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Note

Some additional observations on the analysis of glyceryl ethers and chromatographically related lipids

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Our continued experience with the Vitride reduction of glyceryl ethers and with paper chromatography (PC) as the analytical mechanism for differentiating the products has revealed certain features of the technique which could cause difficulties in interpretation. We have consequently made some modifications which successfully circumvent these complications.

It has long been known that the DAW (diisobutyl ketone–acetic acid–water) PC system, because of its high acetic acid content, is not suitable for the chromatography of extracts containing lyso plasmalogens which are progressively cleaved as the chromatographic run proceeds. We were alert to the possibility that the alk-1-enyl glyceryl ethers, derived from plasmalogens by Vitride reduction, could be similarly labile but generally found little evidence that this had occurred, *i.e.*, the DAW results appeared to be compatible with the Gelman glass fiber paper chromatograms. Recently, however, a major discrepancy was apparent in some preparations, that is, they showed a marked quantitative reduction in the alk-1-enyl component in the DAW chromatograms not substantiated by the Gelman chromatograms. It now appears that some batches of diisobutyl ketone (2,4-dimethyl-6-heptanone) contribute to an excessively dirty solvent front and seem to modify the chromatographic mixture so that the component recognized as alk-1-enyl glyceryl ether was noticably diminished. As compared to the Gelman chromatograms the sphingomyelin-ceramide reduction product(s), visualized by the PAS (Periodic Acid-Schiff) reaction, was also diminished and it seemed that the alkyl ether spot had also increased so that the alk-1-enyl–alkyl ratio was variously distorted. This effect appeared to be corrigible, at least in part, by reducing the DAW chromatographic run, at room temperature, to 30 min. A more suitable solution was to devise a safer chromatographic system. Since the mixture of isooctane, isopropyl acetate and isopropanol provided a reliable differentiating medium with the Gelman silica gel impregnated glass fiber paper it was presumed that by increasing the isopropanol content a mixture could be obtained which would be suitable for the SG-81 paper. This was found to be true and in this way we retained the desirable features of the SG-81 paper for Analytrol quantitation.

We had already commented[1] on the use of the specific alk-1-enyl cleavage reagent, $HgCl_2$, and will provide some evidence that under certain conditions difficulties of interpretation can arise; one has to do with the complexing of the vinyl ether prior to cleavage and the other with the Vitride-sphingomyelin product.

There are two major disadvantages to the Gelman chromatograms: (1) they are easily overloaded so that bleeding of the alk-1-enyl fuchsin product occurs and (2) the fragility of the glass fiber paper precludes use of the Analytrol although charred chromatograms of many types have been successfully quantitated by others using different densitometric equipment. The lower loading capacity of the Gelman paper contributed to our latent recognition of the variable diminished PAS staining of the DAW chromatograms. Only when the visually assessed alk-1-enyl–alkyl ratios for the two chromatographic systems were markedly different it was apparent that problems in quantitative interpretation were possible.

MATERIALS AND METHODS

The chromatographic system, TII, for use with the Whatman SG-81 silica gel impregnated paper consists of isooctane (2,2,4-trimethyl pentane)–isopropyl acetate–isopropanol (250-10:25). The mixture is usable for about 3 days before the R_F values start falling off; a 20-cm chromatographic run takes about 2 h at room temperature. The chromatographic conditions, staining, preparation of extracts, various cleavage reactions, including Vitride reduction, have already been described[1,2]. Insufficient Vitride is detectable at the time the methanol is added when the reaction is to be terminated, if no evolution of gas (hydrogen) occurs it is likely that incomplete reduction had occurred. When this happens the sample can be simply recycled by removing the solvent and again adding benzene and Vitride. When phosphatide is present in the sample the odor of phosphine will be recognized on opening the tube after completion of the Vitride reduction. The presence of free aldehyde, absence of the sphingomyelin-ceramide reduction products (when these lipids are present in the lipid sample) and exclusive presence of the "3rd" spot are all potential indicators of incomplete Vitride reduction.

RESULTS AND DISCUSSION

The general R_F relationships, SG-81/TII, of lipids pertinent to this study are shown in Fig. 1; a similar graphic presentation of the DAW and Gelman data appeared in the previous report[1]. Although the relative compactness (*cf.* Fig. 2) of the DAW spots contributes to a more precise densitometric reading by the Analytrol the optical densities of the PAS-stained batyl alcohol (α-glyceryl ether of stearyl alcohol) standards, over the range $1–4 \times 10^{-4}$ mmoles, are about 25% less in the DAW than in the TII chromatograms. The poorer separation of the "3rd" and "4th" spots from the alkyl glyceryl ether in the DAW system contributes to the misreading of the Analytrol tracings. The ceramides are not as well separated from the monoglycerides and "3rd" and "4th" spots on the SG-81/TII system as they are on the Gelman silica gel glass fiber chromatograms run in a 250:10:5 ratio of the same TII solvents. Not all of the substances, detected as discrete spots on the chromatograms, have yet been identified; *e.g.*, some extracts show a spot just above the alk-1-enyl glyceryl ether obtained by Vitride reduction which is detectable by charring of Gelman chromatograms (*cf.* Fig. 3).

The *in situ*[1] application of aqueous $HgCl_2$ to the origin, prior to the chromatographic run, in the DAW system results in the movement to the solvent front of the

free fatty aldehydes derived from cleavage of alk-1-enyl ether lipids. In both the Gelman and TII system, however, plasmalogen containing extracts usually show three Schiff-positive (and Hg^{2+}-positive) spots which occupy the middle range of the chromatogram (*cf.* Fig. 3) and seem to represent various alk-1-enyl Hg^{2+} adducts and may be reflecting the chain length and degree of unsaturation of the alk-1-enyl group. Methanolic HCl (0.1 *N*) is less specific but serves the same purpose of releasing free fatty aldehyde from plasmalogens without the complications of Hg^{2+} complexing. We do not have an explanation for the disappearance of the sphingomyelin-Vitride product in chromatograms pretreated with aqueous $HgCl_2$ as described above (Fig. 4).

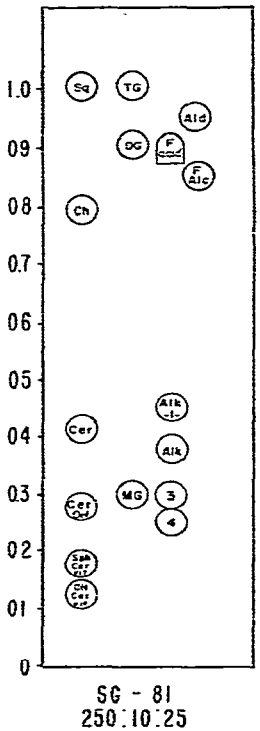

Fig. 1. Diagrammatic representation of the R_F distribution of various natural lipids and Vitride derivatives. Each column is listed from top to bottom. Left: squalene, cholesterol, ceramide, hydroxy ceramide, and the Vitride products of sphingomyelin, ceramide and hydroxy ceramide. Middle: triglyceride, diglyceride, monoglyceride. Right: fatty aldehyde, fatty acid (*e.g.*, palmitic), fatty alcohol, α-alk-1-enyl glyceryl ether, α-alkyl glyceryl ether, "3rd" and "4th" spots referred to in the text.

The "3rd" spot seen in egg yolk extracts[1] can now be explained. The native benzene and chloroform–methanol extracts of freeze-dried yolk already contain this substance which increases on alkaline hydrolysis, except when the saponification is prolonged at elevated temperatures. The substance is monoglyceride and it accumulates during the saponification as a derivative of the abundant yolk triglyceride. When an inadequate quantity of Vitride is used the monoglyceride also increases as was observed in our earlier experience. When the extract sample is treated with enough

Vitride as described here this spot is no longer present in chromatograms of egg yolk, nor is the intermediate diglyceride, and the proper reduction process proceeds. However, the "3rd" spot of cat heart extracts persists as does the "4th" spot of guinea pig heart. Neither of these has been further characterized nor has the nature of their parent lipid(s) been determined.

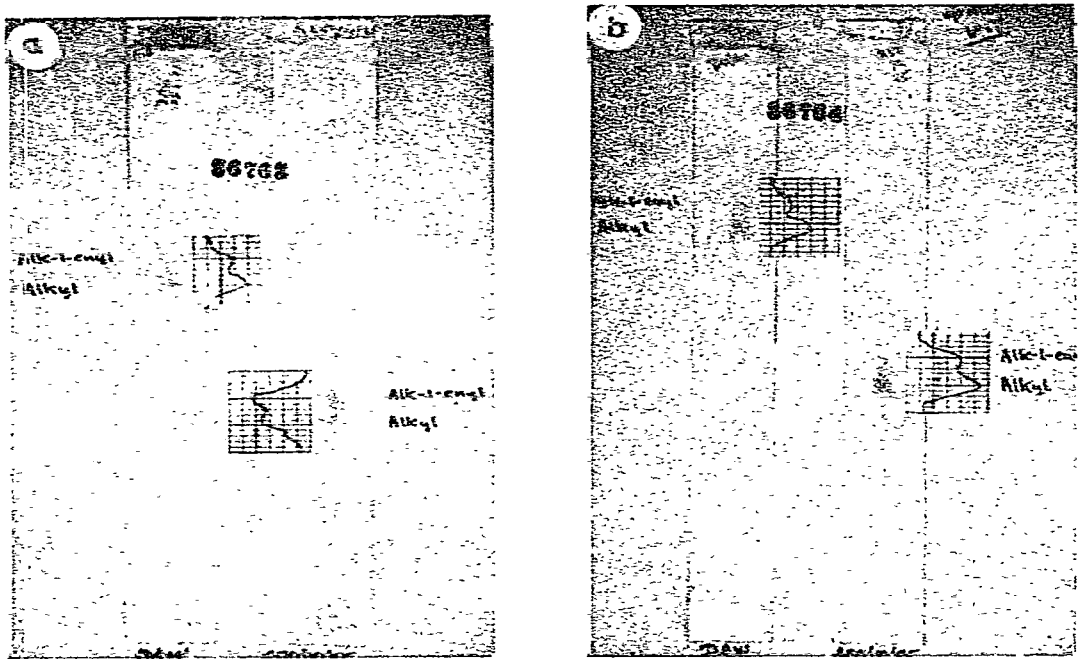

Fig. 2. These are PAS-stained SG-81 chromatograms of Vitride reduced extracts from (a) dog ovary, and (b) dog duodenal epithelium. The left hand chromatogram of each pair was run in DAW and the right hand pair in TH. The accompanying Analytrol tracings show the quantitative relationships between the alk-1-enyl glyceryl ethers (upper spots) to the alkyl glyceryl ethers (lower spots). Because of the nature of the photographic process the demonstration of chromatographic evidence is often less than ideal (*i.e.*, the sensitivities of the film and paper, duration of exposure and kind of development, etc. all contribute to the contrast of the final print) the Analytrol tracings of these chromatograms are a more accurate representation of the quantitation than might be apparent from the photograph of the chromatogram.

Exposure of chromatograms of Vitride reduced tissue extracts to OsO_4 vapors results mainly in the staining (blackening) of the fatty alcohol spots, the glyceryl ethers remain essentially negative. This same oxidation potential of ethylenic bonds can result in reactions with the Schiff reagent which could be confused with a positive plasmal or PAS reaction. Peracetic acid (vapor) is a dangerous reagent in this regard and unnecessary exposure to strong illumination appears to accelerate these reactions. Consequently, the preparation of chromatograms and their subsequent visualization reactions should proceed uninterruptedly in order to retain the high degree of specificity inherent in the procedures described.

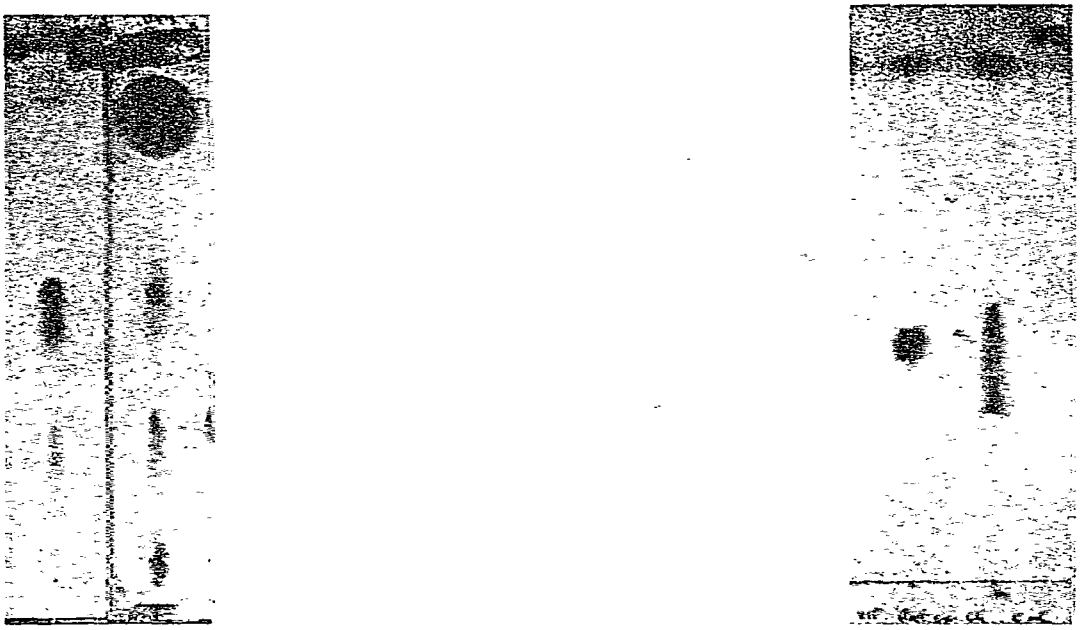

Fig. 3. Gelman glass fiber chromatogram comparing the PAS reaction (left) with H_2SO_4 charring (Vitride reduction of dog duodenal epithelium). The two glyceryl ethers and the sphingomyelin-Vitride product account for the three spots on the PAS-stained chromatogram.

Fig. 4. SG-81/TII chromatogram of Vitride treated rat spinal cord extract stained with the Schiff leucofuchsin reagent showing the single alk-1-enyl spot at the left and the multiple spots at the right resulting from the *in situ* application of $HgCl_2$ at the origin prior to the chromatographic run.

REFERENCES

1 M. H. Hack and F. M. Helmy, *J. Chromatogr.*, 107 (1975) 155.
2 M. H. Hack and F. M. Helmy, *An Introduction to Comparative, Correlative Histochemical Principles*, Gustav Fischer, Jena, 1974, p. 45–54.