Journal of Chromatography, 107 (1975) 155–166

CHROM. 8067

CHROMATOGRAPHIC ANALYSIS OF THE NEUTRAL AND PHOSPHATIDE GLYCERYL ETHERS FROM VARIOUS BIOLOGICAL SOURCES

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(Received October 3rd, 1974)

SUMMARY

A method of analysis is described which permits the facile evaluation of the neutral glyceryl ether lipids (mild alkaline hydrolysis) and total glyceryl ether lipid (Vitride reduction) thereby allowing an assessment of the phosphatide contribution. The alkyl and alk-1-enyl glyceryl ethers are chromatographically resolvable on Gelman glass-fiber type SG and Whatman SG-81 and detected by the periodic acid-Schiff and plasmal reaction, respectively. The attending analytical problems and interpretation are discussed with examples from a number of unicellular organisms and animal tissues.

INTRODUCTION

Recent years have shown credible advances in the chemical understanding of the glyceryl ether lipids which were first discovered by Feulgen and Voit in 1924 in histochemical preparations and named plasmalogens because of their cytoplasmic location[1]. Their alkyl counterparts are not detectable by histochemical means and were first recognized in the non-saponifiable fraction of certain animal tissues and are now known to have been derived *(in vitro)* from more complex lipids[1]. Numerous neutral lipid and phosphatide alkyl and alk-1-enyl glyceryl ethers have been described and their chemical and biochemical features are being intensively studied[1,2]. The increasing interest in their physiological significance invites the development of techniques which permit the ready recognition of the various types as correlated with the total lipid spectrum. We propose to describe here some of our experiences with lipid extracts from numerous biological sources and with a number of analytical procedures adapted to paper (and glass-fiber) chromatographic analysis of their glyceryl ether components.

MATERIALS AND METHODS

Chromatographic procedures

The general chromatographic techniques have been described extensively else-

where[3,4]. We used 15 × 45 cm Pyrex cylinders containing approximately 250 ml of solvent in which the SG-81 or Gelman paper was suspended for ascending chromatography. The Whatman SG-81 silicic acid impregnated paper (13 × 25 cm) was first washed by immersion in chloroform–methanol (1:1) followed by acetone in order to remove the yellow oxidation products which gradually accumulated in these papers. When protected from air and light (wrapped in aluminum foil) these washed papers remained clean for several weeks; they could be safely rewashed following longer storage. The practical shelf life of these papers is at least several years. For the purposes of these studies a slightly modified Marinetti and Stotz[5] diisobutyl ketone–acetic acid–water (120:70:12) (DAW) mixture was found to be satisfactory for the resolution of the glyceryl ethers without interfering with its practical separation of various phosphatides and glycolipids. The DAW mixture was usable for about five days and a chromatographic run took 2–4 h, at room temperature, according to the purpose of the run (*cf.* Fig. 1).

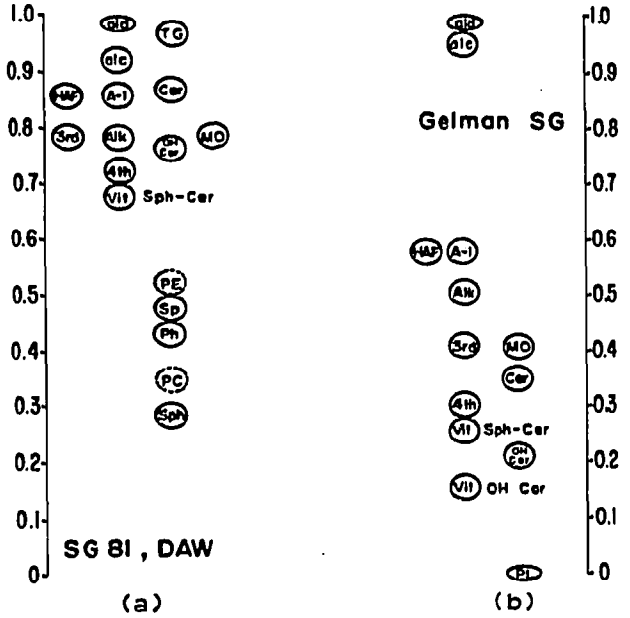

Fig. 1. Distribution patterns of the various lipids pertinent to this study. From top to bottom they are: (a) fatty aldehyde, triglyceride, fatty alcohol, alk-1-enyl a-glyceryl ether, ceramide, alkyl a-glyceryl ether, hydroxy ceramide, monoleate, 3rd and 4th appear to be other alkyl glyceryl ethers, Vitride product of sphingomyelin and ceramide, phosphatidylethanolamine, sphingosine, phytosphingosine, phosphatidylcholine, and sphingomyelin; (b) the alkyl and alk-1-enyl glyceryl ethers are easily differentiated from the ceramides and their Vitride products.

Gelman ITLC type SG (silica gel) impregnated glass fiber paper (10 × 20 cm) was used directly as received from the supplier and did not require pre-washing. The solvent mixture for resolution of the glyceryl ethers consisted of 2,2,4-trimethyl pentane–isopropyl acetate–isopropanol (250:10:5); a chromatographic run took

about 20 min and a single loading of the chromatographic jar with solvent was usable for two to three days. Suitably protected from air and light the shelf life of these papers is also several years.

Silicic acid impregnated cellulose and glass-fiber paper chromatography has some clear advantages over TLC particularly with regard to the plasmalogens (alk-1-enyl) and periodic acid-Schiff (PAS) visualizing reagents where extensive washing with H_2SO_3 is required in order to retain the specificity of these reactions.

Spot tests

The glyceryl ethers were specifically detectable by the PAS reaction and the alk-1-enyl glyceryl ethers were explicitly differentiated by the plasmal reaction. The PAS reaction consists of a 7-min immersion of the air-dried chromatograms (*i.e.*, drying time was 5 min for the Gelman and 15 min for the SG-81, prolonged drying invites spurious staining due to oxidation and there is consequently an opportunity for misinterpretation) in 4×10^{-3} *M* aqueous periodic acid followed by at least four changes of water and one of 0.05 *M* sulphurous acid. The carbonyl groups produced by the periodic acid oxidation of the glyceryl ethers were then visualized by immersion in Schiff's leucofuchsin (in H_2SO_3), 7–10 min, followed by repeated washing in 0.05 *M* sulphurous acid to remove the excess leucofuchsin; the magenta Schiff–aldehyde complex was color-fast and the air-dried chromatograms were permanent. The catalytic cleavage of the vinyl ether linkage (alk-1-enyl) of plasmalogens by aqueous $HgCl_2$ with the production of free fatty aldehyde, which were subsequently coupled with Schiff's reagent, represents a very specific reaction for the detection of alk-1-enyl glyceryl ethers. Consequently 0.05 *M* $HgCl_2$ (1–2 min) was substituted for the periodic acid in the above procedure.

The uniform structure of the SG-81 paper permitted quantitative estimation of the stained chromatograms by using the Beckman-Spinco Analytrol with B-2 cam and 560-nm filters (*cf.* Figs. 2 and 3).

Biological materials

During the course of this investigation we have examined the glyceryl ether content of several hundred extracts obtained from various tissues from twelve different mammals (including some fetal specimens), different fish, amphibia, reptiles and three unicellular organisms including both green and white forms of *Euglena gracilis*. We will illustrate and comment on some of the observations as they bear on the general problem of glyceryl ether analysis and on how they contribute to our understanding of their biological role.

The lipid extracts were prepared from freeze-dried tissue by extraction with chloroform–methanol (2:1) or benzene–methanol (1:1) using 2 ml per 100 mg dry weight of tissue; for the most part such extracts remained essentially unchanged for many months when refrigerated and protected from light.

Commercially available standard lipid substances, both natural and synthetic, of high purity are readily available from several sources and were used here to supplement our own extract data and to help establish the R_F values of Fig. 1; care had to be exercised because some of the specimens were found to be of questionable purity and had to be rejected.

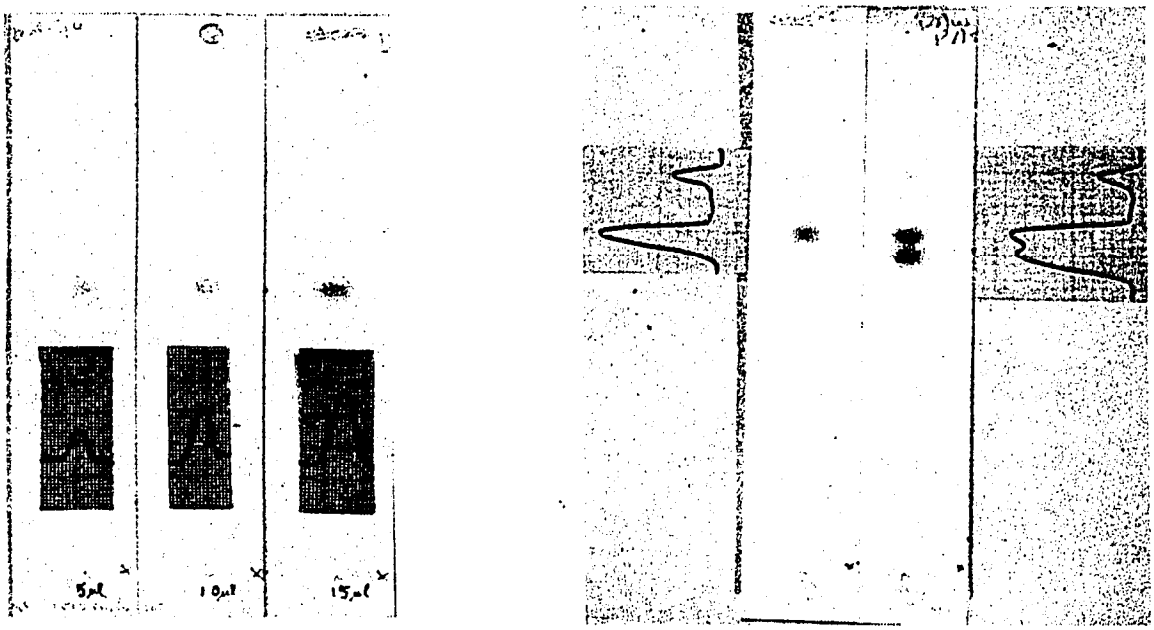

Fig. 2. PAS-stained chromatogram (SG-81, DAW) and Analytrol curves of the a-alkyl glyceryl ether butyl alcohol, 5, 10, and 15 μl of a standard containing 2.5×10^{-5} mmoles/μl. The response is essentially linear.

Fig. 3. Chromatogram and Analytrol tracing of ground-squirrel kidney ether lipids (Vitride reduction) comparing the alk-1-enyl Feulgen plasmal reaction (left) with the PAS reaction (right). The upper, smallest, spot is solvent front lipid, the other two spots are alk-1-enyl and alkyl glyceryl ether, respectively, as illustrated in Fig. 1a.

Formation of glyceryl ethers

Of the specimens studied by us only human amniotic fluid was found to contain a-glyceryl ethers[6] without prior treatment of the lipid by saponification, acetolysis or reduction.

Saponification. One to two milliliters of extract were transferred to 13 × 100 mm screw-cap tubes (PTFE seal) and the solvent removed at 40° by a stream of nitrogen. The lipid was then dissolved in 1 ml benzene and 1 ml 0.5 *M* NaOH in methanol was added, the tube well mixed and left to stand at room temperature for 7 min. Six milliliters of water were then added, the tube well shaken and centrifuged. This clearly separated the benzene containing the glyceryl ethers derived from neutral lipid sources. If gelation occurred it was dispelled by adding one to two drops of 2-butanol.

Acetolysis. One to two milliliters of extract were dried as above, 0.5 ml of a freshly prepared mixture of glacial acetic acid and acetic anhydride (3:2) were added and the tube capped and heated at 130° for at least 8 h. After cooling, 6 ml of water and 1 ml of benzene were added and the tube was well shaken. Following brief centrifugation the overlying benzene was then quantitatively transferred to another 13 × 100 mm tube and the solvent removed by nitrogen as before. Deacetylation was

then accomplished by heating at 60° for 3 h in 1 ml 0.5 *M* NaOH in methanol. On cooling, 6 ml of water and 1 ml of benzene were added, mixed well and centrifuged. The overlying benzene contained the alkyl glyceryl ethers derived from both neutral lipid and phospholipid sources and the free fatty aldehyde derived from the corresponding alk-1-enyl lipids.

Vitride reduction. The total glyceryl ether of tissue extracts can be assayed following reduction by Vitride[7] [bis(2-methoxyethoxy-aluminum hydride), Eastman-Kodak No. 13112 (70% in benzene)]. This reagent resembles $LiAlH_4$ in its general properties but is more handy to use. The lipid from 1–2 ml of extract, thoroughly dried by removing the solvent as above, was re-dissolved in 1 ml benzene. Four to eight drops (Pasteur pipette) of the Vitride were added and the tubes capped and heated at 40° for 1 h. The reaction was terminated by adding 0.5 ml of methanol and 6 ml of water with vigorous mixing. After centrifugation the overlying benzene was found to contain the alkyl and alk-1-enyl glyceryl ethers derived from both neutral lipid and phospholipid sources. When this study was approaching completion we learned that Snyder[2] had already applied the Vitride reagent to the study of glyceryl ethers.

Glyceryl ether assay

Analysis of the above glyceryl ether samples, in benzene, was accomplished by chromatography on Whatman SG-81 and Gelman type SG silica gel impregnated media as described above. Usually there was sufficient sample in 10–20 μl (*i.e.*, approximately 10^{-4} m*M* glyceryl ether) for a very adequate assessment of the neutral lipid contribution (the 7-min saponification), the total alkyl ether contribution (acetolysis) and the total alkyl and alk-1-enyl ether contribution (Vitride reduction). The two chromatographic situations described clearly resolved the a-alkyl from the a-alk-1-enyl glyceryl ethers and between the two systems it was possible to avoid confusion with ceramides (*cf.* Fig. 1).

RESULTS

Saponification

Although deacylation of all the glycerides and phosphoglycerides occurs with saponification only the alkyl and alk-1-enyl diacyl glycerides are assayed by this complete procedure because of their specific chromatographic properties. The lyso compounds derived from the phosphoglycerides are, in addition, poorly soluble in benzene; therefore, this procedure was found to serve as a generally fair assessment of the neutral lipid glyceryl ether content. Whenever interests warrant it, the solvent front materials must be further resolved by chromatography in less polar systems for their possible di- and tri-ether components[3], as only the alk-1-enyl analogues would be specifically visualized (plasmal reaction). Fig. 4 provides an example of its application to cat placenta. Human amniotic fluid had already been shown to contain free a-glyceryl ether[6] and saponification revealed that there were acyl-substituted alkyl ethers as well.

As the figures show, a-alk-1-enyl glyceryl ethers run ahead and are well resolved from a-alkyl glyceryl ethers with only the alk-1-enyl analogues visualized by the plasmal reaction. A notable exception of this is human amniotic fluid and to a

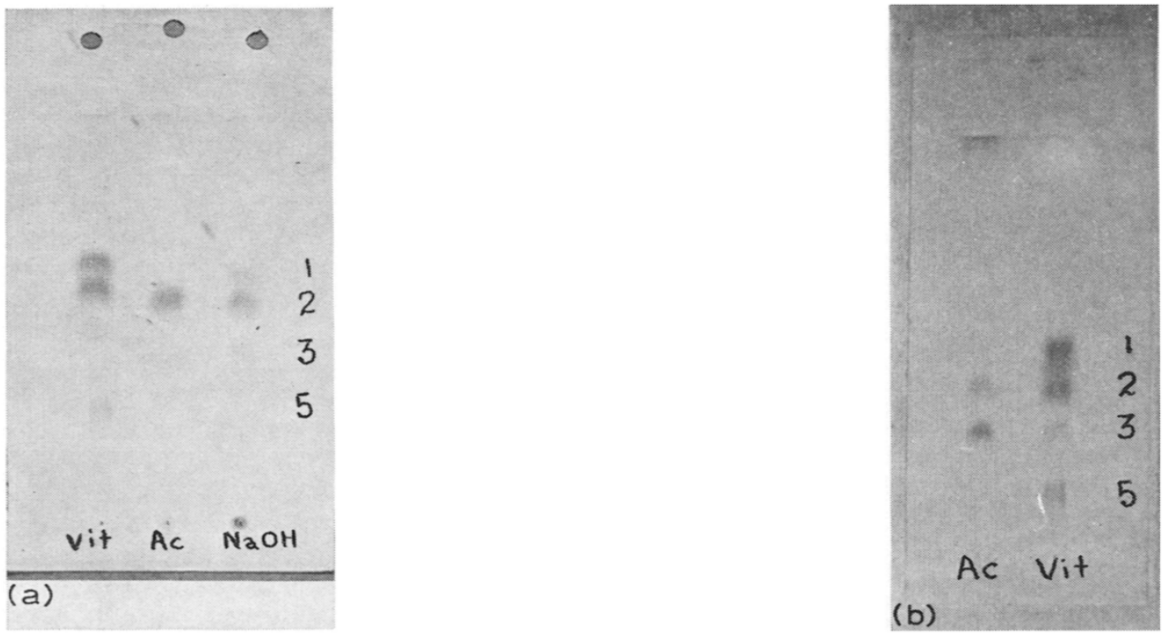

Fig. 4. The derived glyceryl ethers from cat placenta are shown in these chromatograms (Gelman system, PAS stain). (a) From left to right are Vitride, acetolysis, and saponification. (b) Better photographic contrast is seen in this wet chromatogram, the fatty alcohol of the Vitride sample is seen near the solvent front as a white, relatively hydrophobic, spot. Spot 1 is alk-1-enyl, spot 2 alkyl. Spot 3 is referred to in the text as "3rd" spot. Spot 5 is the Vitride product of sphingomyelin-ceramide.

less quantitative extent *Euglena*, where the DAW (R_F value 0.85) and Gelman (0.58) spots were negative to the plasmal reaction yet occupied the same position in the respective chromatographic systems as the a-alk-1-enyl glyceryl ethers.

The Analytrol data revealed that, contrary to expectation, only one equivalent of fuchsin–aldehyde reaction product resulted from staining of the alk-1-enyl ethers by the PAS reaction. That is to say that the optical density of the alk-1-enyl spot was identical whether stained by the plasmal reaction or by the PAS reaction, each representing a different reaction site. The PAS, because of its acidity, was capable of also reacting as a plasmal visualizer but somewhat more slowly. The a-alkyl ether, as a 1,2-glycol, only reacts to the PAS. These facts made visual assessment of these chromatograms much more direct.

Finally, there was an occasional relatively weak staining of the "3rd" spot by leucofuchsin alone, unrelated to either the mechanism of the plasmal reaction or to the PAS reaction which we cannot yet satisfactorily explain. This occurred more frequently in the acetolysis and Vitride samples than in the saponification samples.

Acetolysis

This procedure was found to have two major defects which interfered with its usefulness as an assay technique. Firstly, because of the hydrolysis of the alk-1-enyl lipids the plasmalogens could be less readily assessed. Secondly, the completeness of the hydrolysis and subsequent deacetylation required conditions not yet fully under-

stood. Because of these factors this technique was not explored as much as necessary for a fuller comprehension of the results. It is, however, capable of complete ester cleavage of both neutral glycerides and phosphoglycerides, quantitatively yielding the alkyl ethers as measured against those derived by Vitride reduction. Some extracts provided more difficulties than others. The primary confusion arose from the variable proportion of a-alkyl glyceryl ether and the "3rd" spot which sometimes behaved as though it was a preparative precursor of the normal alkyl ether as seen in the figures. These difficulties have not yet been resolved, however, our simplification of the technique appears to have merit. It remains a likely possibility that the "3rd" spot has biological reality and deserves further study.

Vitride reduction

We have found Vitride reduction of glycerides and phosphoglycerides to be an essentially instantaneous process, yet, there are a number of reasons why it is advantageous to prolong the reaction for 60 min as was done here. Vitride is an efficient desiccant so that the driest conditions possible must be employed in introducing the reagent to the sample. Repeated openings of the stock bottle were tolerated but the consequent slow decomposition of the Vitride renders the reagent increasingly less effective in the desired reduction of the complex lipid mixtures encountered in extracts of animal and plant tissues. This gradual deterioration passes through a phase where free-radical colors are very readily formed, *e.g.*, red with vitamin A alcohol, green (blue) with carotene, yellow with linoleate, orange with linolenate, lavender with arachidonate, while oleate remains uncolored. These colors are lost on addition of methanol and generally the original color of the extract was restored although there could be bleaching of yellows as with egg-yolk extracts. The Vitride gradually loses the ability to reduce sphingomyelin and the ceramides to their PAS detectable derivatives identified in the chromatograms as Vit Sph-Cer (*cf.* Figs. 1 and 4–7). When this point was reached, reduction of alk-1-enyl lipid was incomplete and the "3rd" spot became quantitatively dominant even to the extent of exaggeration. There was an increasing amount of white interface material which seemed to interfere with the recovery of the reduced lipid. As with acetolysis, extracts containing relatively little ether lipid, *e.g.* egg-yolk, appeared to be more prone to yield this "3rd" spot, thereby distorting the evidence that it does have some biological reality. The chromatographic and spot test evidence showed that the Vitride reduction product of sphingomyelin and ceramide is not a spingosine (*cf.* Fig. 1). It is both PAS positive and ninhydrin positive, however, in this instance it was necessary to heat develop the ninhydrin chromatogram; a relatively small amount of sphingosine was sometimes formed. Some tissues, *e.g.* male snake kidney, showed multiple spots in this region suggestive that they were derived from sphingomyelins or ceramides with phytospingosine or other long-chain bases and/or hydroxy or branched-chain fatty acids (*cf.* Fig. 7). Neither the ceramide hexosides nor psychosine were degraded by Vitride. The major result of incomplete Vitride reduction was to give the appearance of increased alkyl ether content, derived, in part, by the relatively slower cleavage of alk-1-enyl phosphatides.

From the foregoing account of our chemical observations it should not be unexpected to find that the biological data we sought were not always adequately consistent until we had finally learned to recognize the signs of inadequate Vitride

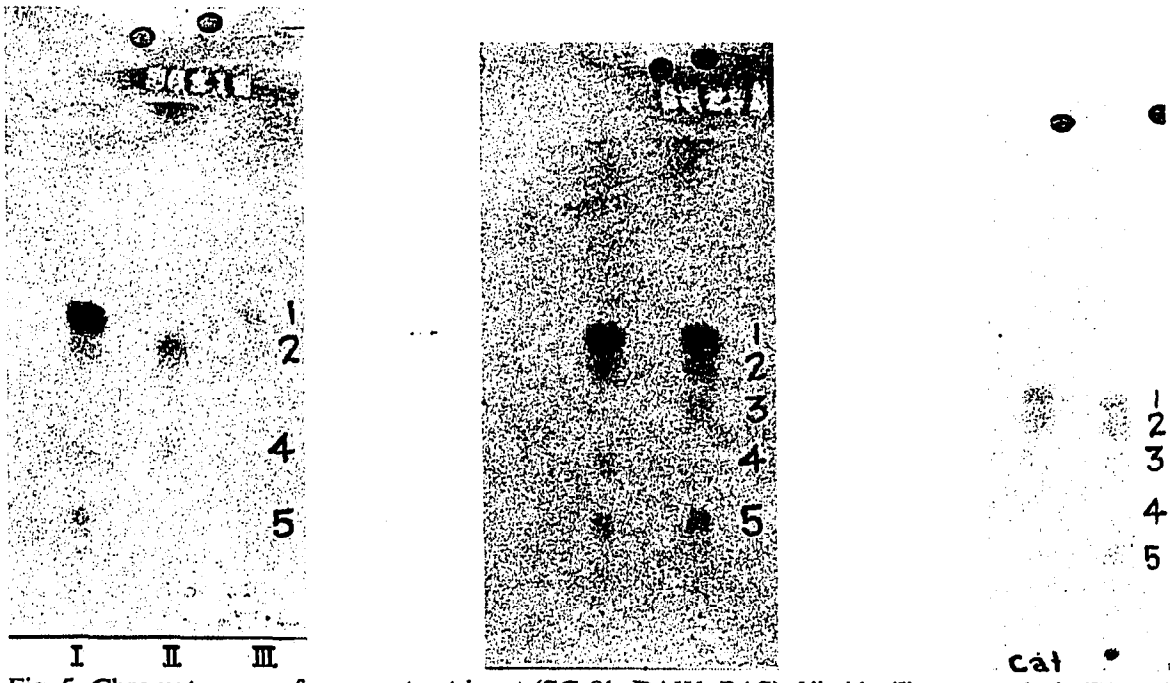

Fig. 5. Chromatogram of pregnant cat heart (SG-81, DAW, PAS). Vitride (I) *vs.* acetolysis (II) and human amniotic fluid acetolysis (III) sample. Spot numbering as for Fig. 4. Spot 4 is referred to in the text as "4th" spot.

Fig. 6. A comparison of the derived glyceryl ethers (Vitride preparations) from pregnant cat heart (left) and pregnant guinea-pig heart (right); PAS-stained SG-81, DAW. The essential difference is the restriction of the "3rd" spot to guinea-pig and the "4th" spot to cat. Numbering is consistent with above figures.

Fig. 7. Vitride reduction of cat placenta (left) as compared with male blue-racer snake kidney (*Coluber*) revealing the multiple spots, five, derived from sphingolipid. Gelman, PAS.

reduction and acetolysis. Most of the tissues we examined had relatively little neutral lipid glyceryl ethers as revealed by the 7-min saponification, notable exceptions were some specimens of pregnant cat and dog ovary, which had a substantial proportion of alkyl analogues (*cf.* Fig. 8) and mouse and rat preputial glands with both alkyl and alk-1-enyl well represented.

Consistent with our earlier comparative lipid biochemical studies, wide quantitative and qualitative variations among different species of animals were observed, some of which are reported below.

Heart. We had previously observed[8] that although adult mammalian cardiac muscle contained roughly equal amounts of ethanolamine and choline phosphatide plasmalogens the hearts of fetal and new born animals (and chick) had only the ethanolamine plasmalogen and that with subsequent growth of the animal the choline plasmalogen appeared in increasing amounts. The present studies have verified the relatively low alk-1-enyl content of fetal mammalian heart, which increased rapidly with age. In addition, with cats, dogs and guinea-pigs, the fetal specimens had more alkyl glyceryl ether than alk-1-enyl and although the alkyl ether concentration

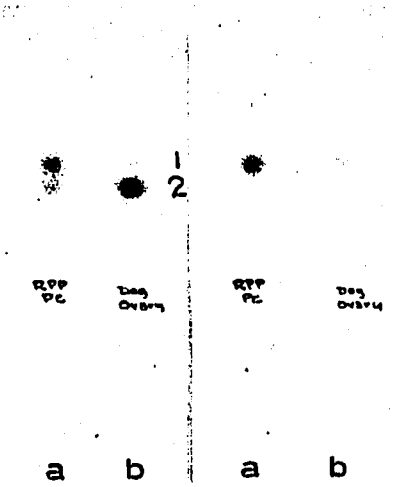

Fig. 8. Comparison of PAS (left) and Feulgen plasmal staining (right) of two Vitride samples, (a) choline phosphatide of rat preputial gland isolated from an aluminum oxide column and (b) pregnant dog ovary. The exclusive differentiation of the alk-1-enyl glyceryl ether by the plasmal reaction is evident SG-81, DAW.

increased with growth the alk-1-enyl increased even more rapidly so that adult mammalian cardiac muscle contained more alk-1-enyl than alkyl glyceryl ethers, mainly as phosphatide. By contrast, adult rat was nearly exclusively alk-1-enyl (*cf.* Fig. 9) and certain fish, amphibian and reptile hearts generally had more alkyl than alk-1-enyl glyceryl ethers, in this way resembling fetal mammalian heart. The phosphatidylcholine (PC) of fetal dog heart, isolated from a column of Al_2O_3, contained relatively little alk-1-enyl as compared with its alkyl ether content and as contrasted with the phosphatidylethanolamine (PE) isolated from the same extract which was mostly alk-1-enyl. In contrast to the other mammalian samples reported above, armadillo heart contained somewhat more alkyl than alk-1-enyl ethers. Yet, here too, the PE isolated from a silicic acid column showed a higher alk-1-enyl than alkyl content.

Liver. The major contribution the liver makes to lipid metabolism is widely recognised as is its generally very low and unexplained level of plasmalogens. Contrasting, however, with this general observation (among mammals) is the liver of guinea-pig, which has concentrations of parenchymal cell phosphatide plasmalogen readily observed in tissue sections by the Feulgen plasmal reaction (barely detectable in rat liver). Vitride reduction revealed that the alkyl glyceryl ethers of guinea-pig were about twice that of the alk-1-enyl and although the concentration was much less this relative proportion was also true for rat liver. The liver of newborn chick was mostly alkyl whereas that of the adult chicken had about equal amounts of alkyl and alk-1-enyl. The glyceryl ether content of the amphibian *Amphiuma* (liver) was mostly of the alkyl type. The PE from cyclostome liver, isolated from a column of silicic acid, contained little alk-1-enyl as compared to alkyl, and the PC contained alkyl as the dominant type. The PE from armadillo liver was dominant in alk-1-enyl.

Erythrocytes. There are some unique (*i.e.*, not understood) variations in the glyceryl ether phosphatide pattern of vertebrate erythrocytes (RBC) already observed

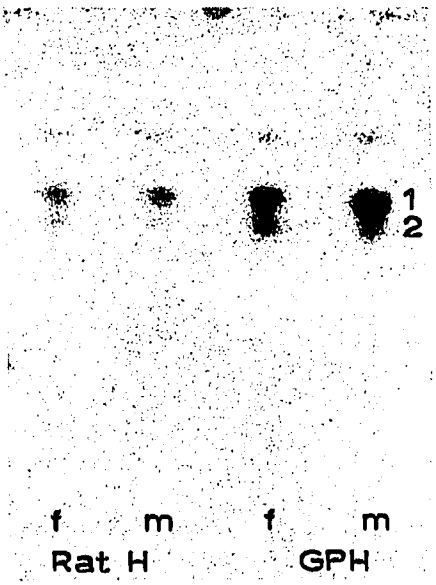

Fig. 9. Chromatogram (SG-81, DAW, PAS) of fetal and maternal rat heart (left pair) and fetal and maternal guinea-pig heart (right pair). In both cases the fetal specimens have relatively more alkyl (lower spot) than alk-1-enyl glyceryl ether as derived from Vitride reduction. There is a marked quantitative increase in alk-1-enyl in the adult rat heart specimen attributable mainly to the choline phosphatide plasmalogen.

by others and confirmed, in part, by our current observations. Cow (bovine) RBC are the most remarkable in this respect with alkyl PE as a very dominant lipid ingredient. The glyceryl ether content of pig RBC was low consisting of approximately equal amounts of alkyl and alk-1-enyl phosphatide types while those of man were quantitatively greater and contained relatively more alk-1-enyl phosphatide. The RBC of dog and chimpanzee contained more alkyl than alk-1-enyl and those of chicken (nucleated) had about equal amounts of the two phosphatide types.

Unicellular organisms. Certain microorganisms, flagellates, etc. have provided abundant useful biochemical information including important details about lipid metabolism. We have examined the glyceryl ethers of three species of interest to us earlier (*cf.* ref. 3), *viz.*, an anaerobe, *Clostridium butyricum*, and two flagellates, *Blastocrithidia culicis* and green and etiolated forms of *Euglena gracilis*, Z strain. Only the *Clostridium* and *Blastocrithidia* had been shown to possess plasmalogens and Vitride reduction revealed that they contained very little alkyl glyceryl ether lipid. *Euglena* is a plant and does not appear to have plasmalogens but does yield alkyl glyceryl ether after Vitride reduction. There was also a very small quantity of the human amniotic fluid type that chromatographically resembled a-alk-1-enyl glyceryl ether with more in the green than in the etiolated form; wax esters were more abundant in the white form.

Commercial phosphatides, etc. It is common practice to employ purified synthetic or natural lipid samples as reference substances in various sorts of chromatography and other assay methods. Not infrequently it was discovered that the samples were not homogeneous, especially those derived from natural sources. Most glycero-

phosphatides exist largely as the diacyl type but are variously mixed with alkyl and alk-1-enyl types in accordance with the animal and tissue of origin. Most PE samples contained the plasmalogen analogue as reported here for our own isolated samples. An exception was a sample labeled "bovine", which had a relatively high content of alkyl ether and in this respect was more like the PE from bovine RBC and from egg yolk. In none of the above preparations was the diacyl content of these phosphatides directly assessed although it would be a reflection of the amount of fatty alcohol formed from Vitride reduction.

Analytrol quantitation. In this preliminary report we have only established the feasibility of the Analytrol analysis of the SG-81, PAS and plasmal stained chromatograms. It is clear that had analyses been possible for all of the data reported here, it would have been much more simple to present and, more than likely, more precise correlations could have been made.

The chromatogram as a permanent record. The fuchsin aldehyde complex, as revealed in these chromatograms, is not as photographically sensitive for reproduction purposes as are H_2SO_4-charred TLC preparations so that the figures are by no means as clear and convincing as the actual chromatograms. The best photographic reproduction was achieved from the fresh wet chromatograms but it was not practical to photograph every chromatogram during the course of an investigation such as this. Our usual procedure was to spray the dried, stained chromatogram with one of the commercially available photographic matte lacquers and to mount the chromatogram on an $8\frac{1}{2} \times 11$ in. white card and to number the chromatograms consecutively. The mounted chromatogram and identifying number was then photographed, when needed, in either color or black and white. We have found a ring strobe light (electronic flash) to be the most suitable illumination for this photography.

DISCUSSION

Most chemical procedures have certain shortcomings, often related to specificity, completeness, and side reactions, which detract from their general usefulness. Those involving the glyceryl ethers are no exception although we believe there is some advantage to have methods which permit some degree of direct visualization of the products as is generally possible with the procedures described here. Quantitation, without the opportunity to follow the contribution of other substances, as with the complex mixtures present in tissue extracts, can be deceptive. For example, the involvement of sphingomyelin and ceramides as described here could easily become an undetected source of contamination under less visible circumstances, particularly when non-specific visualizing agents are used such as H_2SO_4 charring of TLC plates. Silylation of glycerol ethers and the sphingosines could produce derivatives which may defy resolution or recognition under some chromatographic conditions (*e.g.*, GC). The situation of the human amniotic fluid glyceryl ether described here is not new to chromatographic investigation wherein its chromatographic mobility under two sets of conditions belies its explicit chemical nature. Such examples clearly point to the need to be especially on guard when dealing with the complex, diverse, and numerous organic compounds which represent biological systems. In this way we may protect our metabolic interpretations from gross error.

The correct determination of the structure of plasmalogens and the recognition

of a corresponding family of alkyl analogues quickly led to the suspicion that the two groups of substances must be metabolically linked by a dehydrogenase. Recent *in vivo* data[9–11] validate this deduction and greatly enlarge the metabolic picture with regard to the ether lipids and brings them into more prominent focus as to having significant biological meaning. The correlation with sphingosine metabolism[9,10] is particularly pertinent to our present observations.

From the observations made here it would seem that the magnitude of the alkyl dehydrogenation capacity would be one of the *in vivo* limiting factors, but one capable of change (*e.g.*, regulation) as during cardiac muscle growth (Fig. 9). The possibility that the alkyl–alk-1-enyl pair serve in an oxidation–reduction system in some metabolically important way is yet to be demonstrated. On the other hand, and in addition, the evidence could be interpreted to mean that the acyl, alkyl, and alk-1-enyl families of lipids have a general physiological equivalence and can replace each other under certain circumstances.

REFERENCES

1 H. Debuch and P. Seng, in F. Snyder (Editor), *Ether Lipids —Chemistry and Biology*, Academic Press, New York, 1972, pp. 1–24.
2 F. Snyder, in F. Snyder (Editor), *Ether Lipids —Chemistry and Biology*, Academic Press, New York, 1972, pp. 122–156, 284.
3 M. H. Hack and F. M. Helmy, *An Introduction to Comparative, Correlative Histochemical Principles*, Gustav Fischer, Jena, 1974, pp. 45–54.
4 M. H. Hack, *J. Chromatogr.*, 5 (1961) 531.
5 M. Kates, in G. V. Marinetti (Editor), *Lipid Chromatographic Analysis*, Vol. 1, Marcel Decker, New York, 1967, pp. 1–39.
6 F. M. Helmy and M. H. Hack, *Proc. Soc. Exp. Biol. Med.*, 110 (1962) 91.
7 *Eastman Org. Chem. Bull.*, 42, No. 3 (1970).
8 M. H. Hack and F. M. Helmy, *Comp. Biochem. Physiol.*, 16 (1965) 311.
9 W. Stoffel, D. LeKim and G. Heyn, *Hoppe-Seyler's Z. Physiol. Chem.*, 351 (1970) 875.
10 W. Stoffel and D. LeKim, *Hoppe-Seyler's Z. Physiol. Chem.*, 352 (1971) 501.
11 R. Wood and K. Healy, *Biochem. Biophys. Res. Commun.*, 38 (1970) 205.