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V. Derivatives of Dihydric Alcohols and New Types of Neutral Lipid*

V. A. Vaver, N. V. Prokazova, A. N. Ushakov, G. A. Popkova, and L. D. Bergel'son

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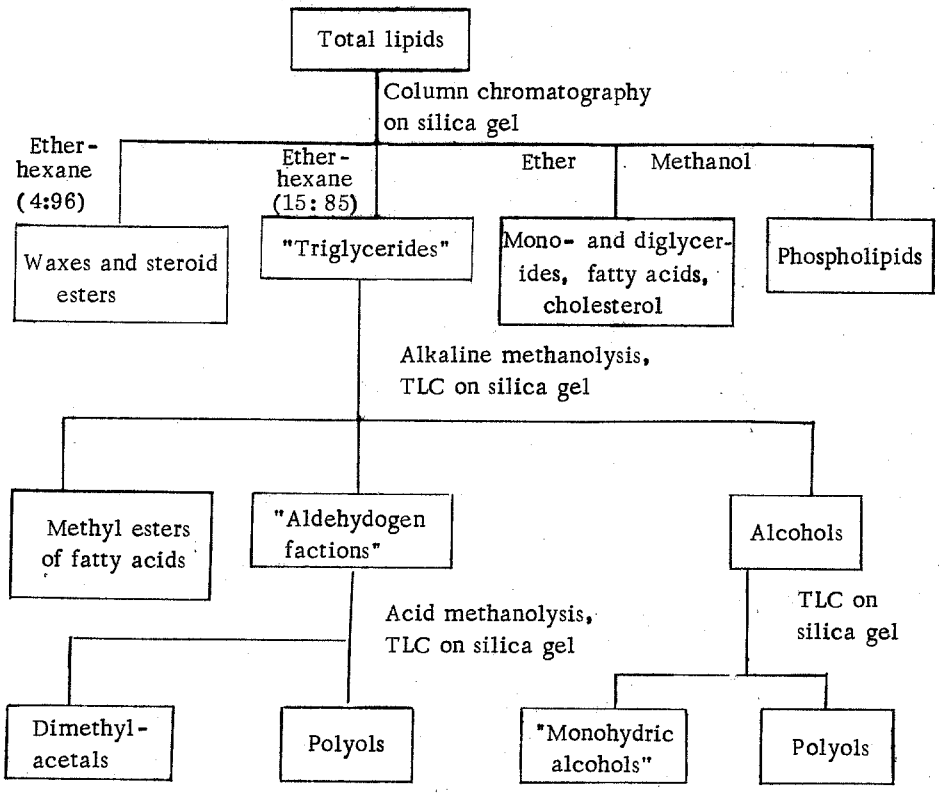
From the beginning of the last century, when Chevreul established that fats consisted of esters of higher carboxylic acids, the conviction has become established that the only polyhydric alcohol occurring in neutral fats is glycerol. In view of this, in numerous investigations of neutral lipids attention has been directed mainly to their fatty acid composition. As a result, the methods of fatty acid analysis have been brought to perfection, while the study of the alcohol components of lipids has advanced only slightly. Nevertheless, isolated communications have begun to appear in the literature which show the presence of a series of dihydric alcohols in fats. Thus, the neutral lipids of mycobacteria [1] and the lung tissue of mammals [2] have been shown to contain ethylene glycol, meso-butane-2, 3-diol has been found in the seed oil of the far-eastern plant Coix lachryma [3], and propane-1,3-diol in the phospholipids of rat liver [4] and sea urchin eggs [5]. Although data of this type have always been considered rare anomalies in the chemistry of the lipids, it has not been possible to exclude the likelihood that diols are also present in lipids isolated from other sources and that they have remained undetected because of the imperfection of the methods of investigation used.

Consequently, some years ago, a systematic study of the alcohol fractions of neutral lipids of plant, animal, and microbial origin was begun in the lipid chemistry laboratory of the Institute of the Chemistry of Natural Compounds AS USSR using gas-liquid and thin-layer chromatography. The very first experiments [6, 7] showed that even purified fractions isolated by chromatography of the neutral lipids on silica gel [8] were extremely inhomogeneous not only with respect to their fatty acid composition but also with respect to their alcohol composition. In the neutral hydrolyzates of such fractions we always found small amounts of ethylene glycol and various C_3 and C_4 diols. On the basis of the results obtained the hypothesis was put forward that a new type of neutral lipid consisting of esters of dihydric alcohols and fatty acids is present in the fats of animals, plants, and microbes [7].

Chart of the Investigation of Diol Lipids



TLC = Thin-layer chromatography

*For communication IV, see E. V. Dyatlovitskaya, K. P. Greshnykh, and L. D. Bergel'son, Prikladnaya biokhim. i mikrobiol. [Applied Biochemistry and Microbiology], no. 6, 1965.

The present paper gives the results of a detailed investigation of the "triglyceride" fractions isolated by chromatography on silica gel [8] from three sources: corn seeds, soil yeast Lipomyces sp. No. 40, and rat liver (see chart).

Table 1

Thin-layer Chromatography of the Products of the Alkaline Methanolysis of the "Triglycerides" of "Odesskaya-10" Corn, of the Soil Yeast Lipomyces sp. No. 40, and of Rat Liver

Source	Amount of "triglycerides" mg	Fractions									Total of fractions	
		Methyl esters			"Aldehydogen fractions"			Alcohols				
		R_f	mg	%	R_f	mg	%	R_f	mg	%	mg	%
Corn	261	0.60	228.1	87.4	0.19	5.7	2.2	0.00	21.6	8.1	255.4	97.7
Soil yeast	230	0.60	162.0	70.5	0.18	25.0	11.0	0.00	23.0	10.0	210.0	91.5
Rat liver	250	0.60	120.0	48.0	0.20	70.0	28.0	0.00	14.2	5.6	204.2	81.7

Table 2

Gas-chromatographic Identification of Polyols Liberated by Alkaline Methanolysis of the "Triglycerides" of "Odesskaya-10", of Soil Yeast Lipomyces sp. No. 40, and of Rat Liver

Polyols	Relative retention volumes							
	Acetates of the polyols (polyethyleneglycol succinate, 10%; 150°C)*				Trimethylsilyl esters of the polyols (silicone elastomer SKTV, 10%, 100°C)			
	Corn	Soil yeast	Rat liver	Synthetic samples	Corn	Soil yeasts	Rat liver	Synthetic samples
Ethylene glycol	0.09(0.1)	—	—	0.09	0.13	—	—	0.13
Butane-1, 3-diol	0.12(0.02)	0.13(0.02)	—	0.12	0.24	0.25	—	0.25
Butane-1, 4-diol	0.27(0.04)	0.24 Traces	0.25(Traces)	0.24	0.40	0.42	0.43	0.44
"C_5 diol"	0.36(0.07)	0.36(0.2)	0.36(0.1)	0.36**	0.79	—	0.75	0.81***
Glycerol	1.00(1.0)	1.00(1.0)	1.00(1.0)	1.00	1.00	1.00	1.00	1.00

*The relative amounts of acetates (with glycerol triacetate taken as 1.0) are given in parentheses.

**Diacetate of pentane-1, 5-diol

***1, 5-Bis-(trimethylsiloxy)-pentane

The "triglyceride" fractions were subjected to alkaline methanolysis [9] and the products so obtained were separated by thin-layer chromatography on silica gel (Table 1). Three groups of substances were isolated: non-polar (methyl esters of fatty acids), highly polar (alcohols), and those intermediate in polarity, which we shall subsequently call the "aldehydogen fractions."

The highly polar fractions consisted mainly of polyhydric alcohols and, in some cases, contained in addition to the latter a small amount of substances corresponding in chromatographic behavior to the higher monohydric alcohols (Fig. 1). These impurities were separated by thin-layer chromatography, after which the polyol fractions were actylated or treated with hexamethyldisilazane [10]. The mixture of acetates or trimethylsilyl esters formed were analyzed by means of gas-liquid chromatography (Table 2, Figs. 2 and 3, a).

As can be seen from Table 2, all the polyol fractions that we studied contained, in addition to glycerol, a small amount of butane-1, 4-diol; the soil yeasts and maize seeds were also found to contain butane-1, 3-diol, and the polyol fraction of the corn "triglycerides" contained ethylene glycol.

Since the diols mentioned are liberated by the alkaline methanolysis of the "triglyceride" fractions, they are evidently present in the initial lipids in the form of esters which are not separated from the triglycerides by chromatography on silica gel. Experiments on the thin-layer chromatography of synthetic dioleoylbutane-1, 4-diol and

triglycerides in fact showed that although these substances possess different R_f values, they are not appreciably separated under the conditions of thin-layer chromatography (Table 3).

Thus, the "triglyceride" fractions of the lipids of corn, soil yeast, and rat liver contain, in addition to glycerol esters, non-glyceride lipids consisting of the diol esters (I) and (II):

$$\underset{\text{I, } n = 2,4}{R\text{COO}(\text{CH}_2)_n\,\text{OCO}R'};\quad \underset{\text{II}}{R\text{COOCH}_2\text{CH}_2\text{CH}(\text{OCO}R')\text{CH}_3}.$$

It must be mentioned that there were also unidentified peaks on all the chromatograms of the acetates or trimethylsilyl esters of the polyols obtained from the "triglyceride" fractions (cf. Figs. 2 and 3). It is therefore not excluded that these fractions contain, in addition to the non-glyceride lipids (I) and (II), esters of other polyhydric alcohols. Thus, the gas-liquid chromatograms of the polyol acetates exhibit relatively large peaks with a retention volume of 0.36, coinciding with the peak of pentane-1, 5-diol diacetate (Figs. 3 and 4a). However, in the chromatograms of the corresponding trimethylsilyl esters the positions of the "pentanediol" peaks differ from that of the peak of 1, 5-bis-(trimethylsiloxy)-pentane (rentention volume 0.81; cf. Table 2), which does not permit us to consider these peaks to be definitively identified. Since it is possible that the corresponding polyol might be one of the isomeric pentanediols (or a mixture of them) we shall provisionally call it a "C_5 diol."

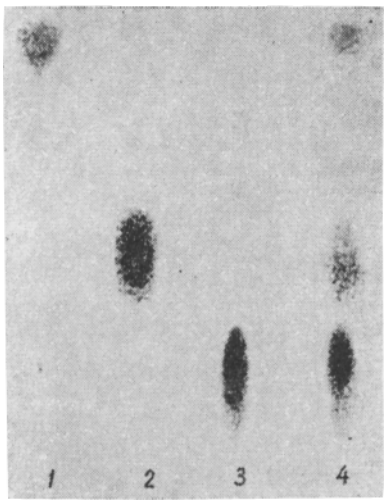


Fig. 1. Thin-layer chromatography of the alcohols obtained by the alkaline methanolysis of "Odesskaya-10" corn. 1) n-Dodecan-1-ol; 2) ethylene glycol; 3) glycerol; 4) alcohols liberated by the alkaline methanolysis of the "triglycerides."

Concerning the "aldehydogen fractions," their R_f values on thin-layer chromatography on silica gel (almost coinciding with the R_f value of distearoylglycerol) and their IR spectra (strong absorption bands of HO groups at 3400-3500 cm^{-1} and the absence of a strong absorption of CO groups in the 1700-1800 cm^{-1} region), and also their stability in an alkaline medium permit the assumption that they are nothing other than esters of polyhydric alcohols containing free HO groups.

When heated with a solution of hydrogen chloride in methanol, each of the aldehydogen fractions gave a mixture of substances that could be separated into two zones by preparative thin-layer chromatography on silica gel. In all the cases investigated, the nonpolar zone evidently consisted of a mixture of dimethylacetals, since its R_f value did not differ from that of the dimethylacetal of palmitaldehyde, and on heating with an acidic solution of p-nitrophenylhydrazine a mixture of hydrazones was formed with λ_{max}^{EtOH} 395 mμ [11].

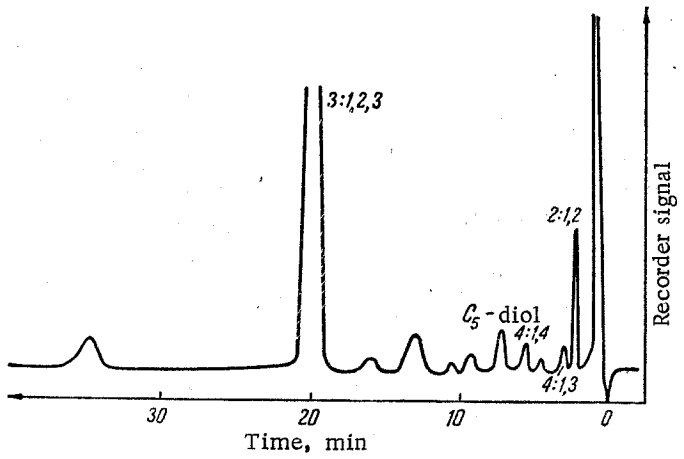


Fig. 2. Gas-liquid chromatogram of the acetates of the polyols of the "triglyceride fraction" of "Odesskaya-10" corn. First figure – number of C atoms; figures after the colon – positions of the hydroxyl groups in the diol molecules.

The more polar fraction was a mixture of alcohols, and this was acetylated or treated with hexamethyldisilazane, as mentioned above, and investigated by gas-liquid chromatography (cf. Table 4 and Fig. 3, b). As can be seen from

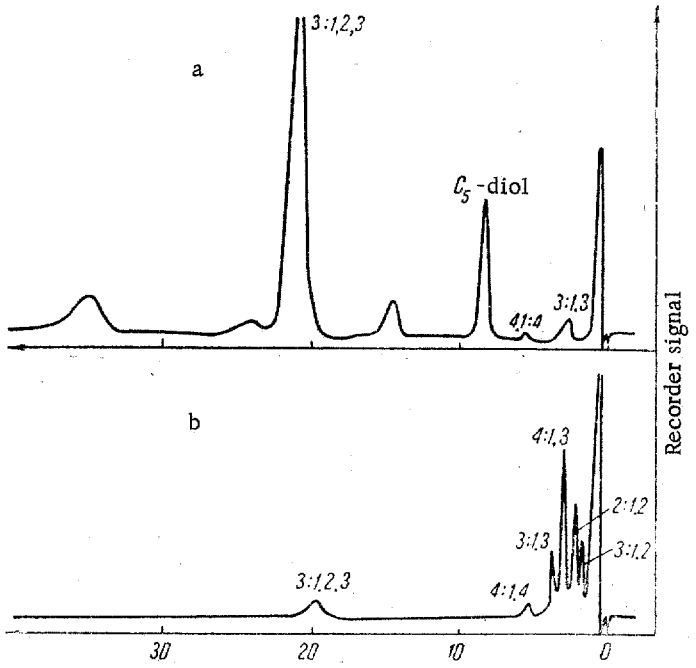


Fig. 3. Gas-liquid chromatography of the acetates of the polyols of the "triglyceride" fraction of the soil yeast Lipomyces sp. No. 40. a) Polyols liberated on alkaline methanolysis; b) polyols contained in the "aldehydogen lipids." (Designation of polyols as for Fig. 2).

Table 4, the hydrolyzates of the "aldehydogen fractions" contained, in addition to glycerol, ethylene glycol, propane-1, 2-diol, propane-1, 3-diol, butane-1, 3-diol, butane-1, 4-diol, and a C_5 diol.

The results obtained justify the assumption that the "aldehydogen fractions" contain vinyl esters of diols of the type (III) and (IVa or b):

$$\underset{\text{III, } n=2,\ 3,\ 4}{HO(CH_2)_n OCH{=}CHR};\quad \underset{\text{IV}a\text{ , } n=1,2}{CH_3CH(CH_2)_n OCH{=}CHR}\ (OH);\quad \underset{\text{IVb, } n=1,2}{CH_3CH(CH_2)_n OH}\ (OCH{=}CHR)$$

This conclusion is confirmed by the results of the hydrogenation of the "aldehydogen fractions" over a nickel catalyst in alcohol. In contrast to the initial substances, their hydrogenation products do not form acetals on treatment with methanolic hydrogen chloride. The IR spectra of the hydrogenated products differ from the spectra of the "aldehyde fractions" by a marked decrease in intensity and a change in the positions of the absorption bands in the 1250-1100 cm^{-1} region, which is characteristic for the conversion of a vinyl ester into a dialkyl ester [15].

The vinyl esters (III) and (IVa or b) are evidently formed in the alkaline treatment of the "triglycerides" as a consequence of the saponification of esters of the type of (V) and (Va or b).

$$\underset{\text{V, } n=2,\ 3,\ 4}{R'COO(CH_2)_n OCH{=}CHR}$$

$$\underset{\text{VIa, } n=1,2}{CH_3CH(CH_2)_n OCH{=}CHR}\ (OCOR');\qquad \underset{\text{VIb, } n=1,\ 2}{CH_3CH(CH_2)_n OCOR'}\ (OCH{=}CHR)$$

Consequently, the "triglyceride" fractions of corn seed, soil yeast Lipomyces sp. No. 40, and rat liver contain not only diacyl derivatives of the diols (I) and (II) but also a new type of lipid, the non-glyceride plasmalogens (V) and (VIa or b).

Table 3

R_f Values of Glycerol Trioleate and Butane-1, 4-diol Dioleate in Silica Gel Thin-layer Chromatography

System	Glycerol trioleate	Butane-1, 4-diol dioleate	A mixture (1:1) of glycerol trioleate and butane-1, 4-diol dioleate
Petroleum ether-ethyl-ether-acetic acid (85:14:1)	0.50	0.46	0.48 }*
Chloroform	0.42	0.58	0.50 }
Chloroform-benzene (7:3)	0.37	0.15	0.25 }

*One spot

The relative amounts of the diols and glycerol formed in the alkaline methanolysis of the "triglyceride fractions" and in the acid methanolysis of the "aldehydogen fractions" were determined by gas-liquid chromatography of the acetates (cf. Tables 2 and 4). Although the results obtained may not accurately reflect the ratio of the various lipids in the materials investigated (since differential losses of the individual components during the preparation of the acetates are not excluded), they nevertheless show that the neutral fats always contain 2-4% of diols together with the glycerol.

Table 4

Gas-chromatographic Identification of Polyols Contained in the "Aldehydogen fractions" of "Odesskaya-10" Corn, Soil Yeast Lipomyces sp. No. 40, and Rat Liver

Polyols	Relative retention volumes							
	Acetates of the polyols (polyethyleneglycol succinate, 10%, 150°C)*				Trimethylsilyl esters of the polyols (SKTV, 10%, 100°C)			
	Corn	Soil yeasts	Rat liver	Synthetic samples	Corn	Soil yeasts	Rat liver	Synthetic samples
Propane-1, 2-diol	—	0.07(0.9)	0.07(0,08)	0.07	—	0.15	0.14	0.13
Ethylene glycol	0.08Traces	0.09(3.0)	—	0.09	0.13	0.12	0.12	0.13
Butane-1, 3-diol	0.11 (0.02)	0.12(5.0)	0.12(0.15)	0.12	0.25	0.24	0.26	0.25
Propane-1, 3-diol	—	0.15(0.7)	0.15(0.11)	0.15	0.18	0.19	0.18	0.19
"C_5 diol"	0.24(0.06)	0.24(0.25)	0.24(1.25)	0.24	0.42	0.41	0.40	0.44
Butane-1, 4-diol	0.36(0.02)	—	0.35(0.75)	0.36**	0.78	—	0.75	0.81***
Glycerol	1.00(1.00)	1.00(1.00)	1.00(1.00)	1.00	1.00	1.00	1.00	1.00

*The relative amounts of acetates (with glycerol triacetate taken as 1.0) are given in parentheses.

**Diacetate of pentane-1, 5-diol

***1, 5-Bis-(trimethylsiloxy)-pentane

We have found lipids of the two types mentioned in various other sources (for example, in the neutral lipids of sunflower seed oil, sheep fat, cod liver [7], and egg yolk). Thus, these form new classes of neutral lipids that are widely distributed in nature. We propose for these compounds the general name "diol lipids."

Experimental

Extraction of the lipids. The neutral lipids of corn were extracted with n-hexane from the vacuum-dried comminuted seeds of the variety "Odesskaya-10" of the 1963 crop.

The total lipids of the soil yeast Lipomyces sp. No. 40* were extracted from the lyophilized mass with a mixture of chloroform and methanol (3:1). The extract was washed with a saturated solution of sodium chloride, and the chloroform layer was separated off and evaporated to constant weight under vacuum.

The total lipids of rat liver were obtained by extracting fresh homogenized liver with a mixture of chloroform and methanol (2:1) by Folch's method [12].

Isolation of the "triglycerides." The extracted lipids (1-3 g) were chromatographed on a column (45 × 700 mm) of grade KSK silica gel (100-150 mesh) by a modified Barron-Hanahan method [8]. The lipids were eluted successively with hexane-ether (96:4), hexane-ether (85:15), ether, and methanol, the lipid content of the eluate being followed by thin-layer chromatography on silica gel. Evaporation under vacuum of the hexane-ether (85:15) eluates gave the "triglyceride" fractions.

Methanolysis of the "triglyceride" fractions and separation of the methanolysis products. The methanolysis was carried out by a modification of Luddy's method [9]. A solution of 230-250 mg of the "triglycerides" in 3 ml of petroleum ether was mixed with 20 ml of a 0.4 N solution of potassium methoxide in methanol, and the mixture was boiled for 2 hr. After cooling, the reaction mixture was neutralized with 0.5 N methanolic sulfuric acid and evaporated under vacuum, and the residue was extracted with a mixture of chloroform and methanol (95:5) (6 × 5 ml).

The methanolysis products were separated by means of preparative thin-layer chromatography in the hexane-ether (85:5) or hexane-ether-acetic acid (89:10:1) systems [14] on plates (18 × 18 cm) with 18 g of silica gel prepared from sodium silicate [13] and fixed with 1 g of gypsum. The plates were dried in the air (10-12 hr) and were activated for 1 hr at 110°C. It was possible to separate 250-300 mg of the mixture of methanolysis products on a single plate. To detect the separated zones, the edge of each plate was sprayed with 50% sulfuric acid or a solution of phosphomolybdic acid in ethanol. The mixture of methyl esters of the acids (R_f 0.6) was eluted with ether, and the "aldehydogen fractions" (zones with R_f 0.2) and the alcohols (R_f 0.0) with methanol.

The alcohol components of the "triglyceride" fractions were chromatographed on plates (13 × 18 cm) with 6 g of silica gel free from iron salts in the chloroform-methanol (85:15) system in the presence of decan-1-ol, ethylene glycol, and glycerol as reference samples (cf. Fig. 1). The substances were detected by means of an ammoniacal solution of silver nitrate. In those cases where substances with R_f values of monohydric alcohols were present in the mixture of alcohols, the alcohol fractions were subjected to preparative thin-layer chromatography on the same plates and in the same system, and to define the separated zones the edge of each plate was sprayed with an ammoniacal solution of silver nitrate. Under these conditions the "monohydric alcohols" were separated completely and the diols and glycerol incompletely (see Fig. 1). Consequently, the individual zones were extracted with methanol after which the extracts of the diols and triols ("polyols") were combined and investigated by gas-liquid chromatography.

Acetylation of the polyols. The polyols isolated by means of thin-layer chromatography (5-10 mg) were acetylated with a mixture of 0.5 ml of glacial acetic acid and 0.25 ml of acetic anhydride, in the presence of a catalytic amount of 60% perchloric acid. The reaction mixture was boiled for 30 min, cooled, treated with 0.25 ml of absolute methanol, and again heated to the boil. The excess of methanol and the methyl acetate were evaporated off under vacuum, and the residual acetic acid was distilled off at atmospheric pressure through a small column in the form of the azeotropic mixture with benzene. Finally, the benzene solutions of the acetates were heated with activated carbon, filtered, and evaporated under vacuum to small bulk.

Trimethylsilyl ethers of the polyols. Mixtures of the polyols (5-10 mg) were dissolved in 0.2 ml of hexamethyldisilazane, boiled for 30 min in a flask with a reflux condenser in the presence of a catalytic amount of hydrogen chloride or trimethylchlorosilane [10], and cooled; 0.5 ml of absolute methanol was added, and after 15 minutes the mixture was again evaporated to dryness. The residues were extracted with methylene chloride (5 × 2 ml); the extracts were centrifuged to eliminate ammonium chloride, and evaporated to minimum volume immediately before gas chromatography.

Gas-liquid chromatography. The gas-chromatographic analysis of the acetates and trimethylsilyl ethers was carried out on a Pye chromatograph with a β-ionization detector. The packed part of the column was 1200 × 5 mm, the size of the sample was 0.05-0.1 μl, the argon flow rate was 50-60 ml/min, and the detector voltage 100 V. The stationary phases (see Tables 2 and 4) were supported on Chromosorb W with particle dimensions of 60-80 mesh.

Acid methanolysis of the "aldehydogen fractions" and the separation of the methanolysis products. The "aldehydogen fractions" (25-30 mg) isolated by means of thin-layer chromatography as described above, were boiled for 30 min with 2.5 ml of a 5% solution of hydrogen chloride in absolute methanol. After cooling, the mixture was evaporated and separated on plates (18 × 18 cm) with 6 g of silica gel prepared from sodium silicate [13] in the hexane-ether

*Strain taken from the collection of the department of soil microbiology of Moscow State University, cultured on a nutrient medium containing 4% of glucose, 0.1% of ammonium sulfate, 0.2% of magnesium sulfate, 0.1% of potassium hydrogen phosphate, and 0.3% of yeast autolyzate.

(9:1) system. Elution with ether of the zone with R_f 0.65 gave 20.23 mg of a mixture of acetals. Elution with methanol of the starting zone gave a mixture of polyhydric alcohols, which were acetylated or treated with hexamethyldisilazane, as described above. The results of the gas-chromatographic analysis of the mixtures of acetates and trimethylsilyl esters are given in Table 4.

Summary

1. Two new types of neutral lipid have been found in the fats of animals, plants, and microorganisms: diacyl derivatives of various dihydric alcohols and monacyl derivatives of their 1-alkenyl derivatives ("diol plasmalogens").

2. Of the diols contained in the neutral lipids of corn seed, soil yeast (Lipomyces sp. No. 40), and rat liver, the following have been identified: ethylene glycol, propane-1, 2-diol, propane-1, 3-diol, butane-1, 3-diol, and butane-1, 4-diol.

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Institute of the Chemistry of Natural Compounds AS USSR