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OCCURRENCE, SYNTHESIS AND BIOLOGICAL EFFECTS OF SUBSTITUTED GLYCEROL ETHERS

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CONTENTS

I. INTRODUCTION

Glycerolipids with ether-linked aliphatic moieties have attracted increasing interest and have during recent years been the object of extensive chemical and metabolic studies. Two major groups of ether-linked lipids, *viz.* alkyl ether lipids and alk-1-enyl ether lipids, have been shown to be present in the living cells of most animal tissues. A high content of ether lipids has been found in malignant tumors (cf ref. 13).

Glycerolipids containing methoxy-substituted *O*-alkyl groups were first isolated from Greenland shark liver oil.[9] This article will mainly deal with the isolation, identification and synthesis of methoxy-substituted glycerol ethers, but will also present some studies on their biological effects. The identification of glycerol ethers with a hydroxyl group in the 2-position of the long alkyl chain will also be described.

II. ISOLATION AND CHARACTERIZATION OF SUBSTITUTED GLYCEROL ETHERS

A. *Methoxy-substituted Saturated and Monounsaturated Glycerol Ethers*

When determining the content of glycerol ethers in the unsaponifiable fraction of liver oil from Greenland shark (*Somniosus microcephalus*) by chromatography on silicic acid columns, elution patterns of the type demonstrated in Fig. 1 were obtained.[9] Peak I represents a mixture of glycerol ethers with chimyl, batyl and selachyl alcohol (hexadecyl, octadecyl and octadecenyl glycerol) as principal components, whereas the material in peak II had characteristics differing from those of the ordinary glycerol ethers. Thin layer chromatography (TLC) (Figs. 2a and b) showed that the material in peak II had

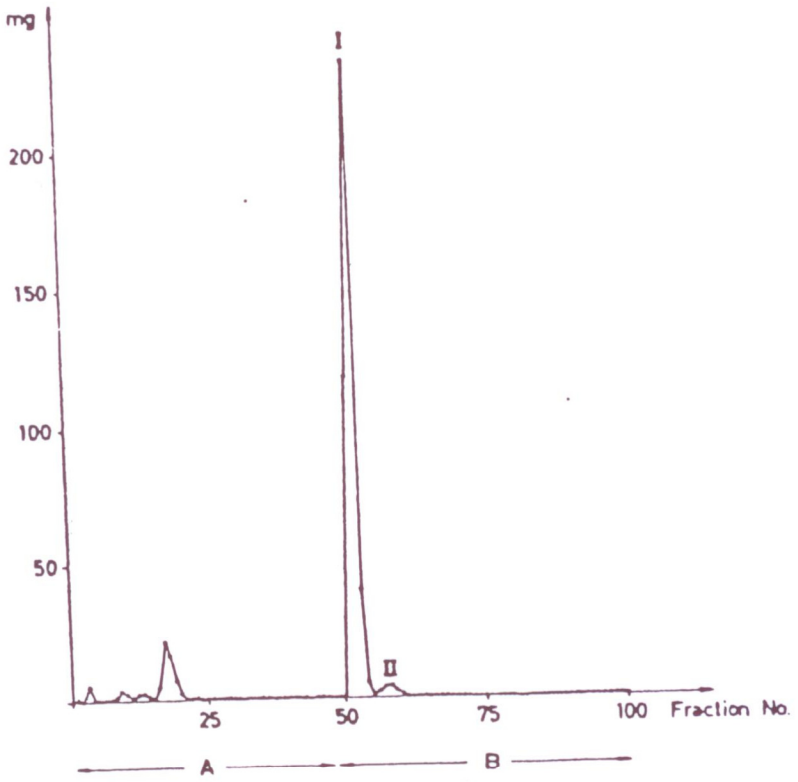


FIG. 1. Chromatography of nonsaponifiable material (467 mg) from Greenland shark liver oil on a silicic acid column (35 g). Eluting solvents: 5% diethyl ether in light petroleum, b.p. 60–80°C (A) and diethyl ether (B). Fraction volume: 20 ml. (Figs. 1–5 reproduced from Hallgren and Ställberg[9] by permission of *Acta Chem. Scand.*).

a somewhat lower R_f value than the ordinary glycerol ethers in peak I. The main part of the peak II material consisted of unsaturated components (Fig. 2b). The infrared (IR) spectra of the materials from peaks I and II were practically identical with a strong absorption band at $\sim 1100\,cm^{-1}$, where the absorption of the peak II material was even somewhat stronger. The IR spectrum thus indicated that also the unidentified peak II material consisted of glycerol ethers. The nuclear magnetic resonance (NMR) spectrum showed a signal at $\delta = 3.3$ ppm indicating a methoxy substituent. A very slight band in the IR spectrum at $2830\,cm^{-1}$ supported this assumption. As for the ordinary glycerol ethers, treatment of the peak II material with acetone in acid solution gave isopropylidene derivatives, showing that the attached long alkyl chain was bound to the glycerol in the α-position.

Gas liquid chromatography (GLC) of the isopropylidene derivatives of the substituted glycerol ethers before and after hydrogenation indicated two homologous series, one of which was saturated, the other unsaturated. The relative retention times of the four main components of peak II on a polar and a nonpolar stationary phase are summarized in Table 1.

The mass spectra of the dominating components 1, 2 and 4 of Table 1 are shown in Figs. 3–5. The fragmentation patterns could be explained by structures with a methoxy group attached to the 2-position of the long carbon chains, calculated from the glycerol ether bond. Mass spectrometry (MS) also indicated that the molecular weights of components 1–4 before hydrogenation were 384, 386, 398 and 412 and after hydrogenation 386, 386, 400 and 414.

The positions of the double bonds in the two dominating unsaturated components were determined by oxidative splitting of the double bonds and gas chromatographic and mass spectrometric analysis of the esterified acids formed. The presence of methyl dodecanoate and methyl tetradecanoate showed the double bond positions to be between carbon atoms nos. 12 and 13 and between nos. 14 and 15, respectively, calculated from the free ends of the long hydrocarbon chains.

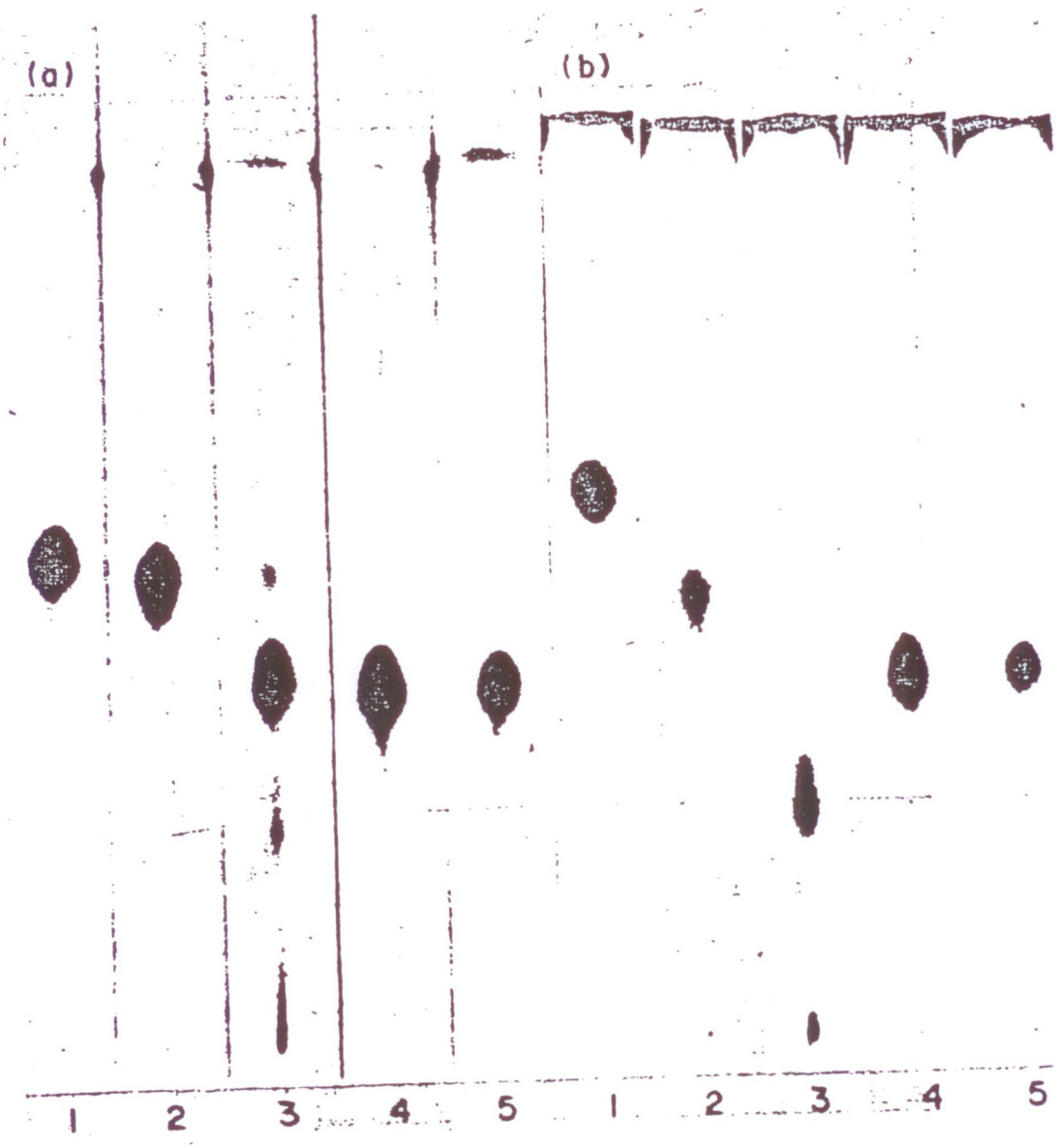


FIG. 2. (a) Thin layer chromatograms on Silica Gel G and (b) on silver nitrate impregnated silica gel G with trimethylpentane–ethyl acetate—methanol, 50:40:7, as solvent. (1) Batyl alcohol. (2) Ordinary glycerol ethers isolated by silicic acid column chromatography (peak I, Fig. 1). (3) Glycerol ethers eluted after the ordinary ones during the silicic acid column chromatography (peak II, Fig. 1). (4) *Ditto* after hydrogenation. (5) Synthetic 1-*O*-(2-methoxyhexadecyl)glycerol.

TABLE 1. Retention Times of the Isopropylidene Derivatives of the Glycerol Ethers from the Peak II Material of the Silicic Acid Chromatogram (Fig. 1) Relative to the Isopropylidene Derivative of Octadecylglycerol

Glycerol ethers (as isopropylidene derivatives)	Retention at 218°C on Apiezon L (1%)		Retention at 194°C on polyethylene glycerol succinate (15%)	
	Before hydrogenation	After hydrogenation	Before hydrogenation	After hydrogenation
Octadecylglycerol	1.00	1.00	1.00	1.00
Component 1	0.59	0.68	1.30	1.21
Component 2	0.68	0.68	1.21	1.21
Component 3	0.84	0.97	1.67	1.55
Component 4	1.19	1.38	2.17	2.02

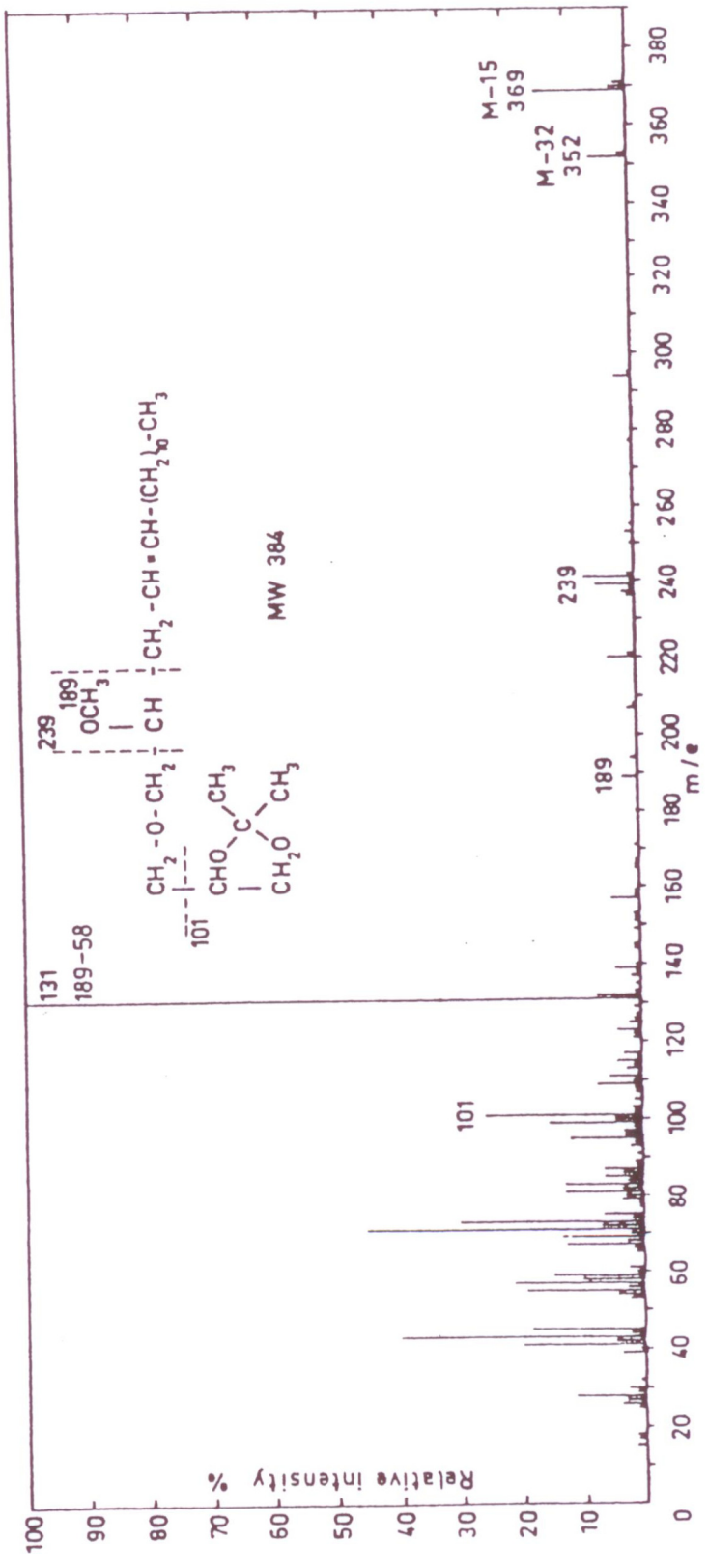


FIG. 3. Mass spectrum of 2,3-*O*-isopropylidene-1-*O*-(2-methoxy-4-hexadecenyl)glycerol from Greenland shark liver oil.

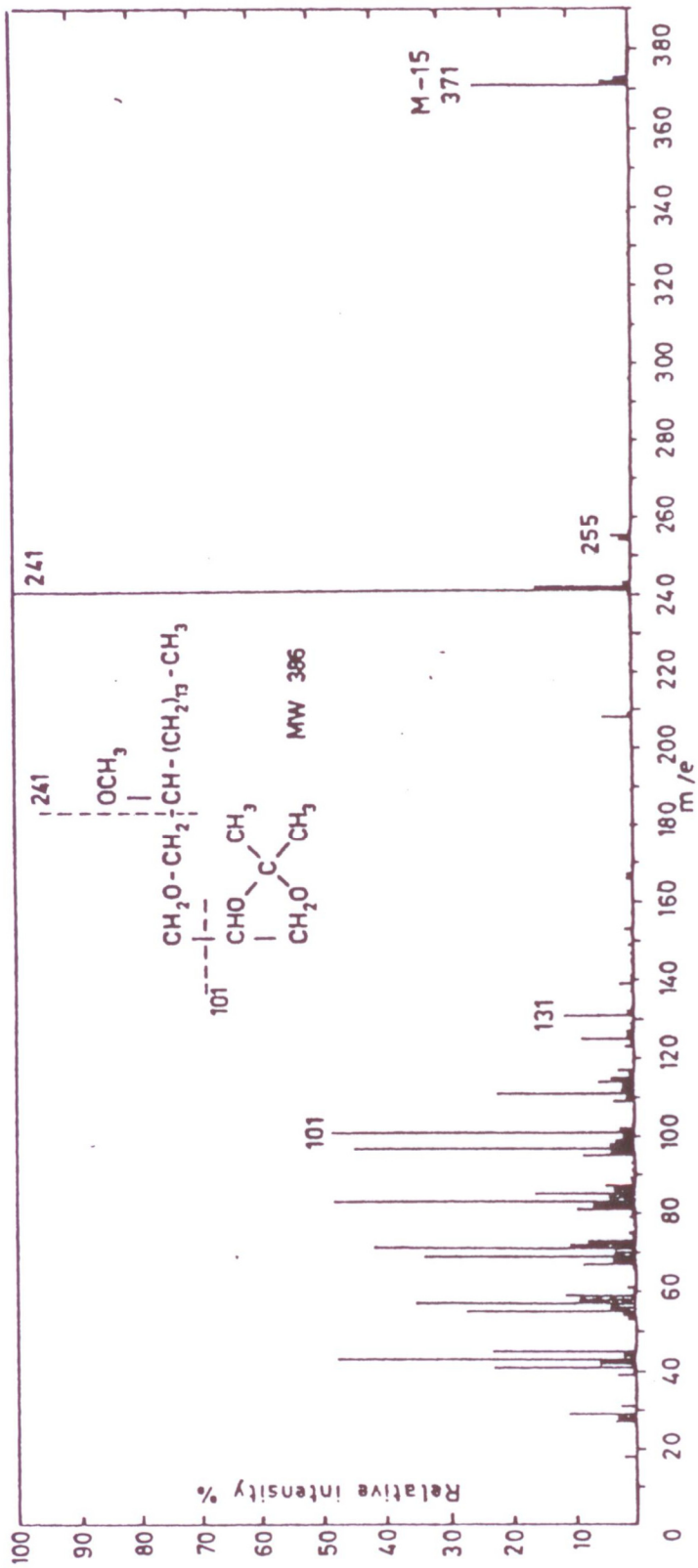


FIG. 4. Mass spectrum of 2,3-*O*-isopropylidene-1-*O*-(2-methoxyhexadecyl)glycerol from Greenland shark liver oil.

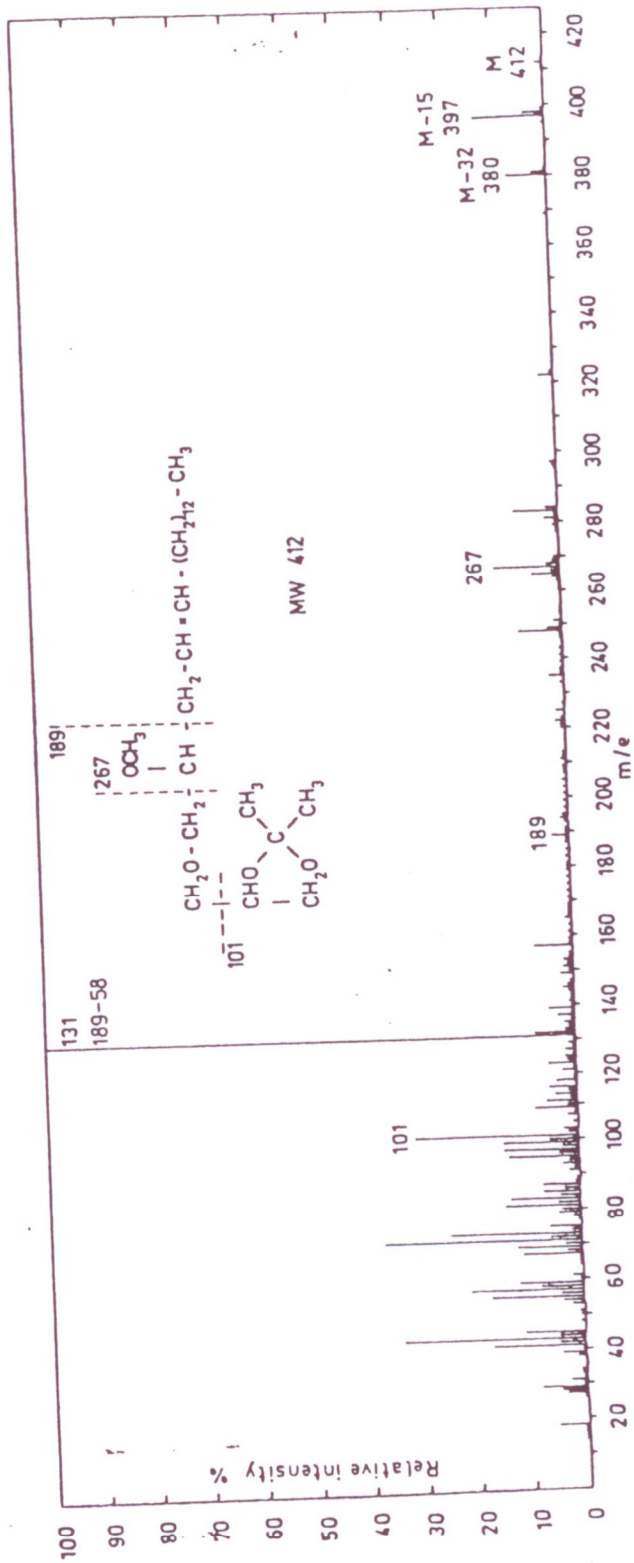


FIG. 5. Mass spectrum of 2,3-*O*-isopropylidene-1-*O*-(2-methoxy-4-octadecenyl)glycerol from Greenland shark liver oil.

The IR absorptions of the mixture of mainly unsaturated glycerol ethers at 1410 and 1655 cm^{-1} indicated a *cis*-form of the double bond. Thus, the compounds 1–4 of Table 1 would be the isopropylidene derivatives of:

1-*O*-(2-methoxy-4-*cis*-hexadecenyl)glycerol;
1-*O*-(2-methoxyhexadecyl)glycerol;
1-*O*-(2-methoxy-4-*cis*-heptadecenyl)glycerol, and
1-*O*-(2-methoxy-4-*cis*-octadecenyl)glycerol.

To confirm the structures, 1-*O*-(2-methoxyhexadecyl)glycerol and 1-*O*-(2-methoxy-4-*cis*-hexadecenyl)glycerol were synthesized. (See Section IV A). The synthetic compounds and the corresponding components from shark liver oil were identical with respect to TLC, MS, IR, NMR and to GLC on several different phases.

In addition to the four components mentioned, small amounts of other homologs were present in the shark liver oil. Table 2 gives the composition of the mixture of the methoxy-substituted as well as the unsubstituted glycerol ethers, determined by GLC and MS. The glycerol ether with a 4-hexadecenyl chain is predominant in the mixture of methoxy-substituted compounds, whereas a glycerol ether with a 9-octadecenyl chain constitutes the largest component of the unsubstituted glycerol ethers. A polyunsaturated component (See Section II B) was only found in the methoxy-substituted glycerol ethers. The methoxy-substituted glycerol ethers constituted about 4% of the total glycerol ether content of the shark liver oil.

B. *A Methoxy-substituted Polyunsaturated Glycerol Ether*

The mixture of methoxy-substituted glycerol ethers described above contained a small amount of a compound which was slightly more polar than the main part.[11] This compound was enriched to about 85% by repeated chromatography of the material (as isopropylidene derivatives) on silicic acid columns.

Analysis by combined GLC–MS of the compound before and after hydrogenation gave the molecular weights 458 and 470, respectively, consistent with the existence of six double bonds. A weak band at 1640 cm^{-1} and a strong band at 700 cm^{-1} in the IR spectrum indicated *cis* double bonds. That the compound was a 2-methoxy-substituted glycerol ether was shown by the MS fragmentation pattern of the hydrogenated isopropylidene derivative. The pattern was the same as for 1-*O*-(2-methoxyhexadecyl)-2,3-*O*-isopropylideneglycerol and 1-*O*-(2-methoxyoctadecyl)-2,3-*O*-isopropylideneglycerol.

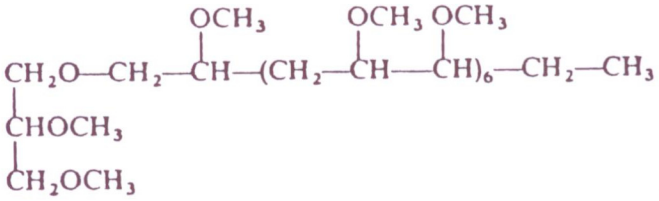
TABLE 2. Composition of Unsubstituted and Methoxy-substituted Glycerol Ethers from Greenland Shark Liver Oil

Alkyl chain*	Methoxy-substituted glycerol ethers %	Ordinary glycerol ethers %
14:0	0.5	2.8
15:0	tr	0.5
16:0	14.6	10.6
16:1	53.7	12.1
17:0	1.1	0.5
17:1	3.3	1.9
18:0	1.7	5.8
18:1	21.0	62.0
19:0	0.1	tr
19:1	0.6	0.3
20:0	0.3	0.1
20:1	tr	2.8
22:0	tr	tr
22:1	tr	0.6
22:6	3.1	

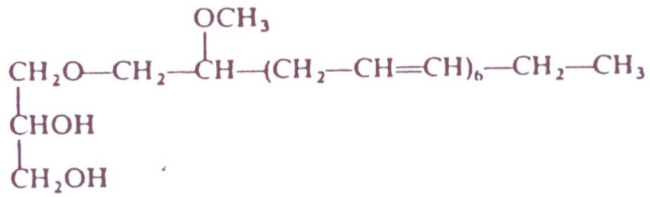
*The first figure denotes the number of carbon atoms in the long carbon chain and the figure after the colon the number of double bonds.

The NMR spectrum of the polyunsaturated compound had multiplets at about δ = 2.1, 2.8 and 5.3 ppm, which could originate from CH_2-groups adjacent to one doubly bonded carbon atom, CH_2-groups between two doubly bonded carbon atoms and CH=CH groups adjacent to CH_2-groups, respectively.

To settle the positions of the double bonds, the polyunsaturated compound was converted to a polymethoxy compound, which was analyzed by combined GLC–MS.[12] The conversion was carried out by *cis*-addition of osmium tetroxide to the six double bonds giving six osmate ester groups, which by reductive cleavage were split to six *vic*-diol groups. Methylation of these twelve hydroxy groups and the two of the glycerol part of the molecule should give a compound with fifteen methoxy groups and a molecular weight of 818. The mass spectrum did not give any molecular ion peak but showed fragment ions originating from both ends of the molecule. The mass spectrum was in accordance with the structure



which for the polyunsaturated compound implies the structure



C. *Hydroxy-substituted Saturated and Monounsaturated Glycerol Ethers*

Trace quantities of a mixture of compounds, more polar than the methoxy-substituted glycerol ethers were isolated from the unsaponifiable fraction of shark liver oil. These compounds were identified as 2-hydroxy-substituted glycerol ethers[10] by the following data. By TLC, the material had the same R_f-values as synthetically prepared 1-*O*-(2-hydroxy-4-hexadecenyl)glycerol and 1-*O*-(2-hydroxyhexadecyl)glycerol.[15] Combined GLC–MS analyses of the isopropylidene derivatives of the components in the mixture gave, for the predominating compound, a mass spectrum which was practically identical with that of synthetic 1-*O*-(2-hydroxy-4-*cis*-hexadecenyl)-2,3-*O*-isopropylideneglycerol (Fig. 6). The retention times at GLC were also identical. Hydroxy-substituted tetradecyl, tetradecenyl, hexadecyl and octadecenyl glycerol ethers were also identified by GLC–MS.

III. THE OCCURRENCE AND COMPOSITION OF METHOXY-SUBSTITUTED GLYCEROL ETHERS IN LIPIDS FROM MAN AND ANIMALS

It was considered of interest to find out whether the methoxy-substituted glycerol ethers, which had been found in Greenland shark liver oil, are of more common occurrence. Various biological materials from other marine animals[8] and from mammals,[7] principally man, were therefore analyzed for their content of methoxy-substituted glycerol ethers. The contents of unsubstituted glycerol ethers were determined in the same materials. The materials from marine animals were herring and Baltic herring fillets, mackerel fillets, the edible parts of marine crayfish, shrimps and sea mussels, a commercial sample of cod liver oil and liver oil from cod caught in the Baltic sea. Fresh-water crayfish was also studied. As it had been shown that human milk had a comparatively

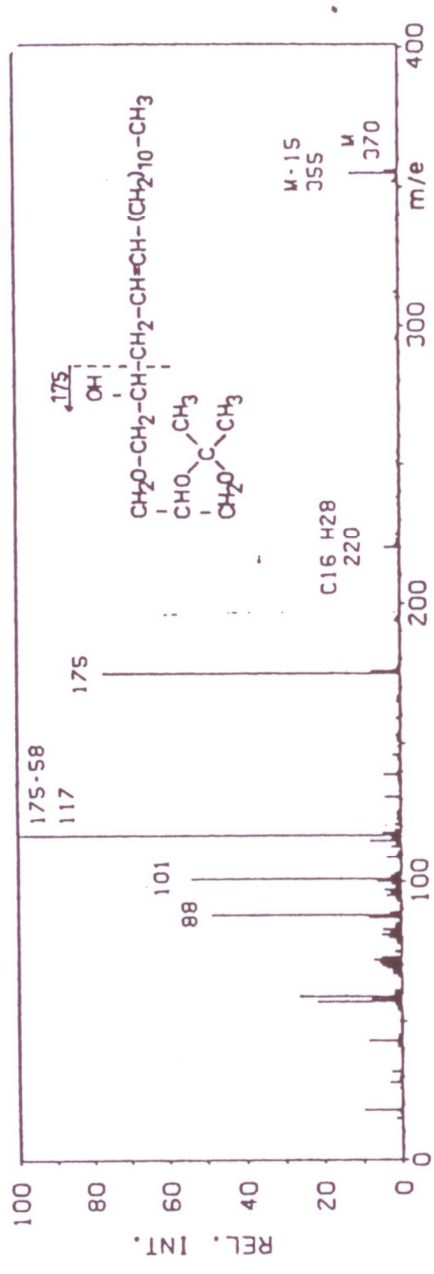


FIG. 6. Mass spectrum of synthetic 1-*O*-(2-hydroxy-4-*cis*-hexadecenyl)-2,3-*O*-isopropylideneglycerol. (Reproduced from Hallgren and Ställberg[10] by permission of *Acta Chem. Scand.*).

high content of glycerol ethers, methoxy-substituted glycerol ethers were sought in milk from different mammals. Human milk from different periods of lactation, cow's milk and sheep's milk were chosen for the study. The high content of glycerol ethers in red bone marrow and tumors was the reason for selecting human red bone marrow, red blood cells, blood plasma and a uterine carcinoma for determination of their content of methoxy-substituted glycerol ethers.

The methoxy-substituted glycerol ethers and the unsubstituted ones were isolated from the unsaponifiable fractions of the neutral lipids and the phospholipids by chromatography on silicic acid columns. The glycerol ethers were converted to their isopropylidene derivatives and rechromatographed on silicic acid columns or on thin-layer plates. When present in more than trace quantities, the two classes of glycerol ethers were estimated by weighing. The composition of the mixtures of the methoxy-substituted and the unsubstituted glycerol ethers was determined by gas chromatography and mass spectrometry. In a few cases, ethanol was used instead of methanol in the saponification, extraction and chromatographic procedures to exclude the possibility that the methoxy-substituted glycerol ethers might be artifacts formed by methanol treatment.

2-Methoxy-substituted glycerol ethers were found together with the unsubstituted ones in the neutral lipids as well as in the phospholipids of all the materials studied. In the different milk samples, as well as in the various materials from man, only trace quantities of methoxy glycerol ethers were found. The content was judged to be larger in the phospholipids than in the neutral lipids. In the marine animals, the percentage of methoxy glycerol ethers was higher, especially in the phospholipids, than in the mammalian tissues (Fig. 7). The highest content was found in the phospholipids from sea mussels and marine crayfish (0.47 and 0.35%, respectively). The content of unsubstituted glycerol ethers in the lipids of herring, Baltic herring and mackerel fillets is more or less comparable to the amounts found in mammalian tissues. A somewhat higher content of unsubstituted glycerol ethers was found in crayfish, shrimps and mussels. As compared to shark liver oil, cod liver oil contained only small quantities of glycerol ethers, unsubstituted as well as methoxy-substituted ones.

The same principal components as in Greenland shark liver oil were found in the methoxy-substituted glycerol ethers from the different animal and human tissues studied.[7,8] The dominating components were 2-methoxy-substituted hexadecyl, hexadecenyl and octadecenyl glycerol ethers. The C_{16} compounds amounted to 50–90% of the different mixtures of methoxy glycerol ethers. Particularly high contents of C_{16} compounds were found in human, cow's and sheep's milk. A methoxy-substituted docosahexaenyl glycerol ether, first found in Greenland shark liver oil, was also isolated from other sources, *viz.* human red blood cells, shrimps, mackerel and cod liver oil.

IV. SYNTHESES OF METHOXY- AND HYDROXY-SUBSTITUTED GLYCEROL ETHERS

A. *2-Methoxy-substituted, Saturated and Monounsaturated Glycerol Ethers*

For confirmation of the structures proposed for the substituted glycerol ethers isolated from shark liver oil and also to get material for biological tests, 1-*O*-(2-methoxyalkyl)glycerols and 1-*O*-(2-methoxy-4-*cis*-alkenyl)glycerol were synthesized.[15] The routes, exemplified by the 16:0 and 16:1 compounds, are outlined in Fig. 8, in which the yields in the different steps are also given.

As starting materials, 2-methoxy-substituted esters were needed. The saturated methoxy ester was easily prepared from the bromo ester and sodium methoxide. The syntheses of the corresponding acetylenic esters[14] seemed more time-consuming as the different routes first tried were multi-step malonic ester syntheses. The overall yields were only about 25%. However, it was found that a reaction between an alkyl alkoxyacetate and a substituted propargyl halide, as exemplified in step 1 in route II (Fig. 8), directly

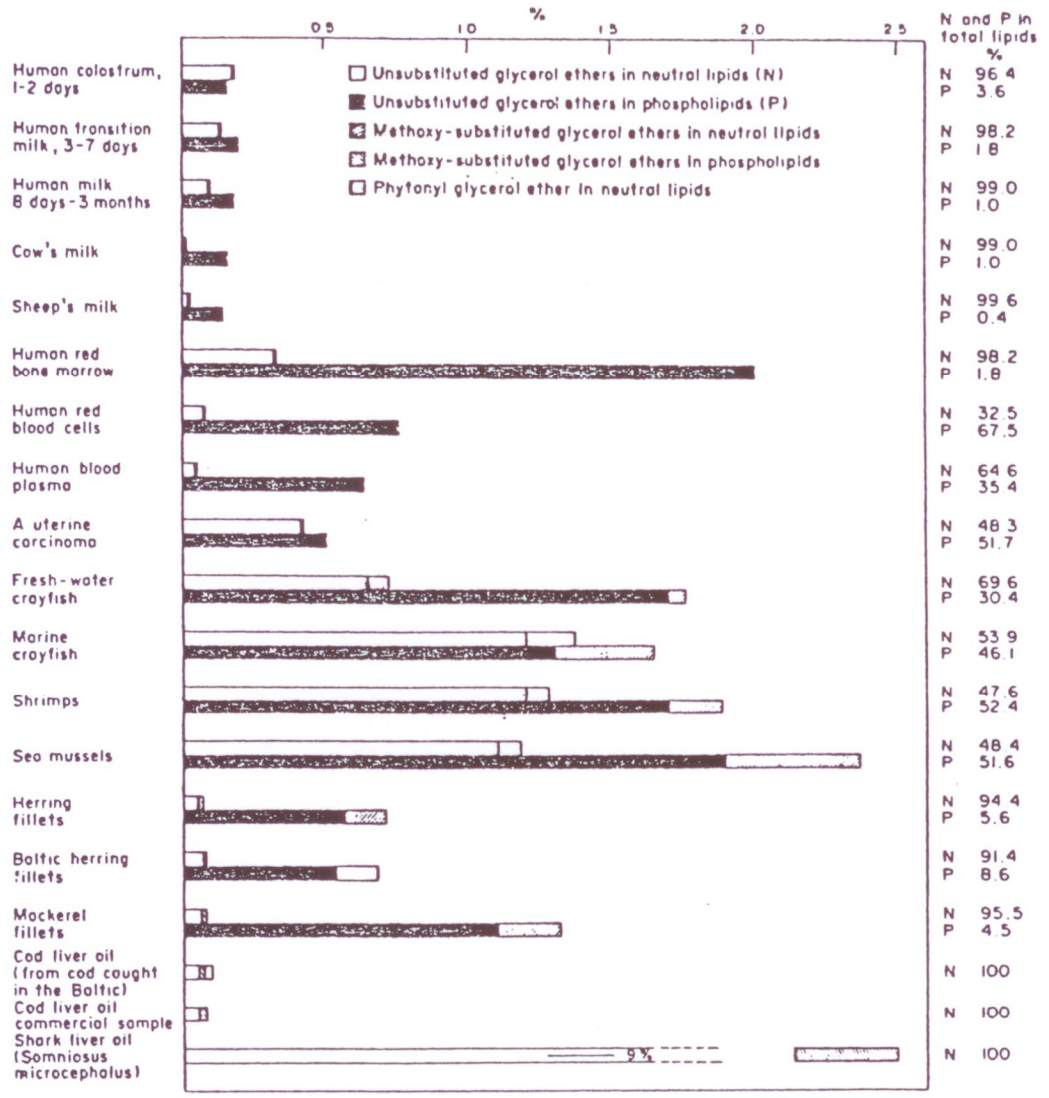


FIG. 7. Unsubstituted and methoxy-substituted glycerol ethers in neutral lipids and phospholipids from various human and animal sources. The contents of neutral lipids and phospholipids in the total lipids are given in the right column.

gave the desired ester of 2-alkoxy-4-alkynoic acid in a good yield. The methylene group of the alkyl alkoxyacetate was in fact reactive enough to be alkylated not only by substituted propargyl halides but also by substituted allyl halides and saturated halides, although in the latter cases to a lesser extent. The alkylations were carried out in tetrahydrofuran or 1,2-dimethoxyethane, with potassium, sodium or sodium hydride as condensing agents and with the alkyl alkoxyacetate in fairly large excess.

The reductions of the esters to alcohols were performed with lithium aluminium hydride in ether solution or with Red Al[5] in hexane (Red Al obtained as a 70% solution of sodium *bis*-(2-methoxyethoxy)aluminium hydride in benzene).

The alkylations[1] of isopropylideneglycerol were performed in heptane with either the *p*-toluenesulfonate or the methanesulfonate of the appropriate substituted alcohol. In most cases, potassium hydroxide was used for the salt formation of isopropylidene glycerol; the water formed was removed by azeotropic distillation. The ketal protecting group on the glycerol moiety of the condensation product was removed by treatment with a mixture of ether and concentrated hydrochloric acid at room temperature.

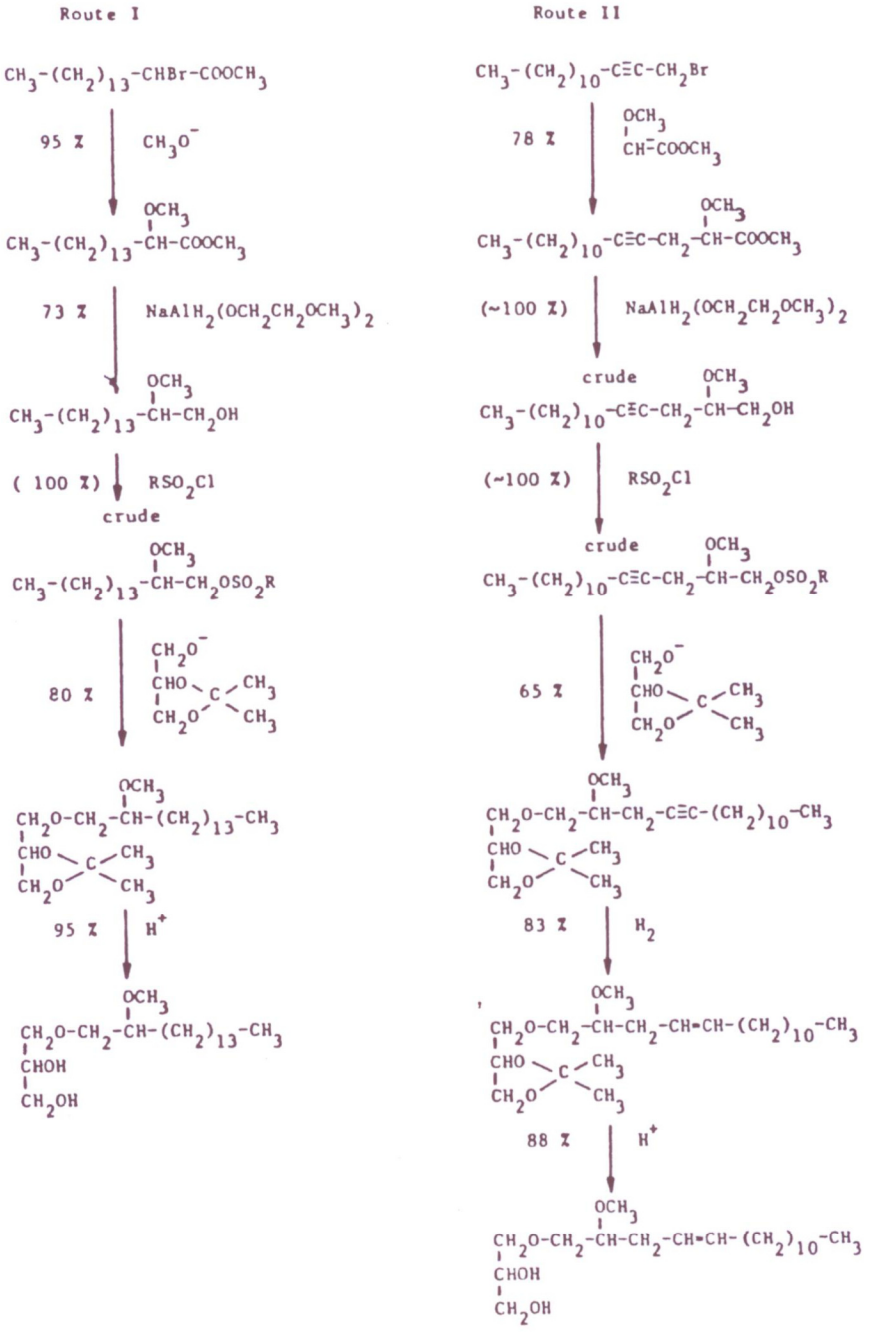


FIG. 8. Flow sheets for the syntheses of 1-*O*-(2-methoxyhexadecyl)glycerol, route I, and 1-*O*-(2-methoxy-4-*cis*-hexadecenyl)glycerol, route II.

The hydrogenation of the acetylenic glycerol ethers (or their isopropylidene derivatives) to the corresponding *cis*-olefinic compounds was achieved using palladium on barium sulfate as catalyst and pyridine as solvent.[6]

The four possible stereoisomers of the methoxy-substituted glycerol ethers have not yet been prepared. They would be of great interest for biological testing and for determination of the configuration of the naturally occurring compounds.

B. *2-Hydroxy-substituted, Saturated and Monounsaturated Glycerol Ethers*

1-*O*-(2-Hydroxyhexadecyl)glycerol[15] was obtained by hydrogenolysis of 1-*O*-2-benzyloxyhexadecyl)-2,3-*O*-isopropylideneglycerol (95% ethanol, 10% palladium on charcoal as catalyst) and removal of the protecting ketal group by acid hydrolysis.

The monounsaturated compound, 1-*O*-(2-hydroxy-4-*cis*-hexadecenyl)glycerol,[10] was obtained by hydrogenation of 1-*O*-(2-benzyloxy-4-hexadecynyl)-2,3-*O*-isopropylideneglycerol in pyridine solution and with 5% palladium on barium sulfate as catalyst.[6] This gave a mixture of benzyloxy- and hydroxy-substituted hexadecenyl compounds, which were separated by chromatography on a silicic acid column. Acid hydrolysis of the appropiate isopropylidene derivative then gave 1-*O*-(2-hydroxy-4-*cis*-hexadecenyl)-glycerol.

The benzyloxy compounds[14,15] needed for the hydrogenations were synthesized in the same way as outlined by routes I and II, Fig. 8, for the methoxy-substituted hexadecyl and hexadecynyl glycerol ethers.

V. BIOLOGICAL EFFECTS OF SUBSTITUTED GLYCEROL ETHERS

In experimental studies, the methoxy-substituted glycerol ethers have demonstrated different biological activities, such as antibiotic effects against bacteria, fungistatic activity against dermatophytes, inhibition of tumor growth and of metastasis formation and stimulation of the immune reactivity.

The mixture of methoxy-substituted glycerol ethers from Greenland shark liver oil as well as synthetic 2-methoxyhexadecyl glycerol (mixture of stereoisomers) had an antibiotic effect *in vitro* against several types of bacteria, especially *Corynebacterium hofmannii*, *Diplococcus pneumoniae*, *Staphylococcus pyogenes* (A) and *Staphylococcus pyogenes* (H Oxford), *Streptococcus pyogenes* and *Streptococcus viridans*. The antibiotic activity was about as strong as that of nitrofurantoin.[3]

The methoxy-substituted glycerol ethers showed a fungistatic and fungicidal activity *in vitro*. The dermatophytes used in these studies were monosporically selected strains of *Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*. In the fungistatic tests, the mixture from Greenland shark liver oil at a concentration of 100 μg/ml inhibited dermatophyte growth in the range of 25–60%. The synthetic compounds, 2-methoxyhexadecyl and 2-methoxyhexadecenyl glycerol, at the same concentration demonstrated an inhibition of 5–15%. In the fungicidal tests, the methoxy glycerol ethers in high concentrations, 1000 μg/ml, inhibited the growth of *T. rubrum* and *E. floccosum*.

The substituted glycerol ethers inhibited tumor growth both *in vitro* and *in vivo*. In the *in vitro* tests, two cell lines were studied, a methylcholanthrene-induced murine sarcoma (MCGI-SS) and a juvenile osteogenic sarcoma (2T). A marked growth inhibition was noted for the mixture of 2-methoxy glycerol ethers from Greenland shark liver oil, different single components from Greenland shark liver oil (2-methoxyhexadecenyl glycerol before and after hydrogenation and 2-methoxyoctadecenyl glycerol) and various synthetic glycerol ethers (2-methoxyhexadecyl, 2-ethoxyhexadecyl, 2-methoxyhexadecenyl, 2-methoxyhexadecynyl, 2-hydroxydodecyl and 3-methoxyhexadecyl glycerol).

In the *in vivo* tests, the effects on the growth of several solid tumors, ascites tumors, leukemias and lymphomas were studied. The glycerol ethers were incorporated into the feed. Growth inhibition was noted on Melanoma B16, and on a methylcholanthrene-induced sarcoma (MCG101) in C57BL/6J mice and on lymphoma LAA in A/Sn mice by synthetic 2-methoxyhexadecyl glycerol and on a spontaneous mammary carcinoma in C3H mice by methoxy-substituted glycerol ethers from Greenland shark liver oil. Spontaneous metastasis formation from a methylcholanthrene-induced sarcoma (MCG1-SS) in CBA mice was inhibited in lymph nodes and lungs by methoxy-substituted glycerol ethers from Greenland shark liver oil and by synthetic 2-methoxyhexade-

cyl glycerol. Metastases induced in the liver by injection of MCG1-SS cells into a tail vein or into the portal vein were inhibited by 0.5% of methoxy-substituted glycerol ethers from Greenland shark liver oil in the feed.[3,4]

The methoxy-substituted glycerol ethers also stimulated the immune reactivity against sheep red blood cells (SRBC) and the cellular immune reactivity tested by graft-versus-host reaction. The number of plaque-forming cells (PFC) was determined mostly in CBA mice and in some studies also in C57BL/6J mice and DBA/2J mice. The mixture of methoxy-substituted glycerol ethers from Greenland shark liver oil or synthetic 2-methoxyhexadecyl glycerol given in a concentration of 0.1, 0.25 or 0.5% of the diet for 4 or 14 days before SRBC significantly increased the number of PFC. The effect on cellular immune reactivity was investigated by means of graft-versus-host reaction by determining spleen indices

(spleen index = spleen weight ÷ body weight of treated mice/spleen weight ÷ body weight of controls)

in F_1 hybrid mice receiving spleen cells from the parental strain. Synthetic 2-methoxyhexadecyl glycerol given in a concentration of 0.1% of the feed for 44 days, significantly increased the spleen indices.[2]

In toxicological studies in rats and dogs, it was found that synthetic 2-methoxyhexadecyl glycerol (mixture of stereoisomers) in high doses is toxic, especially to the lymphatic system and to epithelial cells. The high dose levels were 1 g/kg body weight twice a day for 4 weeks to the rats and 350 mg/kg body weight twice a day for the same time period to the dogs. The substance was administered orally by means of a gastric tube to the rats and in gelatine capsules to the dogs. Degenerative changes (vacuolization and some reduction of lymphocytes) were noted in the spleen and in the lymph nodes. The thymus displayed an involution mostly involving the lymphocytic component. Degenerative changes were observed in the tubular renal epithelium and in the epithelium of the urinary bladder. A degeneration of the testis and the ovary and an atrophy of the prostate and uterus were also found. In spite of these widespread pathological findings, the bone marrow was normal. Thus, the methoxy-substituted glycerol ethers in lower doses stimulate the immune reactivity but in higher doses they are toxic to the lymphatic system.

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