J. Lipid Research, April 1963
Volume 4, Number 2

Metabolism of plasmalogen: II. The determination of alkenyl ethers in the presence of free aldehydes*

H. R. Warner† and W. E. M. Lands

Department of Biological Chemistry,
The University of Michigan,
Ann Arbor, Michigan

[Manuscript received October 2, 1962; accepted January 3, 1963.]

SUMMARY

A rapid and sensitive method for the analysis of alkenyl ethers (plasmalogens) in the presence of free aldehyde is proposed. The method is applicable over the range 0.02–0.2 μmoles of plasmalogen and is particularly useful for following the course of enzymatic or chemical reactions in mixtures containing both free aldehydes and plasmalogens in aqueous media.

Plasmalogens,[1] naturally occurring alkenyl ethers, react with a variety of acidic carbonyl reagents in a manner similar to free aldehydes. This reactivity may be due to rapid hydrolysis of the acid-labile alkenyl ether group, thus producing the free aldehyde. As a result, many of the standard colorimetric procedures are unsuitable for the specific determination of aldehydes in the presence of alkenyl ethers, and, similarly, there are few convenient procedures for determining alkenyl ethers in the presence of free aldehydes. The iodometric method of Norton (1) for the determination of alkenyl ethers is of considerable value, but this method is less sensitive than the reaction for aldehydes with fuchsin-sulfurous acid and is subject to interference from other iodine-reactive compounds (e.g., reducing agents such as sulfhydryl derivatives), if present. More sensitive iodometric methods have been reported recently by Gottfried and Rapport (2) and Williams et al. (3), but these are still not directly applicable to systems that contain mercaptans. The latter are present in many enzymatic reaction mixtures. Although most interfering substances may be removed by washing a lipid extract with water, this process will considerably lengthen the time needed for analysis. Similarly, the method of removing interfering aldehyde by volatilization for 6 hr at 140° and a pressure of $10^{-3}\mu$ (4) is not convenient for rapid assay of many samples. Schmidt et al. (5) described a method for the analysis of plasmalogen in the presence of free aldehydes involving the destruction of the interfering aldehyde by incubation at pH 8.3 and 25° for 16 hr, and subsequent measurement of the alkenyl ether with fuchsin-sulfurous acid. The major objection to this procedure is the length of time required to destroy free aldehydes. For our routine assays, it was of interest to devise a method that maintained the high sensitivity of the fuchsin colorimetric procedure and was sufficiently rapid for the determination of enzymatic rate constants within an hour after the reaction.

The procedure described in this paper involves the use of alkaline peroxide at elevated temperature to destroy the aldehyde; the substituted vinyl ether remains intact. This procedure has been found useful for quantitative determination of the plasmalogen content in mixtures, and for rapidly following the changes in alkenyl ether and free aldehyde content during a reaction when both are present. The method is particularly useful for determining levels of alkenyl ether in the range 0.02–0.20 μmoles.

REAGENTS AND MATERIALS

Mercuric Chloride–Acetic Acid Solution. Dissolve 1 g of mercuric chloride (analytical reagent) in 5 ml

* This work was supported in part by a grant (G-7647) from the National Science Foundation.

† National Science Foundation Cooperative Graduate Fellow.

[1] In this paper the term "plasmalogen" is regarded as a general term for glycerol derivatives that give the plasmal test.

of warm, deionized water, and add 95 ml of reagent glacial acetic acid.

Fuchsin Reagent. Dissolve 0.5 g of basic fuchsin (certified for use in Feulgen Reaction) in 500 ml of deionized water. Filter this solution and add the filtrate to 500 ml of deionized water saturated with sulfur dioxide.

Isoamyl Alcohol. Analytical reagent, free of aldehyde.

Aldehyde Emulsion. The free aldehyde used in this study was produced by treating a pig heart lecithin fraction (34% plasmalogen) with 90% acetic acid for 24 hours at 37°. This treatment resulted in 98% hydrolysis of the alkenyl ether to free aldehyde as determined both by iodine titration and the procedure described in this paper. The resulting mixture was diluted with ether and the acetic acid removed by repeated washing of this solution with 1 M K_2HPO_4. The ethereal solution of aldehyde was stored in the refrigerator. Aliquots of the ethereal solution were added to 0.02 M potassium phosphate, pH 7.0, and the ether was removed under a steam nitrogen. This aqueous lipid emulsion, containing about 1 μmole free aldehyde/ml, was used in the studies reported in this paper.

In order to determine the nature of the aldehydes present in this and other plasmalogen preparations, the ethereal solutions of the aldehydes, obtained after acid hydrolysis of the plasmalogens, were subjected to silicic acid and gas-liquid chromatography. Results of these analyses are shown in Table 1.

Alkenyl Ether. α′(-1-Alkenyl)-glycerylphosphorylcholine (alkenyl-GPC) was prepared from either pig or beef heart lecithin fractions by mild alkaline hydrolysis as previously reported (6). The alkenyl-GPC produced in this way was further purified by chromatography on silicic acid and only the fractions very rich in alkenyl ether were combined. This highly purified plasmalogen was used in the enzymatic experiments and as a reference compound in the aldehyde analysis. The ratio of phosphorus to alkenyl ether was 1.0 in this preparation, and re-chromatography on silicic acid demonstrated that it was essentially homogeneous and that less than 0.4% of the total aldehyde was present as free aldehyde. Assuming an average molecular weight of 480, corresponding to the aldehyde composition given in Table 1, these preparations were consistently only 87–88% alkenyl-GPC on a weight basis, even after prolonged drying at 100° or in a desiccator. The ash remaining after complete combustion accounted only for that expected from the phosphorus in the compound. The excess weight of this preparation might be accounted for by the presence of 4 moles water/mole alkenyl-GPC.

TABLE 1. NATURE OF ALDEHYDES FOUND IN VARIOUS HEART LIPID PREPARATIONS*

Retention Time	Aldehyde	Beef Heart Lecithin	Beef Heart Alkenyl-GPC	Pig Heart Lecithin	Pig Heart Alkenyl-GPC
min		%	%	%	%
2.1		3	8	6	6
2.6		4	7	5	5
3.0	*n*-Pentadecanal	1	4	1	
3.6			2		
4.1	*n*-Hexadecanal	70	67	71	75
4.8		2			
5.4	*n*-Heptadecanal	1	2	3	3
7.1	*n*-Octadecanal	14	9	9	8
8.2		3	2	5	5
8.8	*n*-Nonadecanal	3		2	2

* Gas-liquid chromatography was carried out using argon at a pressure of 15 psi and an 8-ft column packed with ethylene glycol–succinate polymer on a gas-chrom P base. The column temperature was 185° and the retention times, R_T, were calculated assuming $R_T = 0$ for the initial air peak, which barely precedes the solvent peak. The percentage composition of the total aldehydes chromatographed was determined by dividing each peak area by the sum of the peak areas.

Total phosphorus was determined by the method of Bartlett (7), alkenyl ether by the method of Norton (1), free aldehyde by the Fuchsin reaction (6) with fractions separated by silicic acid chromatography, and ester by the method of Lands (8).

Calculated for $C_{24}H_{50}O_6NP$ (μmoles/mg):
P, 2.08; alkenyl ether, 2.08; free aldehyde, 0.00; ester, 0.00.

Calculated for $C_{24}H_{50}O_6NP \cdot 4H_2O$ (μmole/mg):
P, 1.81; alkenyl ether, 1.81; free aldehyde, 0.00; ester, 0.00.

Found (μmoles/mg):
P, 1.80; alkenyl ether, 1.76; free aldehyde, <0.01; ester, 0.05.

EXPERIMENTAL PROCEDURE

In a test tube, add a 0.1-ml sample to be analyzed to 0.5 ml of 1.0 M KOH. Then add 0.5 ml of 1.0 M H_2O_2, mix gently, and heat 10 min in a water bath at 70°. Remove, cool, add 0.5 ml of the mercuric chloride–acetic acid reagent, mix, and then immediately add 4 ml of fuchsin reagent. Mix well and allow the color to develop for 20 min at room temperature. Extract the colored complex with 4 ml of isoamyl alcohol, separate the two layers by centrifugation, and measure the absorbancy of the alcohol layer at 550 mμ.

RESULTS AND DISCUSSION

Nature of Aldehydes in Plasmalogen. The results in Table 1 indicate that in both pig and beef heart lecithin preparations, the predominant aldehyde produced by

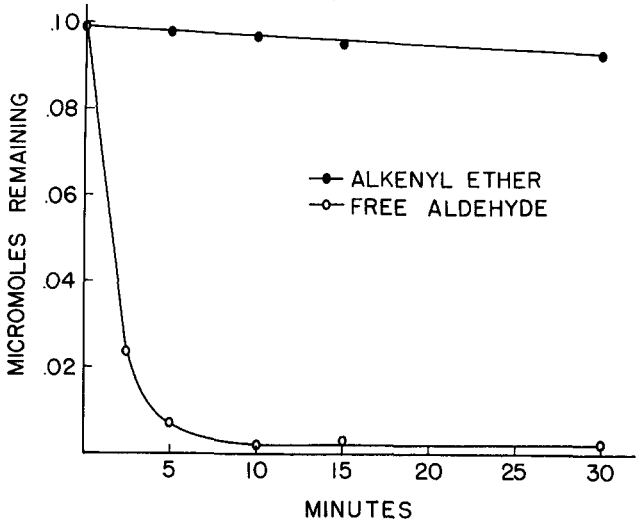


FIG. 1. Dependence of aldehyde destruction on incubation time. Each incubation tube contained 0.5 ml of 1 M KOH and 0.5 ml of 1 M H_2O_2, and either 0.10 μmole of free aldehyde or 0.10 μmole of alkenyl-GPC. The routine procedure was carried out at 70° using the various incubation times as indicated.

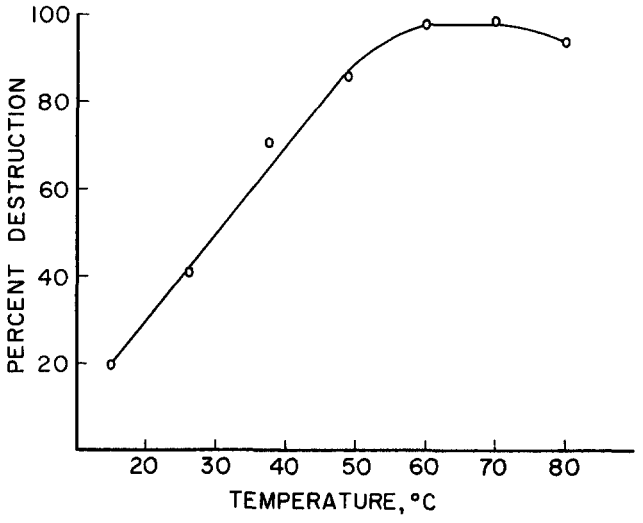


FIG. 2. Effect of temperature on aldehyde destruction. The reaction conditions were identical to those used in Fig. 1. All tubes contained 0.1 μmole of aldehyde and were incubated for 10 min at the given temperature.

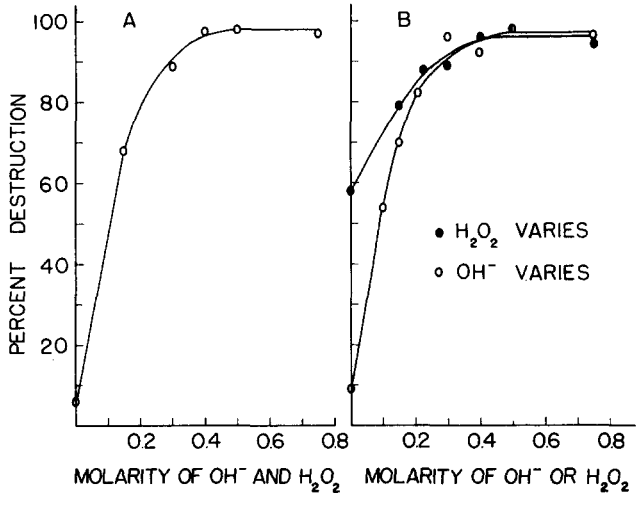


FIG. 3. Effect of KOH and H_2O_2 concentrations on aldehyde destruction. (A) 0.1 μmole of aldehyde was incubated for 10 min at 70° in 1 ml of solution containing equimolar amounts of KOH and H_2O_2 as indicated. (B) 0.1 μmole of aldehyde was incubated for 10 min at 70° in 1 ml of solution containing unequal amounts of KOH and H_2O_2 as indicated. The concentration of the nonvarying component in each series was 0.5 M.

acid hydrolysis has a retention time corresponding to hexadecanal. The next most abundant product appears to be octadecanal. The aldehyde composition of the deacylated derivative does not differ significantly from that of the parent compound. In all cases, hexadecanal and octadecanal account for 75–85% of the total aldehydes present. The remaining products have retention times that apparently correspond to those expected for branched-chain aldehydes, as reported by other workers (9, 10).

Reaction Conditions. The aldehyde emulsion described above was used to determine the most suitable assay conditions (Fig. 1, 2, 3). Figure 1 shows that the destruction of aldehyde was complete within 10 min whereas only 3% of the alkenyl ether was destroyed under similar conditions. The slight decrease in aldehyde destruction at temperatures greater than 70° (shown in Fig. 2) is possibly due to rapid loss of hydrogen peroxide by decomposition at the elevated temperatures. Figures 3A and B indicate that equimolar concentrations of H_2O_2 and KOH at 0.5 M are suitable for complete destruction of aldehyde. The results in Table 2 indicate that neither the individual reagents, the procedures used, nor the process of destroying free aldehyde, significantly altered the color yield from alkenyl ethers. This lack of interference is shown more clearly by the data plotted in Fig. 4. The lower curve represents the color produced from the aldehyde–alkenyl ether mixtures that had been treated with alkaline–peroxide. This curve is essentially the same as that from samples of the alkenyl ether alone. Furthermore, the slope of the upper curve shows that the color produced from the alkenyl ether in mixtures with aldehyde but without alkaline–peroxide treatment is identical to that obtained in the absence of aldehyde. Finally, since the absorbancy is a linear function of alkenyl ether concentration, quantitative determinations may readily be made if a suitable standard is available.

TABLE 2. EFFECT OF REACTION CONDITIONS ON COLOR YIELD

Alkenyl Ether	Free Aldehyde	Conditions*			Absorbancy
		Heat	KOH	H_2O_2	
μmoles	*μmoles*				
0.1	0.1	−	+	+	1.04
0.1	0.1	+	+	+	0.54
0.1	0.0	+	+	+	0.55
0.1	0.0	+	−	−	0 55
0.1	0.0	−	−	−	0.53
0.1	0.0	−	+	+	0.55
0.0	0.1	+	+	+	0.04

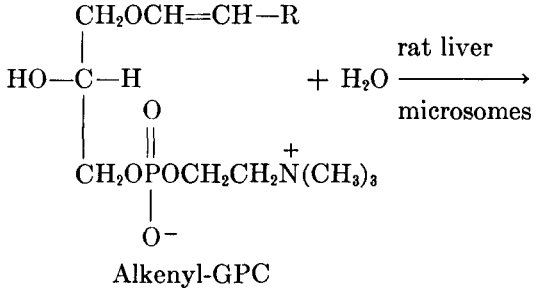
* Where indicated amples were exposed to heating at 70° for 10 min, 0.5 M KOH, and 0.5 M H_2O_2.

Color Development. The fuchsin reagent must be added immediately after the mercuric chloride–acetic acid reagent since the aldehyde resulting from the acid-catalyzed alkenyl ether hydrolysis is slowly destroyed in acidic peroxide solution. The color development proceeds gradually after addition of the fuchsin reagent, and is complete in 20 min. During color development, the solution often becomes slightly turbid; upon long standing, the colored complex may even begin to precipitate. Therefore, it is advisable to extract the color into isoamyl alcohol before measuring the absorbancy.

If only a measure of total aldehyde content is desired, the mercuric chloride–acetic acid solution may be added directly to the dried lipid sample, and this mixture let stand for 15 min at room temperature before adding the fuchsin reagent.

Our experience with a variety of aldehyde derivatives and reaction conditions in the fuchsin analysis shows that identical conditions must be used for standards and samples.

Enzyme Assay. To test the performance of this assay in enzymatic reaction systems, an enzyme-catalyzed hydrolysis of alkenyl-GPC was carried out as previously described (6), in a system containing microsomes, buffer or deionized water, and alkenyl-GPC. Hydrolysis occurred, producing



Alkenyl-GPC

GPC and free aldehyde. The extent of reaction was measured in four ways: (*a*) by using the iodometric method of Norton for alkenyl ether in 1 ml of incubation mixture, (*b*) by using the same method for alkenyl ether in a water-washed chloroform–methanol extract of 1 ml of incubation mixture, (*c*) by measuring appearance of water-soluble phosphorus-containing compounds, and (*d*) by using the alkaline peroxide method described in this paper. The results of these assays using two different levels of enzyme are shown in Fig. 5. As may be seen in the upper four curves, the iodometric and phosphate assays gave similar results, within experimental error, whereas the alkaline peroxide method indicated a slightly lower initial rate of hydrolysis. The good agreement between the phosphate assay and the alkenyl ether assay indicates that there was no measurable accumulation of a cholorform-

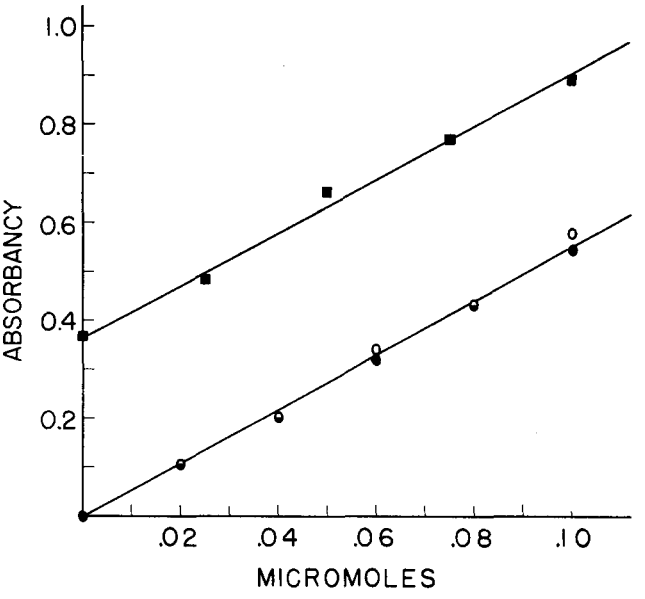
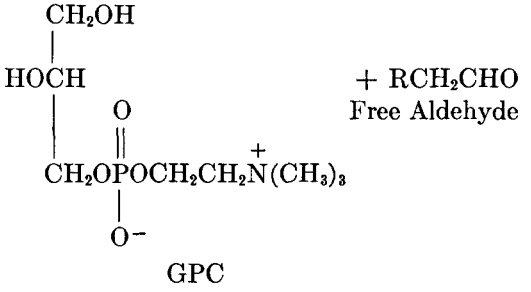


FIG. 4. Effect of aldehyde destruction on determination of alkenyl ether. (○) The incubation tubes contained varying amounts of alkenyl-GPC treated with 0.5 ml of 2 M KOH and 0.5 ml of 2 M H_2O_2 at 70° for 10 min. (●) The conditions were similar to those above except that each tube contained, in addition, 0.07 μmole of free aldehyde. (■) The assay tubes contained varying amounts of alkenyl-GPC plus 0.07 μmole of free aldehyde in 1 ml of 0.1 M potassium phosphate buffer, pH 7.1, and were not incubated.



GPC

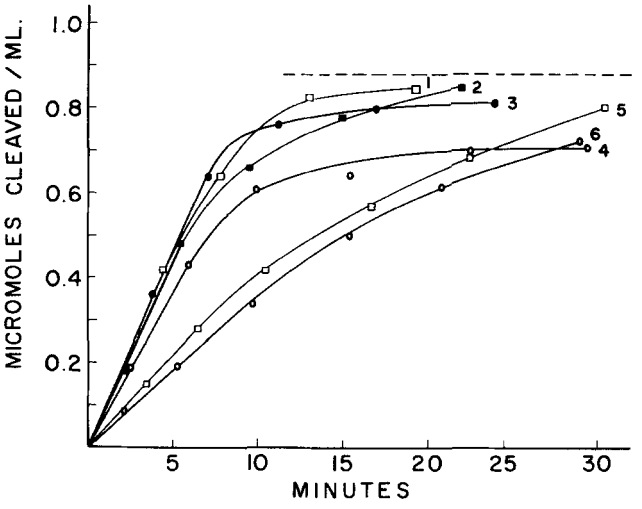


FIG. 5. Comparison of various enzyme assays. A reaction mixture, containing 11 μmoles of alkenyl-GPC and 24 mg of rat liver microsomal protein in a total volume of 12.1 ml was incubated at 37°. At various times, aliquots were removed and analyzed as indicated. (□) Curve 1: 1-ml aliquots were added to 12 ml of chloroform–methanol 2:1. Then 1.4 ml of water was added, and the chloroform layer was analyzed for alkenyl ether by the iodometric method of Norton. (■) Curve 2: 0.1-ml aliquots were added to 4 ml of chloroform–methanol 2:1. After the addition of 1 ml of water, the chloroform and aqueous phases were each analyzed for total phosphorus. (●) Curve 3: 1-ml aliquots were analyzed for alkenyl ether by the iodometric method of Norton. (○) Curve 4: 0.1-ml aliquots were analyzed by the alkaline peroxide assay. In a similar experiment, using one-half as much enzyme, the extent of reaction was measured by the decrease in chloroform-soluble alkenyl ether, Curve 5 (□), and the alkaline peroxide assay, Curve 6 ((○). The dashed line indicates complete hydrolysis of the alkenyl ether.

soluble intermediate during the reaction. On the other hand, an alkali-stable form of aldehyde did appear to accumulate gradually (perhaps as a side-product) in the enzymatic reaction systems in which the aldehyde production was rapid. The two lower curves in Fig. 5 show that rates half as fast were obtained with methods *b* and *d* when the enzyme concentration was halved.

The purification of an enzyme and determination of its kinetic constants are generally easier if the catalytic activity of a protein fraction can be determined before it undergoes spontaneous denaturation. Therefore the alkaline peroxide procedure may be useful for assaying the alkenyl ether hydrolase enzyme during its isolation and purification because the relatively high speed of the assay permits a rapid calculation of initial velocities, which are linearly dependent upon enzyme concentration.

REFERENCES

1. Norton, W. T. *Biochim. Biophys. Acta.* **38**: 340, 1960.
2. Gottfried, E. L., and M. M. Rapport. *J. Biol. Chem.* **237**: 329, 1962.
3. Williams, J. N., Jr., C. E. Anderson, and A. D. Jasik. *J. Lipid Res.* **3**: 378, 1962.
4. Schogt, J. C. M., P. H. Begemann, and J. Koster. *J. Lipid Res.* **1**: 446, 1960.
5. Schmidt, G., B. Ottenstein, W. A. Spencer, C. Hackethal, and S. J. Thannhauser. *Federation Proc.* **16**: 832, 1957.
6. Warner, H. R., and W. E. M. Lands. *J. Biol. Chem.* **236**: 2404, 1961.
7. Bartlett, G. R. *J. Biol. Chem.* **234**: 466, 1959.
8. Lands, W. E. M. *J. Biol. Chem.* **231**: 883, 1958.
9. Schogt, J. C. M., P. H. Begemann, and J. H. Recourt. *J. Lipid Res.* **2**: 142, 1961.
10. Farquhar, J. W. *J. Lipid Res.* **3**: 21, 1962.