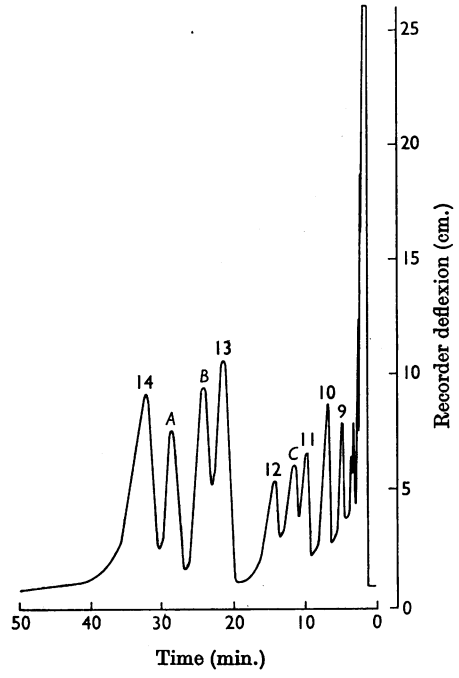

Fig. 4. Gas–liquid chromatogram of methyl esters of dibasic acids resulting from the oxidation with chromic acid of the fatty acid present in carcinolipin. The numbers of individual peaks correspond to numbers of carbon atoms in single dibasic acids; other peaks are lettered: *A*, original fatty acid of carcinolipin; *B*, hexadecanoic acid; *C*, unidentified. The fatty acid of carcinolipin (150mg.) was heated in 15ml. of acetic acid with 500mg. of chromic acid at 68° for 3hr. (Cason *et al.* 1959). Neutral and acid fractions of the reaction mixture were separated and prepared for gas–liquid chromatography as described in the Materials and Methods section.

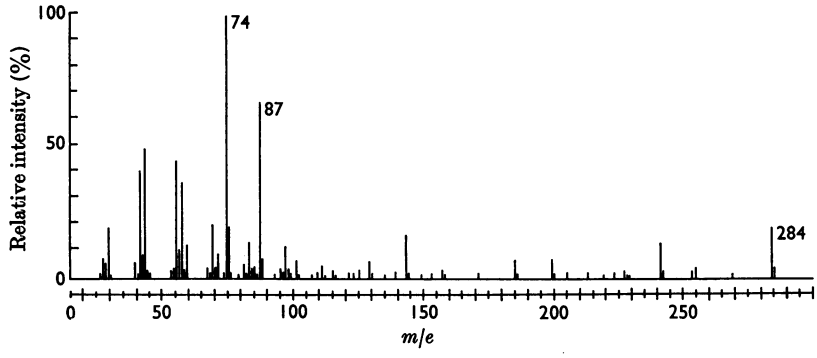

Fig. 5. Mass spectrum of the fatty acid present in carcinolipin.

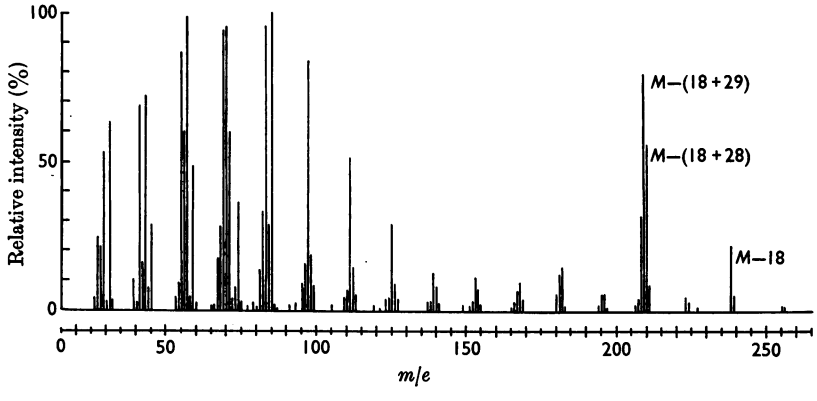

Fig. 6. Mass spectrum of the alcohol prepared by $LiAlH_4$ reduction of the fatty acid of carcinolipin.

quantities of acetone were also identified in the ketone fraction.

Mass spectra of the acid. The mass spectrum of the fatty acid methyl ester was analogous to the spectra of long-chain aliphatic esters (see Fig. 5). The molecular ion at *m/e* 284 indicated the composition $C_{18}H_{36}O_2$ and characterized the compound as an ester of a saturated C_{17} acid. The abundant ions at *m/e* 74 and 87 suggested the presence of unsubstituted methylene groups in α- and β-positions to the methoxycarbonyl group (Budzikiewicz, Djerassi & Williams, 1964; Ryhage & Stenhagen, 1963).

A shorter retention time of the ester than that of methyl margarate in gas–liquid chromatography, as well as the optical activity of the free acid, excluded the possibility of a straight-chain fatty acid. Since the mass spectrum of the methyl ester gave no satisfactory information about the branching, the ester was converted by lithium aluminium hydride reduction into the alcohol.

In the mass spectrum of the alcohol (see Fig. 6), the molecular and $(M-1)$ ions were just discernible at *m/e* 256 and 255 respectively. The fragmentation pattern corresponded to that of anteiso alcohols. The peaks at *m/e* 238 and 210 belong to the fragments $(M-\text{water})$ and $[M-(\text{water}+\text{ethylene})]$ respectively. The most prominent peak of the high mass range of the spectrum at *m/e* 209 belongs to the ion that arises from the ionized molecule by subsequent loss of water and an ethyl side chain.

Comparison with an authentic sample. No depression of melting point was found when the acid was admixed with the synthetic sample of 14-methylhexadecanoic acid. Also, the gas–liquid-chromatographic pattern and mass spectra of both these substances were identical. Cholesterol esters synthesized from both the natural and the synthetic fatty acid showed a closely similar stimulating effect on the incorporation of labelled protein hydrolysate into soluble RNA of rat liver *in vitro*,

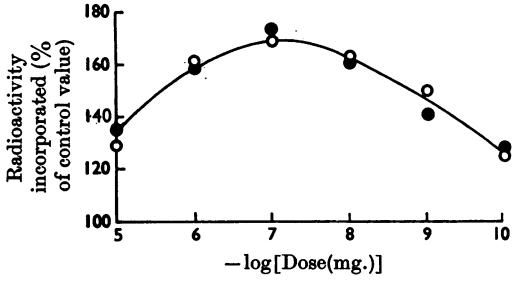

Fig. 7. Effect of cholesterol esters prepared from the natural fatty acid (○) and the synthetic 14-methylhexadecanoic acid (●) and authentic cholesterol on the incorporation of labelled amino acids into soluble RNA *in vitro*. Suspensions of both cholesterol esters in polyethylene glycol (mol.wt. 600) were added to incubation mixtures containing rat liver pH 5 enzymes, ATP and labelled algal-protein hydrolysate. Mixtures were incubated, transfer RNA was isolated and its radioactivity was determined as described by Hradec (1967).

as shown in Fig. 7. This activity was the same as that found with the natural carcinolipin isolated from ox liver by combined liquid–solid and liquid–liquid chromatography as described by Hradec & Sommerau (1968).

All the results reported here thus provide good evidence that carcinolipin is cholesterol (+)-14-methylhexadecanoate.

DISCUSSION

Oxidative degradation of branched-chain fatty acids has so far been used mainly for the confirmation of known structures in the experiments of Cason *et al.* (1959) and Murray (1959). If an unknown substance is being investigated some severe limitations of such methods appear, as shown by our results. No dominant degradation product could be demonstrated in the acid fraction after chromate oxidation. Apparently secondary degradation made the gas–liquid-chromatographic pattern too complicated to allow a conclusion on the type and position of the chain branching. Permanganate oxidation yielded a homologous series of acids with carbon numbers characteristic for branched-chain acids (Horning, Karmen & Sweeley, 1964). However, the possible presence of more than one branched alkyl group could not be excluded, since insufficient information is available on the gas–liquid-chromatographic behaviour of multibranched fatty acids (Abrahamsson *et al.* 1963). The presence of butan-2-one in neutral portions obtained by both oxidative procedures provided good evidence for the presence of a branched methyl group in the anteiso position. However, the yield of ketones after oxidative degradation was very low, and secondary degradations occurred (Cason *et al.* 1959). A higher ketone resulting from the splitting of another chain branch could thus easily have been missed. Although the melting point of the acid was identical with that reported for 14-methylhexadecanoic acid (Weitzel & Wojahn, 1951) the evidence was still far from being conclusive. Only the mass-spectrometric examination gave the ultimate proof of the supposed structure. Examination of both the acid and its alcohol seemed advisable, since the mass spectrum of this latter compound is more characteristic for the anteiso structure than that of the original acid.

At the present time it is difficult to find relations between the chemical structure of carcinolipin settled by experiments reported here and the biological activity of this substance. The cholesterol moiety of carcinolipin could have some relation to the carcinogenic activity of this compound (Hradec & Kruml, 1960). Cholesterol and some of its derivatives were reported to be carcinogenic although there is disagreement about this (for review see Bischoff, 1963).

It has been claimed that lipids are involved in protein synthesis (Hendler, 1961). No such activity has so far been reported for cholesterol esters, however. The presence of a branched-chain fatty acid in the molecule of carcinolipin seems to be essential for its activity in stimulating protein synthesis, since esters of cholesterol with straight-chain fatty acids of comparable chain length are inactive in this respect (Hradec & Sommerau, 1968).

Relatively little is known about the biological significance of branched-chain fatty acids (Lederer, 1964). Although these substances are repeatedly reported to represent minor components of animal fats, their presence in cholesterol esters has not previously been established. In sheep fat such acids represent most of the fatty acids present (Weitkamp, 1945). Hansen, Shorland & Cooke (1952) isolated 14-methylhexadecanoic acid from animal fats. Relatively large quantities of this acid were found in *Bacillus subtilis*, where together with 12-methyltetradecanoic acid it makes up 60% of the total fatty acids present (Kaneda, 1963). Serdarevich & Carrol (1966) isolated the anteiso heptadecanoic acid from lipids of *Listeria monocytogenes*. Grimmer & Jacob (1965) obtained some evidence that it is also present in human blood.

All these reports indicate a rather wide occurrence of 14-methylhexadecanoic acid in biological materials. This fact, together with our results on the stimulating activity of carcinolipin in protein synthesis (Hradec, 1961; Hradec & Štroufová, 1960), seems to support our opinion that this substance may have a physiological role in the biosynthesis of proteins.

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