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DIOL LIPIDS

VII. Separation of Diol Lipids from Triglycerides by Gel Filtration and Countercurrent Separation

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Investigations carried out in our laboratory in the last few years have shown that lipids of various origins contain small amounts of diol analogs of neutral glycerides and glycerophosphatides [1]. Compounds of this type, representing new classes of lipids, have been synthesized [2-4], but it has not been possible to isolate them from natural sources (with the exception of diesters of butane-2,3-diol and ethylene glycol [5]). This is apparently due to the following facts: 1) the amount of diol components in the lipids is small, generally 0.5-2% of the total glycerolipids; 2) the method of adsorption chromatography most widely used for isolating neutral lipids does not permit the neutral diol lipids to be separated from triglycerides, since they form critical mixtures [2]; 3) although it has been shown by reversed-phase thin-layer chromatography that the R_f values of neutral diol lipids and of triglycerides may differ substantially [3], the separation of a mixture of these substances in which the triglycerides greatly predominate is a complex task in view of the fact that the triglycerides "trap" the minor amounts of diol lipids.

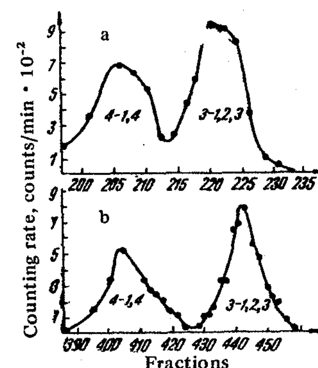

Fig. 1. Separation by countercurrent distribution of a mixture of glycerol tri[1-^{14}C]stearate and butane-1,4-diol di[1-^{14}C]-stearate in the PE-C-F (5 : 2 : 5) system in an apparatus with 121 cells: a) after 257 transfers; b) after 503 transfers; 4) butane-1,4-diol diester; 3) glycerol triester.

This communication is devoted to the development of methods permitting the lipid fractions to be enriched with the diol compounds or be separated completely into triglyceride and diol lipid fractions regardless of their ratio in the initial mixture. As is well known, triglycerides can be separated according to their degree of unsaturation by chromatography on adsorbents containing silver nitrate [6]. Other methods in use permit the separation of triglycerides both with respect to the number of carbon atoms and with respect to the degree of unsaturation. These include gas-liquid chromatography [7], partition chromatography on paper and in thin layers with reversed phases [3,8], and also column partition chromatography between two liquid phases [9,10]. Of the methods mentioned, at first sight the last is the most promising for work on the preparative scale. By this method using the acetonitrile-methanol-heptane system in a column with hydrophobic Celite it has been possible to separate triglycerides differing by a single methylene group or by one ethylenic bond [10]. However, this method is associated with the use of very large amounts of solvent. Thus,

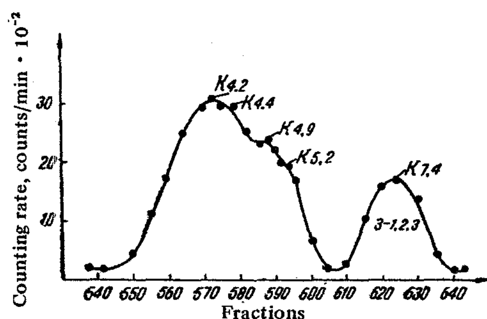

Fig. 2. Separation by countercurrent distribution of a mixture of glycerol tri[1-^{14}C]-stearate and the di[1-^{14}C]stearates of ethylene glycol, propane-1,3-diol, butane-2,3-diol, and butane-1,4-diol, in the H-C-F (5: 2 : 5) system in an apparatus with 103 cells (708 transfers were carried out; fractions 610-640 contain the tristerin).

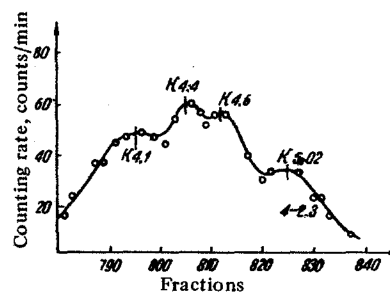

Fig. 3. Separation by countercurrent distribution of a mixture of the di[1-^{14}C]stearates of butane-1,4-diol, ethylene glycol, propane-1,3-diol, and butane-2,3-diol in the PE (bp 40-60° C)--C-F (5 : 2 : 5) system in an apparatus with 121 cells (total number of transfers 988; fractions 820-835, butane-2,3-diol distearate).

to separate 15 mg of a mixture of triglycerides on 150 g of adsorbent required about 32 *l* of a mixture of acetonitrile and methanol (85 : 15) [10]. Such an amount of mobile phase in combination with a low rate of elution would greatly complicate the separation of minor components from relatively large amounts of triglycerides by this method. For example, to isolate 1 mg of a mixture of diol lipids from the triglyceride fraction it would be necessary to work with samples of 100–200 mg, for which hundreds of liters of solvents would be required. Methods of countercurrent distribution and gel filtration could prove to be more convenient for the separation of diol lipids from triglycerides.

It has not been possible to use gel filtration hitherto for the separation of compounds with a limited solubility in water or polar solvents because of the absence of gels of the "Sephadex" type that swell in organic solvents. A communication [11] has recently appeared on the possibility of the partial separation of such compounds as tristearin (mol. wt. 891) and tricaprin (mol. wt. 554) on lipophilic Sephadex LH-20. Although the difference in the molecular weights of the triglycerides and diol lipids [for example, ethylene glycol distearate (mol. wt. 595) and butane dioldistearate (mol. wt. 623)] is still lower, this method could prove useful for the enrichment of natural samples.

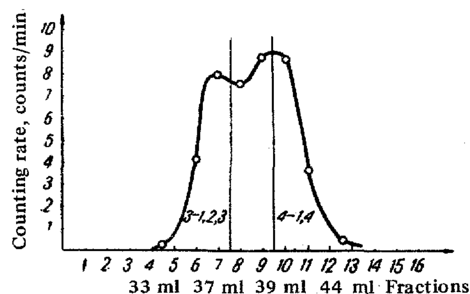

Fig. 4. Separation of a mixture of 13 mg of glycerol tri[1-^{14}C]stearate and 14 mg of butane-1,4-diol di[1-^{14}C]stearate on Sephadex LH-20. 100-ml column, rate of collection of the fractions ~1 ml/2 min, C–T (3 : 2) system (for symbols, see Fig. 1).

Still more promising for the solution of the problem posed is countercurrent distribution. It is obvious that the considerable predominance of triglycerides in the samples to be separated should not have a substantial influence on the separation of the substances by countercurrent distribution, since on working with concentrations far from the level of saturation of the system by the given substance the distribution coefficient does not depend on the concentration.

There are a number of papers in the literature in which the separation of complex mixtures of triglycerides by countercurrent separation into groups of compounds with similar degrees of saturation and numbers of carbon atoms is reported [12–15]. In all cases, systems containing PE or H, NM, nitroethane, nitropropane, and F* were used.

This paper describes attempts to separate triglycerides and diol lipids by gel filtration and countercurrent separation. To accelerate and increase the accuracy of the analysis we synthesized tristearin, and the distearates of ethylene glycol, propane-1,3-diol, and butane-2,3- and -1,4-diols, and also the corresponding oleates, labelled with ^{14}C in the carbonyl carbon.

A trial of systems of solvents similar to those used previously for separation of natural triglycerides [12–14] showed that in such systems as H–NM, H–F, and H–NM–F the distribution coefficients (K) for our oleates were of the order of tens and for the stearates of the order of hundreds. Furthermore, the saturated lipids dissolved only slightly in the individual components of these systems. Thus, the solubilities of butane-1,4-diol distearate and tristearin in hexane at 18° C proved to be 0.06 and 0.1%, respectively. In a further search, we tested and rejected the following systems because of excessively high or low K values (the values for tristearin are given): FA–CC (1 : 1) (K = 0.55); FA–C (1 : 1) (K = 0.06); F–T–CC (4 : 1 : 5) (K = 0.01); FA–T–C (3 : 2 : 3) (K > 500); FA–C–PE (bp 40–60° C) (5 : 2 : 3) (K = 300); FA–CC–H (5 : 2 : 3) (K > 100); heptane–methanol (1 : 1) (K > 100); F–methanol–heptane (3.5 : 1.5 : 5) (K > 100); acetonitrile–H (K ≅ 0). The figures given exclude the possibility of using systems containing only H, NM, F, and FA and show the necessity for introducing a component dissolving lipids well.

It is known that the main requirements to be placed upon a system used for countercurrent distribution is the closeness of the distribution coefficients of the substances undergoing separation to unity and a sufficiently large difference in the values of these coefficients. According to preliminary determinations**, K for tristearin was close to 1 in the PE (40–60° C)–C–F (5 : 2 : 5) system (K = 7)***. In this system, tristearin and butane-1,4-diol distearate (in amounts of 30–40 mg of each component) were separated staisfactorily after only 257 transfers and completely after 503 transfers (Fig. 1a, and 1b). When the stearates from fractions 385–425 were saponified and the alcohols were acetylated [18,19], no triacetin was found by gas chromatographic analysis and no butanediol diacetate was detected in the product from fractions 430–460. The coefficients for tristearin and the butanediol distearate were, respectively, 7.4 and 4.8.

*The following abbreviations are used to denote solvents: H) hexane; PE) petroleum ether; C) chloroform; T) tetrahydrofuran; CC) carbon tetrachloride; NM) nitromethane; F) furfural; FA) formamide.

**The preliminary determination of K was carried out by shaking the lipids and equal volumes of the phases of the system of solvents under investigation in separating funnels.

***Since, in addition to systems consisting of F and H, systems obtained from a mixture of nitroethane and F with PE (1 : 1 : 2) have also been used previously [12, 14], to test the influence of NM on K we studied the system H–C–F–NM (5 : 2 : 2.5 : 2.5). In this system, K for tristearin exceeded 100.

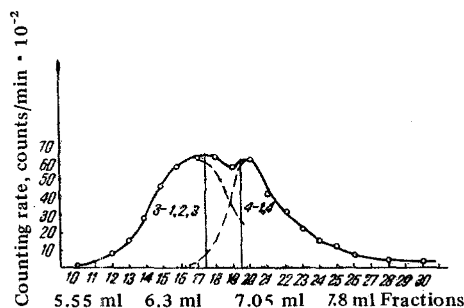

Fig. 5. Separation of a mixture of 10 mg of glycerol tri[1-^{14}C]stearate and 4.2 mg of butane-1,4-diol di[1-^{14}C]stearate on Sephadex LH-20. 15 ml column, rate of collection of the fractions 0.5 ml/30 sec, C–T (3 : 2) system (symbols as for Fig. 1).

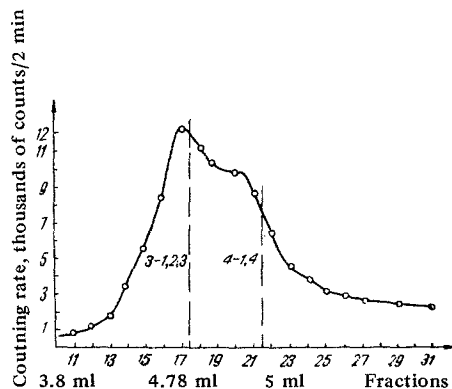

Fig. 6. Separation of a mixture of 10 mg of glycerol tri[1-^{14}C]oleate and 4 mg of butane-1,4-diol di[1-^{14}C]oleate on Sephadex LH-20. 15-ml column, rate of collection of the fractions ~0.14 ml/40 sec, C–T (3 : 2) system (symbols as for Fig. 1).

We used an analogous system in which PE was replaced by H for the separation of tristearin from the distearates of ethylene glycol, propane-1,3-diol, butane-2,3-diol, and butane-1,4-diol, taken in amounts of 30–40 mg. In this case, the mixture of distearates of dihydric alcohols was separated completely from tristearin after 708 transfers (Fig. 2). The gas chromatographic analysis of the products of acetylation of the alcohols formed by the saponification of the stearates showed the complete absence of tristearin in fractions 545–600 and of the distearates of the diols in fractions 610–640. After having separated the tristearin from the mixture of synthetic saturated diol lipids, we attempted to separate the latter into their individual components, reckoning on effecting the separation with a still larger number of transfers. As can be seen from Fig. 3, it was impossible to separate the diol lipids in the diol lipids in the PE–C–F (5 : 2 : 5) system even after 988 transfers. According to gas-liquid chromatography of the acetates of the alcohols, fractions 785–815 contained mixtures of the distearates of butane-1,4-diol, of ethylene glycol, and of propane-1,3-diol, while butane-2,3-diol distearate predominated in fractions 820–835.

It was also impossible to separate triolein from a mixture of the dioleates of the four dihydric alcohols mentioned above. In addition to the solvent systems mentioned above, we used the PE (bp 40–60° C)–C–F (3 : 1.82 : 4) system, in which, according to the results of a preliminary determination, the distribution coefficients were closer to one (for triolein and the dioleates of butane-2,3-diol, propane-1,3-diol, and ethylene glycol, K is, respectively, 5.43, 4.12, 4.08, and 3.17). Furthermore, the latter system is distinguished from the preceding ones by the approximately equal amounts of the phases formed.

In spite of the fact that we were unable to separate triolein from a combination of the oleates of the diols and to separate the diol lipids from one another, we assume that the possibility of countercurrent distribution for the solution of our problem is not yet exhausted. It is obvious that this work requires a further systematic search for more selective systems.

In order to study the possibility of the enrichment of samples of natural neutral lipids with diol lipids, we have studied the separation of tristearin and butane-1,4-diol distearate on a column filled with Sephadex LH-20 in the C–T (3 : 2) system. The use of this mixture of solvents was due to the fact that the Sephadex floats in pure chloroform. Other more polar solvents capable of swelling Sephadex LH-20 cannot be used in this case because of the very low solubility of the stearates in them. It can be seen from Fig. 4 that the stearates, taken in equal amounts, are separated far from completely in the system given. Nevertheless, the gas chromatographic analysis of the acetates of the alcohols obtained from fractions 5–6 and 9–13 show that there was no butane-1,4-diol distearate in the former and no tristearin in the latter. The separation of a mixture consisting of 29% of the same distearate and 71% of tristearin (Fig. 5) led to fractions 10–17 containing about 40% of the total amount of stearates and only 1.4% of the butanediol distearate, while in fractions 20–30 (30% of the total stearates) the ratio of butanediol distearate to tristearin was 46 : 54. Similarly, a mixture of triolein and butane-1,4-diol dioleate (30 : 70) gave fractions 10–17 (30% of the total oleates) containing 5% of the dioleate and fractions 22–31 containing 51% of the dioleate (Fig. 6).

In the experiments mentioned above, mixtures consisting of a diol lipid and a triglyceride in a ratio of 1 : 1–1 : 2 were chromatographed, while in natural oils and fats this ratio is approximately an order of magnitude lower. At such ratios of the natural components an adequate enrichment with the diol lipid can be effected by repeated rechromatography. We have effected such an enrichment, for example, with 20 mg of triolein and 2 mg of butane-1,4-diol dio-

leate. The mixture was separated three times on a column with a volume of 15 ml filled with Sephadex LN-20 in the solvent system given above. After each separation, the eluate was analyzed with respect to its counting rate and the head fractions containing the first (in respect of residence time) half of the main peak of the substance was taken off. The fraction of the mixture remaining after the third separation (4.0–4.4 mg) contained about 40% of butane-1,4-diol dioleate; however, in all the fractions rejected its amount did not exceed 2%.

The results of the investigation carried out have permitted us to give the following general scheme for the isolation of neutral diol lipids from natural sources. First the total neutral lipids containing minor amounts of the diol components must be enriched by the gel filtration method. In this way it is possible to obtain fractions containing the diol lipids and triglycerides in approximately equal proportions. The enriched fractions must be hydrogenated, after which the saturated diol lipids can be separated from the triglycerides by countercurrent distribution. The resulting mixtures of diol lipids may then be analyzed by reversed-phase partition chromatography [3] or by high-temperature gas-liquid chromatography [6]. At the present time we are using this scheme for the isolation of dipole lipids from natural fats.

Experimental

We used standard methods [17] to purify the solvents, the NM, FA, and F being purified by repeated vacuum distillation. The melting points were determined on a Kofler block. The gas-liquid chromatography of the diol and glycerol acetates was carried out on a Khrom-2 chromatograph (Czechoslovakia) under the conditions described previously [18]. Thin-layer chromatography was carried out on plates with a fixed layer of KSK silica gel (150–200 mesh). The revealing agent was a 0.1% solution of morin in ethanol. After the plates had been dried at 100° C (15 min), the spots appeared in UV light. Countercurrent distribution was carried out on automatic apparatuses of the firm Quickfit and Quartz Ltd. (England) consisting of 121 cells each with a capacity of 20 ml (10 ml of each phase) and of 103 cells each with a capacity of 40 ml (20 ml of each phase). The phases for the countercurrent distribution were prepared by shaking a mixture of solvents for 30 min and leaving it to stand for 12 hr. The radioactivity of the samples was determined with a PST-10 end-window counter or with a USS-1 universal scintillation counter.

The tristearin and the distearates of ethylene glycol, propane-1,3-diol, and butane-1,4- and -2,3-diols, and also the corresponding labeled oleates, were synthesized by the reaction of the alcohols with [1-^{14}C]stearoyl and [1-^{14}C]oleoyl chlorides. The stearoyl chloride was obtained by the action of thionyl chloride on the acid with a specific activity of 45.8 μCi/g. The oleoyl chloride was synthesized from oleic acid with a specific activity of 60 μCi/g and oxalyl chloride. According to gas-liquid chromatography, the content of the main substance in the labeled fatty acids exceeded 99.5%.

Synthetic standards of the dipole lipids and triglycerides were obtained by the following general method. At 0° C, a solution of 5.2 mm of acid chloride in 10 ml of absolute ether was added to a mixture of 1.6 mm of the polyol, 0.45 ml of dry pyridine, and 10 ml of absolute ether. The reaction mixture was left overnight at 20° C, boiled for 24 hr, diluted with water, extracted with 0.5 N HCl, and extracted with ether (3 × 10 ml). The ethereal extracts were washed with water, sodium bicarbonate solution, and water again, dried with $MgSO_4$, and chromatographed on a column filled with 60 g of silica gel in the ether–petroleum ether (1 : 5) system. The lipid fraction, a single substance according to thin-layer chromatography (R_f 0.53) was eluted with the same solvent (200 ml). The stearates, recrystallized from hexane, had the following melting points: tristearin 72° C, butane-1,4-diol distearate 70–71° C, butane-2,3-diol distearate 76° C, propane-1,3-diol distearate 64° C, and ethylene glycol distearate 76° C. All the oleates consisted of oils which did not crystallize at room temperature. For countercurrent distribution, the apparatus was filled with the required volume of the phases and the upper phase in the first tube was replaced by a solution of a mixture of labeled lipids in the same phase. Distribution was carried out by recirculating the upper phase for the given number of transfers. Each transfer cycle consisted of shaking for 4 min and settling for 3 min. For quantitative analysis, 0.2 ml of the upper phase was deposited on chromatographic paper (2 × 2 cm) in a current of hot air. The columns were filled with Sephadex by the introduction of a suspension of the swollen gel in the solvent system from a dropping funnel with a wide cock and a stirrer. A solution of the mixture of lipids was deposited on the Sephadex layer in 0.5 ml of chloroform. The resulting fractions were diluted to 2 or 5 ml, and 0.2-ml samples were deposited on chromatographic paper (2 × 2 cm) to determine the counting rate. For the analysis of the polyhydric alcohols, the lipid fractions were saponified with 5% HCl in absolute methanol, the alcohols were isolated by thin-layer chromatography and were acetylated, and the acetates were analyzed by gas-liquid chromatography by the method described previously [18,19].

Conclusions

The possibility, in principle, of separating the minor neutral diol lipids from triglycerides by enriching natural mixtures with dipole lipids on Sephadex and separating the products of their hydrogenation by means of countercurrent distribution has been shown.

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