Characterization and identification of glyceryl ether diesters present in tumor cells

RANDALL WOOD and FRED SNYDER

Medical Division,* Oak Ridge Institute of Nuclear Studies,† Oak Ridge, Tennessee 37830

ABSTRACT The previously unidentified neutral lipid present in tumor tissues has been isolated from Ehrlich ascites cells and unequivocally identified as a lipid class of glyceryl ether diesters containing various degrees of unsaturation, and ranging in approximate molecular weight from 760 to 990. The glyceryl ether diester fraction was shown to be free from neutral plasmalogens (glyceryl diacyl alk-1′-enyl ethers).

The tumor lipid was subjected to saponification, transesterification, and lithium aluminum hydride reduction. The glyceryl monoethers that resulted from deacylation were the 1-isomers ranging in hydrocarbon chain length from C_{12} to C_{24}. The predominant glyceryl ethers were the hexadecyl (49%), octadecyl (21%), and octadecenyl (14%) derivatives. Saturated and mono- and polyunsaturated fatty acids ranging in chain length from C_{12} to C_{24} carbon atoms were esterified to the glyceryl monoether.

Gas–liquid chromatography, thin-layer chromatography, and nuclear magnetic resonance and infrared spectroscopy were used to characterize and identify the intact tumor lipid and its derived products.

KEY WORDS glyceryl ether diesters · glyceryl monoethers · tumor lipids · gas–liquid chromatography · IR spectroscopy · nuclear magnetic resonance · thin-layer chromatography

THE OCCURRENCE OF an unidentified neutral lipid in tumor tissue (approximately 2% of the total lipids) has previously been reported by Snyder, Cress, and Stephens (1) of this laboratory. This report describes the characterization and identification of this previously unidentified lipid isolated from Ehrlich ascites cells.

Abbreviations: GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; TFA, trifluoroacetate; TLC, thin-layer chromatography; TMS, trimethylsilane.

* Under contract with the U.S. Atomic Energy Commission.

† An operating unit of Oak Ridge Associated Universities, Inc.

MATERIALS AND METHODS

Materials

Synthesis, purification, and physical and chemical properties of the isomeric glyceryl monoethers used as standards have been described (2). The synthetic glyceryl ether diesters (glyceryl 1,2-dipalmitoyl 3-octadecyl and glyceryl 1,2-distearoyl 3-hexadecyl ethers), glyceryl diether monoester (glyceryl 1-stearoyl 2-octadecyl-3-hexadecyl diether) and the neutral plasmalogen (glyceryl 1,2-diacyl 3-alk-1′-enyl ether) were made available to us by Doctors Helmut K. Mangold, Wolfgang J. Baumann, and Harold H. O. Schmid of The Hormel Institute. Synthesis and characterization of the glyceryl ether diesters and the glyceryl diether monoesters and the isolation of the neutral plasmalogen from human perinephric fat have been reported (3–5). Methyl esters and thin-layer chromatography (TLC) standard mixtures were obtained from The Hormel Institute. All solvents were glass-distilled and obtained from Burdick and Jackson Lab., Inc. Other reagents used were reagent grade and used without further purification.

Source of Unidentified Lipid

The previously unidentified tumor lipid was obtained from Ehrlich ascites cells grown in the peritoneal cavity of several hundred white Swiss mice (HA/ICR strain) and harvested as described (1). The corresponding lipid obtained from fibroadenomas that occurred in irradiated rats about a year after total-body exposure to 800 R was also examined. The lipids were extracted and the material was isolated by preparative TLC (1).

Thin-Layer Chromatography

Uniform 0.25 mm layers of Silica Gel G were spread on 2 × 20 and 20 × 20 cm glass plates with an applicator (Colab Laboratories, Inc., Chicago Heights, Ill.) we had modified (6). After the chromatoplates had

dried in the air for 30 min they were activated in an oven for 15 min at 110°C. Plates were developed in a chamber of hexane–diethyl ether 90:10. The lipids were made visible by charring according to the procedure of Privett and Blank (7, 8) when the results were to be quantified or documented by photography. When the separated components were to be used for further analyses, we located the components by spraying the plates with 0.2% 2′,7′-dichlorofluorescein in ethanol. Appropriate regions, corresponding to standards, were scraped from the chromatoplates and the lipids were eluted with diethyl ether.

Gas–Liquid Chromatography

An Aerograph Model 200 gas chromatograph equipped with hydrogen flame ionization detectors was used for the analysis of methyl esters, glyceryl ether, and alcohol trifluoroacetate (TFA) derivatives. Analyses were made on 5 ft × 1/8 inch glass columns packed with 15% ethylene glycol succinate–methyl silicone polymer (EGSS-X) coated on 100–120 mesh Gas-Chrom P. A 5 ft × 1/8 inch stainless steel column packed with 5% methyl silicone polymer (SE-30) coated on 60–80 mesh Chromosorb W was also used. Columns were packed and conditioned before use as described previously (9). Detectors were maintained at 230–250°C. Air and hydrogen flow rates were adjusted for maximum detector sensitivity at 80 ml/min of He carrier gas. Injectors were held at 235–240°C.

The chromatographic system used for the analysis of triglycerides and related high molecular weight compounds consisted of an Aerograph Model 500 oven, a Model 328 isothermal temperature controller, a Model 500D electrometer, and a 1.0 mv Brown-Honeywell recorder. Modifications included reduction of the injector and block to a length of 2 inches, location of the carrier gas inlet near the septum, grounding of the "J" negative electrode to the platinized apex of quartz flame tip (0.03 inch I.D.), and the addition of a Brooks Model 8743 flow controller. Analyses were made on a 21 × 0.12 inches O.D. (approximately 0.06 inches I.D.) Pyrex glass column, packed with 3% JXR (Applied Science Laboratories Inc., State College, Pa.) coated on 100–120 mesh Gas-Chrom Q. The column was thermally conditioned for 12 hr at 400°C. Column seals were made with a 1/8 inch Swagelok nut, Teflon "O" ring Swagelok back ferrule reversed, and a Viton "O" ring. These were placed on each end of the column, in the order given, and drawn down tight at 350°C. The column was extended through the injector to within 1/4 inch of the septum. The injector block was maintained at 330°C. The septum holder, containing a silicon rubber disc faced with a Teflon disc, was removed and a 5–10 μg sample diluted in 1.0 μl of carbondisulfide was injected directly into the glass column entrance, but not on the column packing. The septum holder and septa were quickly replaced and the oven temperature was manually programmed at the desired rate. Air and hydrogen flow rates were adjusted to give maximum detector sensitivity at 100 ml/min of He carrier gas.

Infrared Spectroscopy

Infrared (IR) spectra were obtained with a Perkin-Elmer Model 521 grating infrared spectrophotometer with 300 mg KBr discs that contained approximately 1.2 mg of sample. An IR spectrum was obtained for each compound from 2.5 to 40 μ. Regions 2.5–2.7, 3.7–5.27, and 20–40 μ of little or no absorption have not been reproduced in the spectra shown.

Nuclear Magnetic Resonance Spectroscopy

A Varian A-60 high resolution nuclear magnetic resonance (NMR) spectrometer was used to obtain proton resonance spectra. Spectra 1, 2, and 3 were obtained on 24.8, 24.8, and 23.3 mg of sample, respectively, in a solution of deuterochloroform with a 150 μl microcell at room temperature. The single proton resonance peak of tetramethylsilane (TMS) used as an internal standard was assigned the value of 0 ppm of the total magnetic field. Spectra were obtained at the Oak Ridge National Laboratory.

Methods

Methyl esters, alcohols, and the isolated tumor lipid were hydrogenated with palladium chloride as catalyst (10). The unidentified lipid (hydrogenated and unhydrogenated) was reduced with lithium aluminum hydride (11), and the resulting products were isolated by TLC. The chromatoplates were developed in chloroform–methanol 98:2 and the hydrogenolysis products located with a fluorescent spray.

The unidentified material was saponified by refluxing with a several-fold excess of 1.0 N ethanolic KOH for 1–2 hr. After extraction of the nonsaponifiable fraction, the soaps were acidified and the fatty acids extracted. Fatty acids were methylated with an ethereal solution of diazomethane, prepared according to the procedure of De Boer and Backer (12). The unidentified lipid was also transesterified by refluxing in 2% H_2SO_4–methanol for 2 hr. The products (methyl esters and unesterified materials) were separated by the TLC system just described.

Alcohols and glyceryl monoethers were trifluoroacetylated as previously described (9) for GLC analysis.

All hexane and diethyl ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness in vacuo with a rotary evaporator. Samples were kept under

a dry nitrogen atmosphere as much as possible. All samples were stored at −20°C under nitrogen.

RESULTS AND DISCUSSION

Characterization of the Intact Tumor Lipid

Comparison of TLC Behavior with That of Known Lipids

Substantial quantities of a previously unidentified lipid present in a number of tumors, but not observed at comparable chromatographic loads in the normal tissues and fluids of the host, have been reported (1). It was formerly further established by TLC that this tumor lipid was not a methyl ester, but that its R_f value corresponded to that of a glyceryl ether diester. A chromatoplate depicting the TLC behavior of the tumor lipid is shown in Fig. 1. The tumor component isolated from total lipids of Ehrlich ascites cells shows a polarity intermediate between those of sterol esters and triglycerides. The standard neutral plasmalogen, glyceryl diether monoester, and aldehyde migrated higher than the unidentified lipid. The compact glyceryl ether diester spot (lane *3*), representing a saturated member of the homologous series, corresponds to the top of the somewhat elongated tumor lipid spot (lane *2*). The isolated component (lane *2*) comprises a lipid class of wide molecular weight range (Fig. 2) that varies in degree of unsaturation (Figs. 4 and 6, below), which accounts for the elongated TLC spot observed for the tumor lipid (lane *2*).

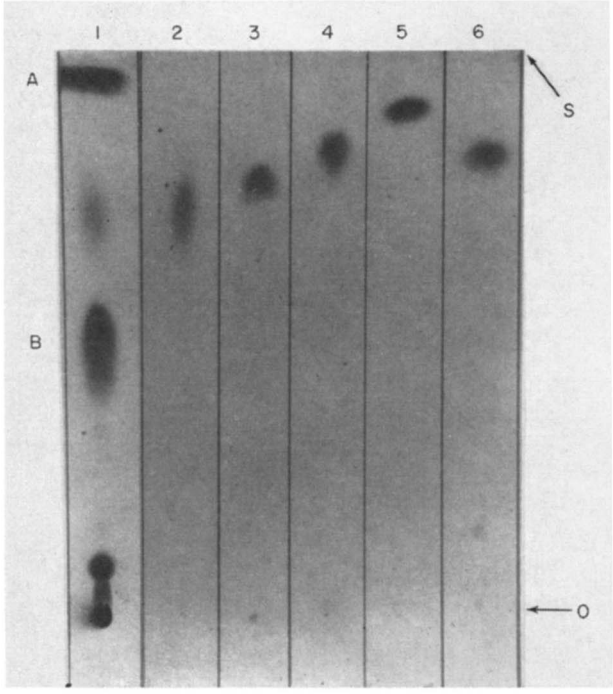

Fig. 1. Thin-layer chromatoplate of the tumor lipid isolated from Ehrlich ascites cells and of known compounds of similar polarity. Development was carried out on Silica Gel G in an unequilibrated chamber with hexane–diethyl ether 90:10. Lane *1*, total lipids from Ehrlich ascites cells (A, cholesteryl esters and B, triglycerides); lane *2*, tumor lipid isolated from Ehrlich ascites cells by preparative TLC; lane *3*, synthetic glyceryl ether diester; lane *4*, a neutral plasmalogen (glyceryl diacyl alk-1′-enyl ether); lane *5*, glyceryl diether monoester; and lane *6*, hexadecanal. The origin and solvent front are indicated by O and S, respectively.

GLC Analysis of Intact Tumor Lipid

A previous attempt to chromatograph the unidentified lipid on a polar column used for methyl ester analysis failed (1). Fig. 2 shows a chromatogram of the intact unidentified lipid obtained with a chromatographic system used for triglyceride analysis. The analysis revealed that the lipid was not a single component, but a homologous series of compounds differing by two methylene units. Tristearin (carbon number 54) added as a standard gave a peak between those designated 54 and 56 in Fig. 2, and this suggested a series of odd carbon number triglycerides. However, methyl ester analysis failed to show any significant amounts of odd carbon numbered fatty acids. A mixture of glyceryl 1,2-distearoyl 3-octadecyl ether and tristearin, which differ in molecular weight by only 14 (the same as would be caused by one methylene unit) was separable with this GLC system.

The glyceryl 1,2-distearoyl 3-octadecyl ether added to the tumor lipid had the same retention time as peak 54. This glyceryl ether diester also eluted with one of the peaks of the tumor lipid isolated from a fibroadenoma from irradiated rats (1). Since the GLC system used could not distinguish between saturated and unsaturated compounds, these results suggested that the unidentified tumor lipid was either a glyceryl ether diester or a neutral plasmalogen.

We have used the same carbon number assignment for the glyceryl ether diesters as is employed for triglycerides (13), although the glyceryl ether diester retention times are shorter because the monoesters have one less oxygen atom. Only qualitative results were obtained because of the high operating temperatures. Hydrogenated samples that were analyzed later, when the column had been thermally conditioned longer and retention times were much shorter (C_{54} before 330°C), gave a similar pattern (Fig. 2) but larger percentages for the higher carbon numbers.

IR Spectra

In Fig. 3 are shown the IR spectra of synthetic glyceryl 1,2-distearoyl 3-octadecyl ether (spectrum *1*), hydrogenated tumor lipid (spectrum *2*), unhydrogenated tumor lipid (spectrum *3*), and the neutral plasmalogen (spectrum *4*). Spectra *1* and *2* show similar absorption. Small observable differences can be attributed to the larger number of molecular species of different chain lengths present in the tumor lipid. Absorption in the

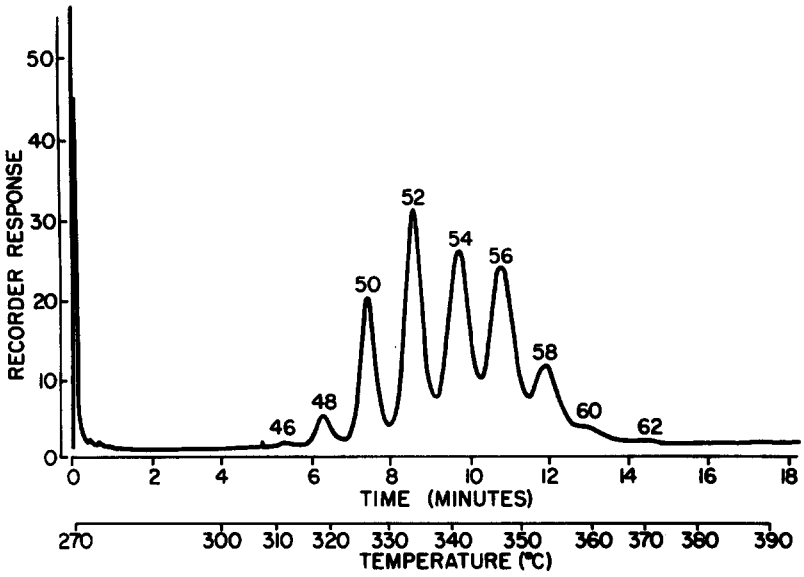

FIG. 2. Gas–liquid chromatogram obtained for the intact unhydrogenated tumor lipid on JXR. Carbon numbers assigned on the basis of glyceryl 1,2-distearoyl 3-octadecyl ether standard chromatographed with the sample.

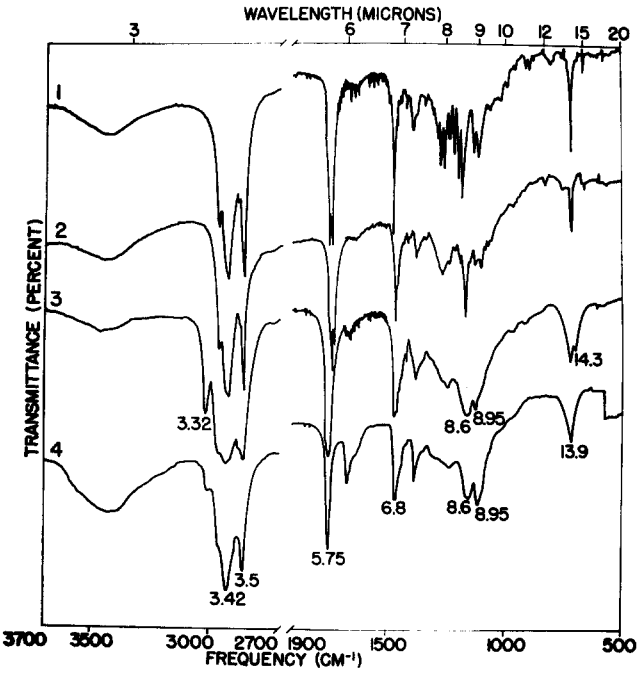

FIG. 3. Infrared spectra of: *1*, synthetic glyceryl ether diester; *2*, hydrogenated tumor lipid; *3*, unhydrogenated tumor lipid; and *4*, neutral plasmalogen.

region of 3.32 μ of spectrum *3* (absent from spectrum *2*) shows the tumor lipid to be highly unsaturated. This was in agreement with methyl ester analysis (see below). A neutral plasmalogen, after hydrogenation, yields a spectrum similar to spectrum *2* (5). Spectra *3* and *4* differ by the intensity of absorption in the region of 6.1 μ, absorption being much stronger for the neutral plasmalogen. The slight absorption in this region of spectra *1*, *2*, and *3* is due to water vapor, which also gives rise to absorption at 2.95 μ. All spectra show ether absorption 8.95 μ and carbonyl absorption at 5.75 μ. Absorption due to the hydrocarbon and ether contributions to the spectra, and a more detailed interpretation of spectrum *4* have been reported (2, 5). Comparison of these IR spectra strongly suggests that the tumor lipid is a highly unsaturated glyceryl ether diester with little or no neutral plasmalogen present.

NMR Spectra

Fig. 4 shows the NMR spectra of the tumor lipid (spectrum *1*), partially hydrogenated tumor lipid (spectrum *2*), and an equal weight mixture of synthetic glyceryl 1,2-distearoyl 3-hexadecyl and glyceryl 1,2-dipalmitoyl 3-octadecyl ethers. The spectra were taken at 5 (Curve *C*), 20 (Curve *B*), and 80 (Curve *A*) amplifications. When differences due to unsaturation are excluded from spectra *1* and *2*, they are identical with spectrum *3*. These differences are due to vinyl proton resonance at 5.35 ppm, methylene protons between two methylene-interrupted double bonds at 2.83 ppm, and methylene protons allyl to a double bond at 2.04 ppm. Chemical shifts agree with those reported (14–16). Resonance in the regions of 5.15, 4.18, 3.5, and 3.4 ppm is due to the presence of a single 2-glyceryl carbon atom proton, a pair of glyceryl protons distal to the ether linkage, a pair of nonequivalent glyceryl protons adjacent to the ether oxygen, and the methylene hydrocarbon protons adjacent to the ether oxygen, respectively. These regions of resonance are prominent in all three spectra with the exception of the

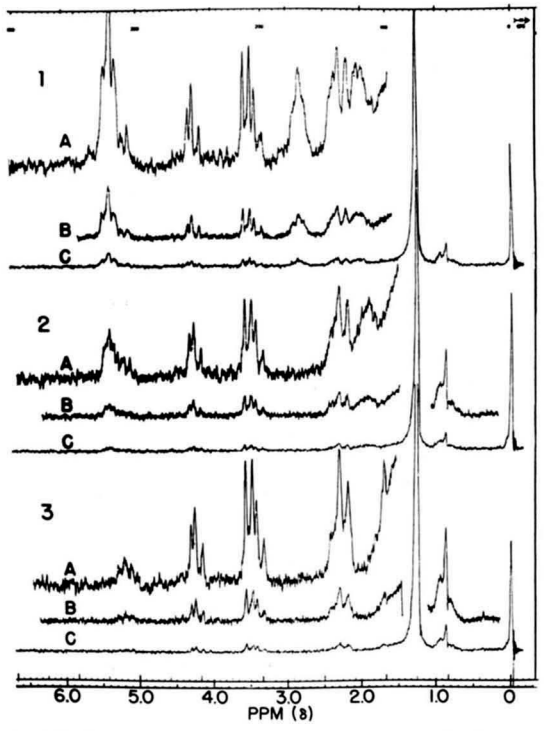

FIG. 4. Nuclear magnetic resonance spectra at 5 (Curve *C*), 20 (Curve *B*), and 80 (Curve *A*) amplification of: *1*, unhydrogenated tumor lipid; *2*, partially hydrogenated tumor lipid; and *3*, mixture of glyceryl 1,2-dipalmitoyl 3-octadecyl and glyceryl 1,2-distearoyl 3-hexadecyl ethers.

quintuplet at 5.15 ppm, which is partially obscured by the vinyl proton resonance at 5.35 ppm. Glyceryl and ether proton chemical shifts are in accord with those reported for free glyceryl monoethers and glyceryl monoether diacetates (2,17). Resonance of methylene protons adjacent to an ester group appeared at 2.25 ppm, hydrocarbon methylene protons occurred at 1.27 ppm, and terminal methyl protons at 0.9 ppm.

Warner and Lands (18) have obtained spectra of methyl *cis*- and *trans*-1-dodecynl ethers, and Craig and Hamon (19) have obtained spectra of 1-glyceryl *cis*- and *trans*-alk-1′-enyl monoethers. The single vinyl proton on C_1 of the hydrocarbon chain appeared as a doublet at approximately 5.9 and 6.2 ppm for the *cis* and *trans* isomers, respectively. We have found this doublet at 5.83 ppm in neutral plasmalogens. Because there is no resonance in this region of the tumor lipid spectrum, the presence of neutral plasmalogens can be discounted. These NMR spectra indicate that the tumor lipid is a glyceryl ether diester containing double bond(s).

Identification of Hydrolysis and Hydrogenolysis Products

TLC

In Fig. 5 is shown a typical thin-layer chromatogram depicting the products resulting from $LiAlH_4$ reductive cleavage of the tumor lipid (lane *3*) and tripalmitin (lane *4*). Alcohol and glyceryl monoether (1-octadecenyl) standards appear in lanes *1* and *2*. The elongated alcohol spot (lane *3*, upper spot) is due to the wide range of chain lengths found in the tumor lipid. This was verified by TLC of mixtures containing alcohols of various chain lengths and by GLC of the alcohol TFA derivatives obtained from the upper and lower regions of the elongated alcohol spot. Quantitative densitometric TLC analysis was carried out on chromatoplates which were developed in hexane–diethyl ether–methanol 80:20:10, then scored below the alcohol region and developed again in chloroform–methanol 98:2. Each component under these development conditions gave a compact spot. The alcohols and glyceryl ethers were found to represent 67.9 and 32.1%, respectively, of the total mass, which was in close agreement with the expected values for a glyceryl ether diester.

Saponification of the tumor lipid followed by acidification yielded two major products with R_f values on TLC equal to those of fatty acids and glyceryl monoethers. This agreed with earlier results (1). Occasionally we ob-

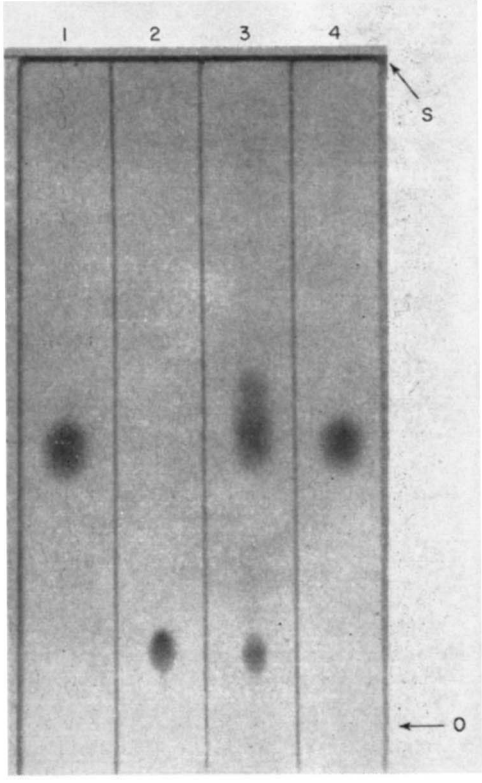

FIG. 5. Thin-layer chromatoplate of the products resulting from $LiAlH_4$ reduction of the tumor lipid (lane *3*) and tripalmitin (lane *4*). Standards shown in lanes *1* and *2* are hexadecanol and a synthetic glyceryl octadecenyl 1-monoether, respectively. Development was carried out on Silica Gel G in chloroform–methanol 98:2. The origin and solvent front are indicated by O and S, respectively.

served other small spots, which were assumed to be partial saponification products.

Acid-catalyzed transesterification of the tumor lipid produced components with R_f values on TLC that corresponded to methyl esters and glyceryl ethers.

Each of the products resulting from $LiAlH_4$ reduction, saponification, and transesterification were isolated by preparative TLC for GLC analyses.

GLC

Alcohols and glyceryl ether TFA derivatives and methyl esters were analyzed on polar and nonpolar columns before and after hydrogenation. Methyl esters and alcohols ranging in chain length from C_{12} to C_{24} were tentatively identified on the basis of retention time data and cochromatography with known standards. Palmitic and stearic were the most abundant saturated acids. Oleic acid was the major monoene. Dienes and higher unsaturates were confined to the longer-chain fatty acids. Only traces of fatty acids with odd-carbon chain lengths were observed. Methyl ester values obtained after saponification and transesterification were close and agreed well with the percentages of alcohol formed after reduction. Hydrogenated methyl ester values obtained by the two methods agreed well, but were somewhat out of line with the hydrogenated alcohol percentages. This discrepancy was later attributed to an artifact produced in the hydrogenation of alcohols.

Analyses of glyceryl ether TFA derivatives by GLC, capable of resolving 1- and 2-isomers (9), identified glyceryl ethers ranging in hydrocarbon chain length from C_{12} to C_{22}. The chromatograms in Fig. 6 show a typical analysis of a synthetic standard and of hydrogenated and unhydrogenated samples from the tumor lipid. Retention time data and cochromatography with isomeric standards revealed only 1-isomers present in the tumor lipid. The 16:0-1 and 18:0-1 (see Fig. 6 for abbreviated nomenclature) components represented more than 90% of the saturated glyceryl ethers. Saturated glyceryl ethers with odd-carbon chain lengths ranging from C_{13} to C_{19} and possibly C_{21} were present. The major unsaturated glyceryl ether was 18:1-1. A number of unidentified components, possibly branched-chain or monounsaturated odd-carbon chain length glyceryl ethers, were present in trace amounts. Similar glyceryl ether composition was obtained for the tumor lipid after $LiAlH_4$ reduction, saponification, and transesterification.

IR Spectra

The IR spectra (Fig. 7) of synthetic 1-glyceryl hexadecyl ether (spectrum *1*) and of hydrogenated glyceryl ethers isolated after transesterification of the tumor lipid (spectrum *2*) are practically identical. The small differences are probably due to the wide molecular weight range present (see Fig. 6). Spectrum *3* (unhydrogenated glyceryl ethers isolated after $LiAlH_4$ reduction of tumor lipid), obtained on a liquid sample, does not show the discrete and detailed absorption bands of the solid samples of spectra *1* and *2*; however, the primary absorption bands are still present. The appearance of a weak carbonyl absorption band at 5.75 μ suggests contamination due to incomplete reduction of

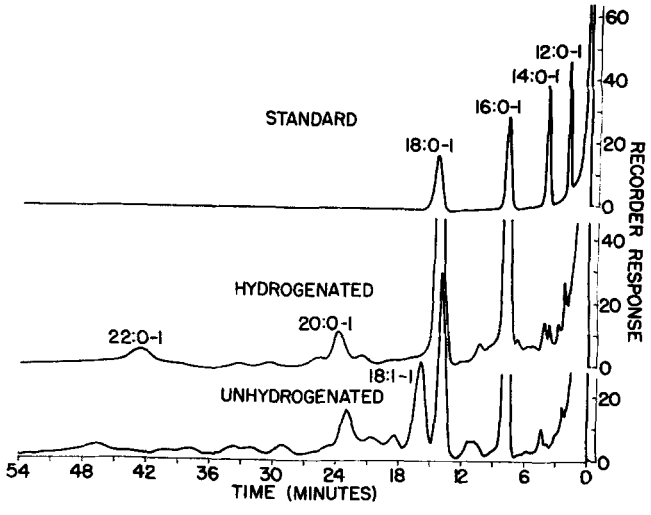

FIG. 6. Gas–liquid chromatograms of a glyceryl ether standard and of hydrogenated and unhydrogenated glyceryl monoethers isolated from the tumor lipid after degradation. Glyceryl ethers were chromatographed as their trifluoroacetate derivatives. The peaks are identified by a three-part number that represents the number of carbon atoms in the hydrocarbon chain, the number of double bonds in the molecule, and to what glycerol carbon atom the hydrocarbon chain is linked (9).

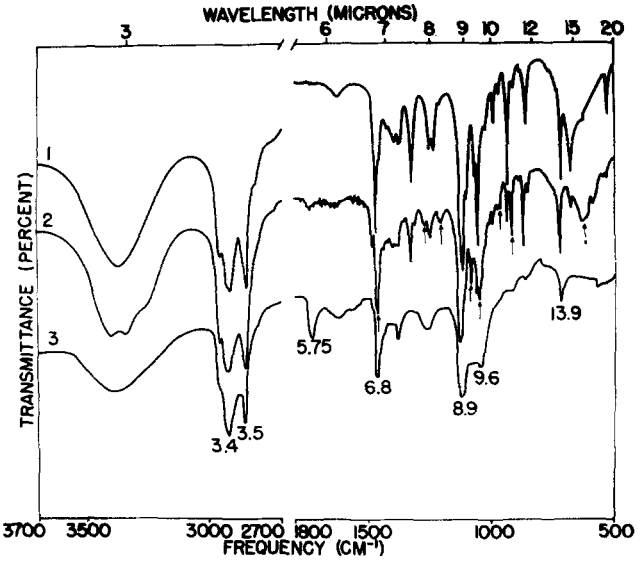

FIG. 7. Infrared spectra of: *1*, a synthetic glyceryl monoether, and *2*, hydrogenated and *3*, unhydrogenated glyceryl ethers isolated from tumor lipid after degradation.

tumor lipid. However, TLC and GLC showed that incomplete reduction seldom occurred. All three spectra show absorption in the regions of 8.9 and 9.6 μ, characteristic of the 1-isomers, previously shown to be distinguishable from the 2-isomers (2).

Identification of Tumor Lipid

These physical and chemical characteristics of the intact tumor lipid and of its hydrolysis and hydrogenolysis products show that the lipid consists of glyceryl ether diesters containing no neutral plasmalogens. We have also established (data not given) that the lipids isolated from a fibroadenoma from irradiated rats, previously analyzed by TLC (1), contained glyceryl ether diesters. The lipids of a human lymphosarcoma and two rat tumors (Fischer R3259 and Walker 256 carcinosarcoma) reported on earlier (1), a number of human tumors (adenocarcinoma of the rectum, carcinoma of the cervix, adenocarcinoma of the prostate, bronchogenic carcinoma, malignant giant cell tumor of bone, pleomorphic sarcoma of soft tissue, carcinoma of bladder, and carcinoma of the ovary), and a mouse KHZ mammary tumor have been shown to contain a lipid component with an R_f value on TLC identical with that of a glyceryl ether diester. However, confirmation of the glyceryl ether diester's presence in each of the tumors by the techniques described in this report has not been made.

We emphasize here that glyceryl ether diesters do not occur solely in tumor tissue. Gilbertson and Karnovsky (20) have reported the percentage of glyceryl ether diesters (neutral plasmalogens included), calculated from the glyceryl ether content of nonsaponifiable neutral lipid, for a number of mammalian tissues. Schmid and Mangold (5) have recently identified glyceryl ether diesters and neutral plasmalogens in human perinephric fat at concentrations (approximately 0.3% of the total lipids) much lower than that observed for tumor tissue (1). The origin and significance of this class of compounds in tumors are not known.

The authors thank Edgar A. Cress and Nelson Stephens for their skilled technical assistance.

Manuscript received 20 March 1967; accepted 22 May 1967.

References

1. Snyder, F., E. A. Cress, and N. Stephens. 1966. *Lipids.* **1:** 381.
2. Wood, R., and F. Snyder. 1967. *Lipids.* **2:** 161.
3. Baumann, W. J., and H. K. Mangold. 1966. *J. Org. Chem.* **31:** 498.
4. Baumann, W. J., and H. K. Mangold. 1966. *Biochim. Biophys. Acta.* **116:** 570.
5. Schmid, H. H. O., and H. K. Mangold. 1966. *Biochem. Z.* **346:** 13.
6. Wood, R., and F. Snyder. 1966. *J. Chromatog.* **21:** 318.
7. Privett, O. S., and M. L. Blank. 1962. *J. Am. Oil Chemists' Soc.* **39:** 520.
8. Privett, O. S., M. L. Blank, D. W. Codding, and E. C. Nickell. 1965. *J. Am. Oil Chemists' Soc.* **42:** 381.
9. Wood, R., and F. Snyder. 1966. *Lipids.* **1:** 62.
10. O'Brien, J. S., and G. Rouser. 1964. *Anal. Biochem.* **7:** 288.
11. Horrocks, L. A., and D. G. Cornwell. 1962. *J. Lipid Res.* **3:** 165.
12. De Boer, Th. J., and H. J. Backer. 1954. *Rec. Trav. Chim.* **73:** 229.
13. Kuksis, A., and M. J. McCarthy. 1962. *Can. J. Biochem. Physiol.* **40:** 679.
14. Storey, W. H., Jr. 1960. *J. Am. Oil Chemists' Soc.* **37:** 676.
15. Purcell, J. M., S. G. Morris, and H. Susi. 1966. *Anal. Chem.* **38:** 588.
16. Hopkins, C. Y. 1961. *J. Am. Oil Chemists' Soc.* **38:** 664.
17. Carter, H. E., D. B. Smith, and D. N. Jones. 1958. *J. Biol. Chem.* **232:** 681.
18. Warner, H. R., and W. E. M. Lands. 1963. *J. Am. Chem. Soc.* **85:** 60.
19. Craig, J. C., and D. P. G. Hamon. 1965. *J. Org. Chem.* **30:** 4168.
20. Gilbertson, J. R., and M. L. Karnovsky. 1963. *J. Biol. Chem.* **238:** 893.