The discovery of plasmalogen structure

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Abstract The sequence of events leading to the formulation of the aldehydogenic chain in plasmalogens as an α,β-unsaturated ether is described.—**Rapport, M. M.** The discovery of plasmalogen structure. *J. Lipid Res.* 1984. **25:** 1522–1527.

Supplementary key words phospholipids • acetal phosphatides • phosphatidal choline • lysophosphatidal ethanolamine • vinyl ethers • hydrogenation • iodination

Celebration of the 25th anniversary of the founding of the Journal gives us pause to consider how radically the lipid field has changed over this period, a change largely due to revolutionary advances in techniques. A quarter of a century ago column chromatography of lipids was still in an early stage of development whereas gas–liquid chromatography and thin-layer chromatography were aborning.

Just preceding this transitional period, I entered the field with an assignment to work on complex lipids with thromboplastic activity and immunological activity. The laboratory chief, Dr. Frank Maltaner, had detected a relation between the former and a nonspecific interference in the serological method (complement-fixation) used to study the latter. A by-product of these studies was the elucidation of the α,β-unsaturated ether structure of aldehydogenic phospholipids, and I thought it might be of interest to return to this subject in a unique way. This unusual structure, which required more than 30 years for correct assignment despite its considerable presence in two major classes of phospholipids in nerve and muscle, still presents a challenge to our understanding of its function. With the present focus of attention on membrane biochemistry, the solution to this puzzle may be within reach, and perhaps this paper will serve to attract an original mind.

In October of 1956, a conference on myelin was held at Washington University School of Medicine. I was invited on the spur of the moment to present a paper on plasmalogens at this meeting, since it was well recognized that these aldehydogenic phospholipids were important components of myelin. I confess that I knew nothing about myelin at the time, but I was riding a rare high to compensate for the years of frustration and seemingly endless failure of the work on immunologically active lipids. It was the intention of the organizers of the meeting to publish a book containing the papers presented at the conference, but after much solicitation and preparation, the effort was abandoned, and the paper never appeared.

Historical interest should have some appropriateness for a volume of the Journal celebrating a time span almost covering the period in which this paper has lain dormant, so here is the paper as presented in October of 1956 with an occasional footnote to clarify inconsistencies or offer apology.[1]

Compounds called acetal phospholipids are present in relatively high concentration in myelin. They were discovered in 1924 by Feulgen and Voit (1) and their history is therefore long; it is also romantic in the sense that their correct structure escaped detection for so many years. I shall review this history briefly and present the evidence which indicates that these compounds are not acetals at all, and strictly are not even aldehyde derivatives; they are α,β-unsaturated ether derivatives of phosphoglycerides. It is still quite correct to use Feulgen's original designation and refer to them as plasmalogens as a generic term, since they do give rise to higher fatty aldehydes on acid hydrolysis.

Feulgen discovered the fuchsin-sulfurous acid reagent in 1914 and used it for staining the nucleus. In 1924, he and Voit reported that in frozen sections, the cytoplasm also stained with this reagent. This reaction was attributed to tissue aldehydes of lipid nature, and it was further determined that untreated tissues did not give the reaction. The aldehydes were liberated by treatment with acid or mercuric chloride, and the precursor was called plasmalogen.

Histochemical studies subsequently undertaken and still actively pursued by a number of workers showed

[1] For a more extensive although uncritical historical review of studies of plasmalogen structure, see reference 27.

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that plasmalogens are widely distributed throughout the animal kingdom. There is general agreement on the high concentrations in myelin of brain and nerve and in the sarcoplasm of muscle, particularly heart muscle. Kidney is strongly staining, whereas liver does not stain at all, and there are many intriguing aspects to the differences in histochemical staining observed among various tissues.

In 1939 Feulgen and Bersin (2) isolated a crystalline plasmalogen from bovine muscle and showed that it was a phospholipid that liberated higher fatty aldehyde on acid hydrolysis and contained glycerylphosphorylethanolamine. Confronted with the problem of assigning structure, it was most natural to put them together as an acetal derivative (**Fig. 1**). This structure accounts for the empirical formula and the alkali stability, but it is not completely satisfactory from the standpoint of acid sensitivity. It is the structure currently found in all texts and reviews. By staying close to the accepted structure of cephalin, and preserving tranquility by maintaining that all of the glycerol hydroxyl groups are bound, it has survived for over 15 years.

In 1951, the crystalline "acetal phospholipid" was again isolated, this time from brain, by Thannhauser, Boncoddo, and Schmidt (3). To them, the assigned structure did not appear incorrect (3, 4) but an interesting development took place. With the objective of studying the metabolism of these substances with isotopic P, it should have been possible to separate plasmalogen P from the P of the other lipids in crude tissue extracts by treatment of such extracts with $HgCl_2$ and weak acid to liberate water-soluble glycerylphosphorylethanolamine. Although this reaction took place with the isolated crystalline plasmalogen, it did not occur with plasmalogens in crude extracts (5, 6). Recalling that the model compound was obtained after alkaline hydrolysis, Schmidt and his co-workers then confirmed that pretreatment of the extracts with alkali did permit the release of water-soluble P after hydrolysis with $HgCl_2$ and acid. Their conclusion was that native plasmalogen differs from crystalline plasmalogen by having an additional lipid group which is split off by alkali.

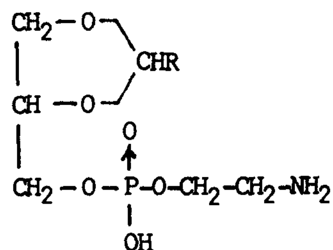

Fig. 1. Structure of plasmalogens as acetal phospholipids proposed by Feulgen and Bersin in 1939 (2) and Thannhauser et al. in 1951 (3) based on analysis of the crystalline phospholipids isolated from muscle and brain.

At this point, I will digress to explain how I became interested in the problem and to trace the individual steps which led to the clarification of structure. The research findings are, of course, all that is important, but the intricate pathways of discovery are often more fascinating and are usually difficult to describe. In connection with some studies I had been carrying out attempting to correlate phospholipid structure and thromboplastic activity, I obtained a specimen of crystalline "acetal phospholipid" from Dr. Schmidt in 1951. This compound was active, and since Dr. Schmidt had just reported that it was probably an artifact, the problem of chemically defining lipids with thromboplastic activity could not be resolved until pure, native plasmalogens were obtainable. This has not yet been accomplished. In 1953, following a visit to Dr. Mead's laboratory in California where I learned of his success in using chromatography on silicic acid to separate serum lipids (7), I decided to study the usefulness of this method to resolve mixtures of phospholipids. For this purpose, the synthetic models which were available were not entirely satisfactory, principally because they contained fully saturated fatty acid chains which made them much less soluble than the corresponding natural products. The simplest experimental model using natural products was also one which offered us a chance to solve two problems simultaneously, and the chromatography of bovine muscle phospholipids was embarked upon, since bovine muscle had been the source of Feulgen and Bersin's crystalline plasmalogen. The initial results were startling. Chromatographic fractionation showed that muscle contained appreciably more choline plasmalogen than ethanolamine plasmalogen (8) (**Fig. 2**). The fractions obtained were homogeneous with respect to the nitrogenous base (**Table 1**).

These fractions were very reactive with fuchsin-sulfurous acid at both 12°C and 37°C, and gave a higher yield of color per atom of phosphorus than the specimen of crystalline "acetal phospholipid" obtained from Dr. Schmidt 2 years earlier (**Fig. 3**). Note also that the natural products were very much more reactive with Feulgen's reagent than synthetic glyceryl acetals, an observation which led Anchel and Waelsch (9) to cast doubt upon the acetal formulation of plasmalogens as early as 1944. At this point, we made what appeared to be a reasonable assumption; namely, that the chromatographically prepared plasmalogen fractions were almost pure. Not only was the color yield high, but it was approximately the same for both the ethanolamine and choline fractions (Fig. 3). For this assumption to be wrong would have required that the ethanolamine plasmalogen be mixed with the same quantity of phospha-

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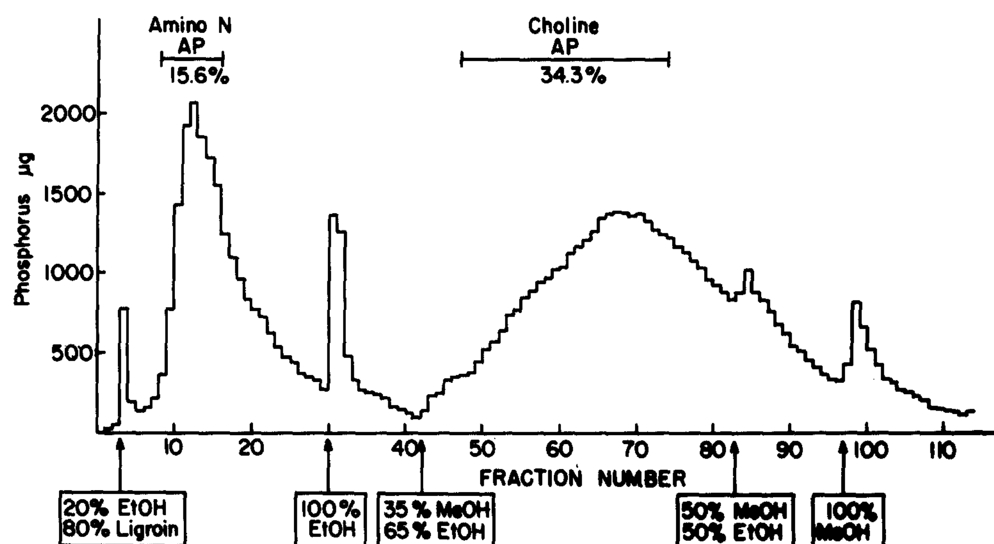

Fig. 2. Chromatographic separation on silicic acid of aldehydogenic phospholipids (AP) from bovine muscle using discontinuous gradient elution. Brackets show fractions that were combined and % of total P applied. Analyses are presented in Table 1.

tidylethanolamine as the choline plasmalogen was with lecithin. Although it was not very likely, it turned out to be the case. In any event, granting that the fractions were not pure, but contained 50% or more of the native plasmalogens, the percentages of nitrogen and phosphorus (Table 1) showed that native plasmalogens contained two fatty chains per atom of P and not one or one and a half, which would have been consistent with certain hypothetical formulations (e.g., an acetal formed by two glycerylphosphorylethanolamine residues, each esterified with one fatty acid).

The next step proved to be the critical one. We observed, on examination of individual chromatographic fractions, that some fractionation was taking place with respect to unsaturation. It therefore seemed advisable to saturate the fraction and rechromatograph it, in order to avoid contending with molecular variations which were unrelated to the problem at hand. Because of its greater solubility, the choline plasmalogen fraction was selected, and catalytic hydrogenation over platinum was effected in alcoholic solution under the mild conditions of atmospheric pressure and room temperature.

TABLE 1. Analysis of aldehydogenic phospholipid fractions isolated by silicic acid chromatography

Fraction Eluted with	N	P	P/N	Amino N/N	Choline/N
	%			*molar ratio*	
20% Ethanol–80% ligroin	1.66	3.66	1.00	1.02	
35% Methanol–65% ethanol	1.73	3.76	0.98	0	0.95

To our surprise, the product no longer gave a positive fuchsin-sulfurous acid reaction. This reaction was the key to the structure, because it meant that the aldehy-

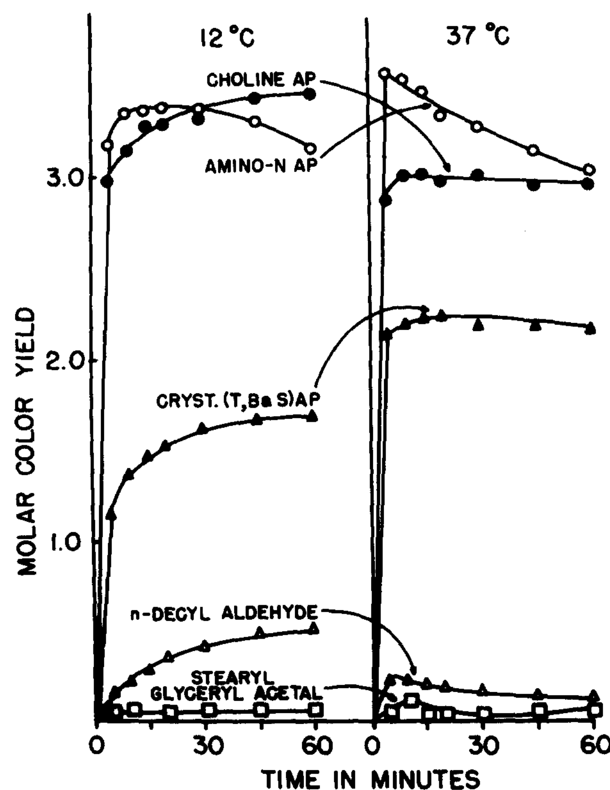

Fig. 3. Fuchsin-sulfurous acid reaction at 12°C and 37°C of aldehydogenic phospholipids (AP), a specimen of the "crystalline brain plasmalogen" obtained from Dr. Schmidt and two related materials.

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dogenic linkage was in some way sensitive to hydrogenation under gentle conditions. The product which resulted was one in which the hydrophobic chain was bound to glycerophosphate and could not be easily hydrolyzed with either acid or alkali; the bond was therefore most likely in the form of a saturated ether, which suggested that the aldehydogenic linkage was an α,β-unsaturated ether. This reaction was independently discovered by Klenk and Debuch in Germany and reported by them in 1954 (10). They had observed it using a brain cephalin fraction containing phosphatidal ethanolamine[2] and much more vigorous reaction conditions: elevated temperature (100°C), mixed catalysts, and hydrogen pressure of 100 atmospheres. They discussed several possible structures which would explain the observation, including both hemiacetal and α,β-unsaturated ether, but in subsequent publications (11, 12) came out strongly for the hemiacetal. Our own work had progressed along several lines which may be rapidly summarized. We developed and applied a quantitative ester group method which permitted us to determine the number of esterified fatty chains in the mixed fractions (13). We established that beef heart lecithin contains about 60% choline plasmalogen, and that Pangborn's procedure gives reproducible preparations (14). We established further that the choline plasmalogen, phosphatidal choline, is hydrolyzed by snake venom lecithinase A at the same rate as phosphatidylcholine, with hydrolysis of one ester group per atom of phosphorus (15, 16). This observation showed that plasmalogens and the usual diacyl phosphoglycerides are very similar in structure, and that the aldehydogenic chain in phosphatidal choline is connected predominantly to the secondary hydroxyl group of the glycerol residue.[3] The structural problem that remained was by far the most challenging.

The properties which any acceptable constitutional formula had to explain were: *1*) the aldehydogenic linkage must be converted by hydrogenation to one that is stable to acid (saturated ether). The reaction product must no longer be aldehydogenic. *2*) Higher fatty aldehyde must be rapidly released by weak acid or mercuric chloride; and, as a corollary, the reaction with fuchsin-sulfurous acid reagent must be very rapid. *3*) The aldehydogenic linkage must be stable to alkali, since plasmalogens remain after alkaline hydrolysis has degraded other phosphoglycerides.

The two formulae receiving serious consideration were the hemiacetal (**Fig. 4A**) by Klenk's laboratory and the α,β-unsaturated ether (Fig. 4B) by mine. There were three properties of choline plasmalogen which did not appear to me to be in harmony with a hemiacetal structure. First, beef heart lecithin (which is about 60% choline plasmalogen) has been extensively used as a serological reagent and shows remarkable stability for several years in alcoholic solution. We might expect a hemiacetal to exchange O-alkyl groups leading to the formation of hemiacetals composed of ethanol and higher fatty aldehyde or, alternatively, to react with ethanol producing acetals, and this does not happen. Second, hemiacetals as found in carbohydrates are readily oxidized by alkaline ferricyanide. This reaction is of a different order of magnitude with plasmalogen (unpublished studies). And third, there is no evidence to indicate that hemiacetals can be catalytically reduced to ethers with ease. I therefore felt that the unsaturated ether, despite the fact that it was a structure previously unknown among natural products, was more acceptable. The original suggestion was made in an annual progress report (17) and inadvertently found its way into the Chemical Abstracts (18), but by 1955 there was sufficient evidence to justify a more formal proposal (19). There are unfortunately no synthetic models with which to study these properties, and since both formulations provided an adequate explanation for the exceptional reactivity of plasmalogen, the problem did not seem to offer much hope for a decisive solution. However, one

```
A                OH
                 |
  O     CH2-O-CH-R
  ||    |
R-C-O-CH      O
        |     ↑      Esterified
        CH2-O-P—  {  nitrogenous
              |      residue
              OH

B                    O
                     ||
             CH2-O-C-R
             |
R-CH=CH-O-CH      O
          |       ↑      Esterified
          CH2-O-P—   {   nitrogenous
                  |      residue
                  OH
```

Fig. 4. Proposed plasmalogen structures: A, Klenk and Debuch; B, our laboratory.

[2] Editor's note: While the terms phosphatidal ethanolamine and phosphatidal choline continue to be used for plasmalogens, the recommended nomenclature is plasmenylethanolamine and plasmenylcholine, respectively. The Journal of Lipid Research follows the 1976 recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1978. *J. Lipid Res.* **19:** 114–128).

[3] In 1956 it was the consensus that snake venom phospholipase attacked the α-acyl chain (*sn*-1) of glycerophospholipids.

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              CH2OH
              |
R-CH=CH-O-CH      O
              |       ↑
              CH2-O-P-O-CH2-CH2-NH2
                      |
                      OH
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Fig. 5. Lysophosphatidal ethanolamine, the crystalline plasmalogen from brain and muscle.[7]

of the most pronounced differences between the hemiacetal and unsaturated ether structures was that reduction of the former to a saturated ether required hydrogenolysis of a carbon-oxygen bond, whereas, with the latter, the reaction was addition to an unsaturated carbon-carbon bond. As noted, the studies on structure were carried out on fractions which contained a considerable proportion of the corresponding phosphatidyl compounds in addition to the plasmalogens. Furthermore, the native plasmalogens themselves contained a fatty acid chain with unsaturated bonds, so that even if pure native plasmalogens were available, considerable ambiguity would still be associated with experiments attempting to distinguish hydrogenolysis from hydrogen addition. A clue to the solution can be found in Fig. 3; namely, the crystalline "acetal phospholipid", containing a single fatty chain which produced saturated fatty aldehyde on hydrolysis, reacted almost as rapidly as the native plasmalogen fractions with fuchsin-sulfurous acid. Therefore, in all probability it contained the same aldehydogenic linkage as native plasmalogens and differed from them solely in lacking the esterified fatty acid; that is, it was probably a lysoplasmalogen, or more precisely, lysophosphatidal ethanolamine (**Fig. 5**). By combining the best features of Feulgen and Bersin's isolation (2) with those of Thannhauser, Boncoddo, and Schmidt (3), and making use of the chromatographic separation already described, we isolated a specimen of the crystalline plasmalogen by procedures more gentle than those previously employed. This crystalline substance gave satisfactory analyses for carbon, hydrogen, nitrogen, phosphorus, glycerol, and ethanolamine (20). It yielded one molecule of higher fatty aldehyde per atom of phosphorus. Hydrogenation over palladium-charcoal catalyst resulted in the uptake of one molecule of hydrogen per atom of phosphorus with complete disappearance of aldehydogenic properties. On bromination, one molecule of bromine was consumed per atom of phosphorus. This result was decisive. Bromination showed that the molecule contained a single unsaturated bond. Hydrogenation of this bond produced the saturated ether linkage. Hydrogenolysis, which would have required hydrogen uptake in excess of that required to saturate the double bond, could therefore not be responsible for the observed results; the evidence for the α,β-unsaturated ether linkage was immeasurably strengthened (20). The element of doubt which may still persist arises solely from the fact that the organic chemist has not yet provided us with sufficient information about this kind of structure. For analogous reactivity we must turn to such remote models as vinyl ethers and special $\alpha\beta$-unsaturated ethers such as dihydropyran. Although these models are not adequate, they do point out the exceptional reactivity present in this structure and offer exciting possibilities for further investigation.

The immediate consequence of this discovery of the true nature of plasmalogen structure may be the design of more specific histochemical reagents that do not depend on hydrolysis and subsequent reaction with fatty aldehyde, but rather on addition to the unsaturated bond. Because of the reactive nature of this bond, it is possible to carry out such additions selectively with respect to normal olefinic unsaturation. Conditions are already known (21, 22) under which iodine may be added to α,β-unsaturated ethers without attacking ordinary double bonds, so that, for example, a simple, although by no means ultimate, histochemical method could be based on addition of radioactive iodine followed by autoradiography. Independent methods for measuring the same cellular constituent may contribute toward clarification of plasmalogen distribution and an understanding of its biochemical functions.

It may be predicted from evidence already at hand that plasmalogens will be found to have a multiplicity of functions.[4] Since the area requiring exploration is large, it is intriguing to attempt to select, from among the many possible chemical reactions, those which may be physiological. Organic chemists have perhaps provided a clue, since dihydropyran and vinyl ethers were recently proposed as reagents to block hydroxyl groups when their regeneration by mild procedures is required at some later time (23, 24). The reaction involves the addition of any hydroxylated compound, ROH, to the double bond to form a mixed acetal (**Fig. 6**). From this combination, the hydroxylated compound can be recovered by mild acid hydrolysis. We may thus look for plasmalogens to enter into reactions, enzymically mediated, with intermediates containing free hydroxyl groups. The mixed acetals which form may undergo several possible reactions: oxidation to produce an ester

[7] The correct structure should have the chain linked to the α-position.

[4] The late Jordi Folch-Pi reproached the author, correctly it seems, for what he called "panplasmalogenism".

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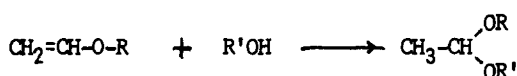

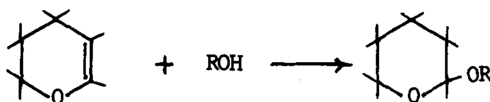

Fig. 6. Reaction of vinyl ethers and dihydropyran with free hydroxyl groups to form mixed acetals.

of higher fatty acid, or hydrolysis leading to regeneration of the hydroxyl group and liberation of both fatty aldehyde and lysophosphoglyceride. The biochemical functions may thus be related to processes of esterification and lipid deposition, active transport across membranes, binding of hormones, and possibly inactivation and release of enzymes via the free hydroxyl groups known to be present at their active sites. The fact that some 20% of the phospholipids in myelin are plasmalogens (2, 25) suggests a more esoteric role in this tissue, one which is related to the special physiological functions and metabolic requirements of brain. The relevance of this exceptional degree of reactivity to hypotheses connecting the activity in nerve to "reversible phosphatide splitting" (26) is immediately apparent, although the detailed mechanism would be altogether different. The unique structure of plasmalogens thus offers us a stimulating, multifaceted challenge to discover its relation to function.

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[5] At the time, this paper was in press.
[6] At the time, this paper was in preparation.

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