JCI The Journal of Clinical Investigation

FATE OF INJECTED PLASMALOGEN IN RABBITS

A. F. Robertson, W. E. M. Lands

J Clin Invest. 1962;41(12):2160-2165. https://doi.org/10.1172/JCI104674.

Research Article

Journal of Clinical Investigation
Vol. 41, No. 12, 1962

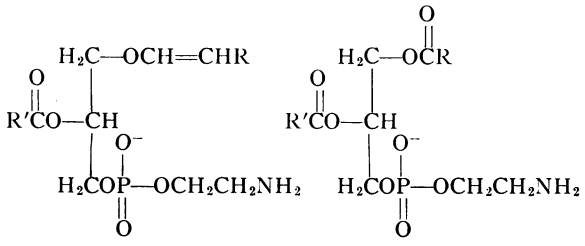
FATE OF INJECTED PLASMALOGEN IN RABBITS *

By A. F. ROBERTSON † AND W. E. M. LANDS

(From the Departments of Biological Chemistry and Pediatrics, University of Michigan, Ann Arbor, Mich.)

(Submitted for publication May 25, 1962; accepted August 15, 1962)

Plasmalogens are a type of lipid that contain an alkenyl ether linked to glycerol in contrast to the ester linkages in phosphatidyl ethanolamine and phosphatidyl choline.



Plasmalogen form of phosphatidyl ethanolamine

Phosphatidyl ethanolamine

The alkenyl ethers react readily in aqueous acids to form aldehydes. Rapport and Lerner have shown that nearly all the aldehydes detected with acidic reagents in lipid preparations from mammalian tissues arise from plasmalogens (1). Plasmalogens constitute up to 70 per cent of the cephalin fraction of brain lipids (2) and 55 per cent of the lecithin fraction of heart lipids (3).

These relatively large amounts of plasmalogen suggest that degenerative diseases of the myocardium or central nervous system might elevate the plasmalogen level in the serum and be of diagnostic or prognostic value. In human brain the plasmalogen content is approximately 25 μmoles per g (wet weight) of white matter (1), and in normal human infants the serum plasmalogen level is 0.03 ± 0.02 μmole per ml. In a 10-kg infant, with a blood volume of 800 ml and a hematocrit of 36 per cent (4), the total plasmalogen in the serum would be approximately 14 ± 10 μmoles. Therefore, the destruction of 1 g of white matter could liberate sufficient plasmalogen to elevate the serum level by 100 per cent or more.

A preliminary survey of hospital patients with encephalitis, Niemann-Pick's disease, or myocardial infarction showed no consistent elevation of serum plasmalogen. This finding had been reported previously for a variety of diseases that did not, however, involve destruction of tissues rich in plasmalogen (5). The lack of correlation between pathological states and serum levels suggested either that plasmalogen is not released immediately upon tissue destruction or that rapid removal of plasmalogen from the blood is preventing its accumulation in the serum to any appreciable extent. In order to investigate the disappearance of plasmalogen from the circulation, an emulsion of phospholipids containing plasmalogen was injected into rabbits and shown to be rapidly removed from the circulation.

METHODS AND MATERIALS

Plasmalogen preparation. Lipids were extracted from 2 kg of fresh beef heart by successive homogenization with 2 L methanol, and 2 and then 1.5 L 50 per cent methanol in chloroform.[1] Chloroform was added to these extracts to make a final solution of 33 per cent methanol in chloroform. The extract was washed with one-fifth volume of water and the water layer removed. The extract was evaporated to dryness and redissolved in 30 ml of 10 per cent methanol in chloroform. Acetone, 800 ml, was added and the solution refrigerated overnight. The acetone-insoluble precipitate was separated by centrifugation, dried, dissolved in ether, and added to a 400-g silicic acid column (Baker, 100–200 mesh) moistened with ether. After removal of the neutral lipids with 1.5 L of ether, phospholipids were eluted with successive mixtures of 1 L of 10 per cent ethanol in ether, 7 L of 75 per cent ethanol in ether, 7 L of 75 per cent methanol in ethanol, and 3 L methanol. Aldehyde determinations of the eluate were used to indicate plasmalogen concentration, and the cephalin and lecithin fractions were collected separately. These fractions

* This project was supported in part by Grant G-7647 from the National Science Foundation.

† Supported by U. S. Public Health Service Postdoctoral Training Grant 2A-5278 (C1) awarded to the Department of Pediatrics, University of Michigan, Ann Arbor, Mich.

[1] All mixed solvents are reported as percentage composition by volume.

were evaporated to dryness, redissolved in 33 per cent methanol in chloroform, and stored at 5° C.

Analysis of material. Analyses were performed on the cephalin fraction (Table I) for amino nitrogen by the method of Lea and Rhodes (6); for aldehyde, with fuchsin sulfurous acid (7); for alkenyl ether, by iodometric titration (8); for esters, by hydroxamate formation (9); and for phosphorus, with ammonium molybdate (10). This fraction was used to prepare the plasmalogen emulsion.

Preparation of emulsion. The cephalin emulsion was prepared immediately before injection by evaporation of 4 ml of the cephalin fraction in 33 per cent methanol in chloroform to dryness *in vacuo* at 40° C. The material was then dissolved in 1 ml of diethyl ether and to it was added 8 ml of a 0.1 N phosphate buffer at pH 7.4 maintained at 37° C. The ether was blown off with nitrogen. Then 0.1 ml of a 10 per cent solution of Tween 20 in phosphate buffer was added. The material was vigorously shaken and sonicated for 30 minutes in a 250-watt Raytheon Sonicator. The emulsion did not change in appearance when kept at 5 to 10° C until 3 to 4 days' time, when a precipitate began to form. The particle size of the emulsified lipids was not determined.

Injection procedure. Adult male rabbits were maintained on Rockland Rabbit Food and water at will. They were weighed immediately before each experiment and anesthetized with sodium pentobarbital in distilled water given intraperitoneally, 38 mg per kg body weight. The external jugular or femoral vein was exposed, opened, and a no. 19 or 22 polyethylene catheter was inserted to a distance of 5 to 8 cm. A three-way stop-cock was placed between the catheter and syringe to prevent air entering the venous system. Blood samples of 6 ml were taken and that volume replaced with 0.9 per cent NaCl injected through the catheter. Injections of 4 ml of the cephalin emulsion containing 20 μmoles of plasmalogen were made through the catheter. Before injection, 4 ml of blood was drawn into the syringe and mixed with the 4 ml of emulsion. After injection, the catheter was flushed with 0.9 per cent NaCl solution. In some cases, the abdominal cavity was opened and at various times a portion of liver, of about 10 gm, was removed distal to a Kelley hemostat. Care was taken not to obstruct the hepatic circulation proximal to the clamp.

Blood analyses. The blood samples were allowed to coagulate, and the serum was separated by centrifugation and frozen until the time of analysis. Lipids were extracted from 0.5 ml of serum by two treatments with 7 ml of 50 per cent methanol in chloroform. The lipid extract was washed with one-fifth volume of water to remove inorganic phosphates and other water-soluble contaminants. The aldehyde content was then determined by the colorimetric method described above.

Whole blood was collected with sodium citrate as an anticoagulant. The plasma was removed after centrifugation and the erythrocytes washed once with 0.9 per cent NaCl. The cells were then hemolyzed by freezing and thawing. An extract was made from the hemolysate as described above for serum.

Liver and lung analysis. Tissue samples were frozen immediately after removal and stored until the time of analysis. Liver and lung samples were weighed after thawing. The lipids were extracted from 5- to 10-g samples by homogenizing the tissue successively with 20 ml of methanol, 20 ml of 50 per cent methanol in chloroform, and 30 ml of 33 per cent methanol in chloroform. Chloroform was added to the combined extract to make a final solution of 33 per cent methanol in chloroform. The extract was then washed with one-fifth volume of water, and the water layer removed. The organic phase was diluted to 50 ml in a volumetric flask and samples taken for aldehyde analysis.

Tissue incubations. Rabbit liver was homogenized in 3 volumes of 0.25 M sucrose containing 0.001 M Versene-9. The total homogenate was incubated with either the cephalin emulsion or α'-(1-alkenyl)-glycerylphosphoryl ethanolamine (alkenyl-GPE) [2] prepared by a modification of the method of Hartree and Mann (11). Alkenyl-GPE, 0.5 to 1.0 μmole, was dissolved in 1 ml of a 0.1 M phosphate buffer at pH 7.1. The tissue homogenate, 0.1 ml, was added and the mixture incubated at 37° C. Samples of 0.1 ml each were withdrawn from the incubation mixture at the specified intervals. The alkenyl ether content was determined with fuchsin sulfurous acid after destruction of the liberated aldehyde by alkaline peroxide (12).

TABLE I

Analysis of cephalin fraction used for preparation of emulsion*

Determination	μmoles per ml	μmoles per μmole of amino nitrogen
Aldehyde	10.4	0.22
Vinyl ether	11.6	0.24
Ester	77.5	1.63
Amino nitrogen	47.5	1.00
Phosphate	40.0	0.84

* Isolated as the acetone-insoluble lipid extract and purified by silicic acid chromatography.

RESULTS

In the rabbits used, the average serum plasmalogen level was 0.009 μmole per ml, ranging from 0.001 to 0.031 μmole per ml. With an average weight of 4 kg and plasma volume of 38 ml per kg ranging from 27 to 48 ml per kg (13), the total plasma plasmalogen varied between 0.15 and 4.71 μmoles. Therefore the injection of 20 μmoles of plasmalogen far exceeded the endogenous plasma

[2] Abbreviations used are: alkenyl-GPE, α'-(1-alkenyl)-glycerylphosphoryl ethanolamine; alkenyl-GPC, α'-(1-alkenyl)-glycerylphosphoryl choline.

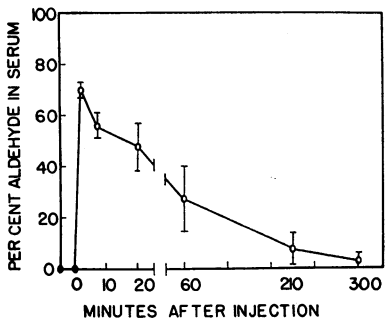


FIG. 1. INJECTION OF PLASMALOGEN EMULSION IN RABBITS. Ordinate represents calculated percentage of injected aldehyde recovered from the blood stream. Plasma volume calculated at 38 ml per kg (13). Open circles indicate the average value for 3 experiments and vertical lines the range of values.

plasmalogen. Figure 1 presents the results of 3 experiments. The percentage of injected aldehyde recovered from the plasma was calculated as follows: % recovery = 100 × μmoles aldehyde per ml serum × kg weight × 38 ml/μmoles aldehyde per ml emulsion × 4 ml. Sixty-five to 75 per cent of the emulsion was circulating 2 minutes after the injection. The material gradually disappeared from the blood, and at the end of 5 hours less than 10 per cent of the injected plasmalogen remained.

When plasmalogen is incubated *in vitro* with defibrinogenated blood, there is only slight decrease in the serum content of plasmalogen (14). The possibility exists, however, that *in vivo,* the transport of plasmalogen into the erythrocytes might explain its disappearance from the serum. With one injected animal, however, in which the usual disappearance of plasmalogen from the serum was demonstrated, there was no significant increase in the plasmalogen content of the erythrocytes.

It was postulated that the emulsified lipid particles might be filtered out in the rich capillary bed of the lungs. For this reason, lungs from control and injected animals were analyzed for aldehyde. In all experimental cases the injected plasmalogen had disappeared from the blood stream when the lungs were removed from the animal. In 7 noninjected animals the normal plasmalogen level was 15 ± 2 μmoles of plasmalogen per 10 g (wet weight) of lung. In the 4 injected animals, the plasmalogen level was identical, at 15 ± 2 μmoles. The total lung weight in our rabbits was close to 10 g. Since the injection was approximately 20 μmoles of plasmalogen, any significant trapping in the pulmonary bed would have been easily demonstrated.

The liver is known to be the principal site of formation of the plasma phospholipids (15). It is also active in removing phospholipids from the bloodstream (16). Because the liver exerts these controls on phospholipid metabolism, analyses of liver samples for aldehyde were performed at various intervals before and after plasmalogen injection. Table II presents the hepatic and serum aldehyde levels of an operated animal in which no injection was made. This experiment shows no significant change in plasmalogen levels due to the operation alone. The liver plasmalogen level in uninjected animals averaged 0.10 μmole per g (wet weight) of tissue with a range of 0.05 to 0.13 μmole per g. Since the liver weight varied from 50 to 100 g, the total plasmalogen content of the liver could vary from 2.5 to 13 μmoles. Therefore, with an injection of 20 μmoles of plasmalogen any significant collection of the injected material ought to be detectable in the liver. Figure 2A shows a progressive increase in the aldehyde content of the liver which is proportional to the decrease in circulating aldehyde. This experiment demonstrates that the liver is capable of removing injected plasmalogen from the blood stream. In a second animal, however, only 17 per cent of the injected plasmalogen appeared in the liver at the end of 3 hours. This could mean that the liver

TABLE II

*Aldehyde content * in liver and serum of noninjected, operated rabbit*

Time (after sham injection)	Aldehyde content	
	In total plasma†	In 10 g liver (wet weight)
minutes	*μmoles*	*μmoles*
0	0.50	1.30
Injection	0.50	1.30
2	0.30	
7	0.30	
20	0.30	1.30
60	0.30	1.50
120	0.30	
180	0.30	1.20

* Fuchsin-sulfurous acid determination of aldehydes in a lipid extract.

† Calculated as μmoles aldehyde per ml serum × kg × 38 (13).

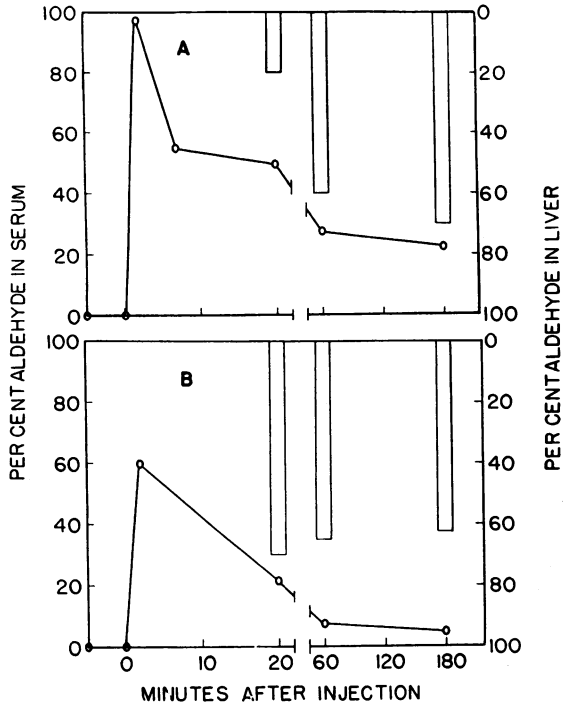


FIG. 2. RECOVERY OF INJECTED PLASMALOGEN FROM SERUM AND LIVER. Open circles represent percentage of injected aldehyde present in serum and bars, in liver.

was metabolizing the plasmalogen as it accumulated. This possibility is accentuated by the results shown in Figure 2B. In this experiment there was rapid removal of the plasmalogen from the blood. At 20 minutes, 79 per cent of the injected plasmalogen had disappeared from the circulation and 68 per cent of the plasmalogen was present in the liver. After 180 minutes, 95 per cent of the injected plasmalogen had been removed from the blood stream but only 62 per cent of the plasmalogen is present in the liver. This suggests that the plasmalogen is being metabolized by the hepatic tissue at a rate equal to or greater than its removal from the blood stream. Support for this possibility comes from the demonstration of an enzyme in rat liver microsomes that will hydrolyze the alkenyl ether linkage of alkenyl-GPC (7). Figure 3 shows the results of incubating rabbit tissue with the cephalin emulsion and alkenyl-GPE. The emulsion of cephalin is not degraded in 1 hour by rabbit liver homogenates, whereas 75 per cent of the alkenyl-GPE is hydrolyzed in that time.

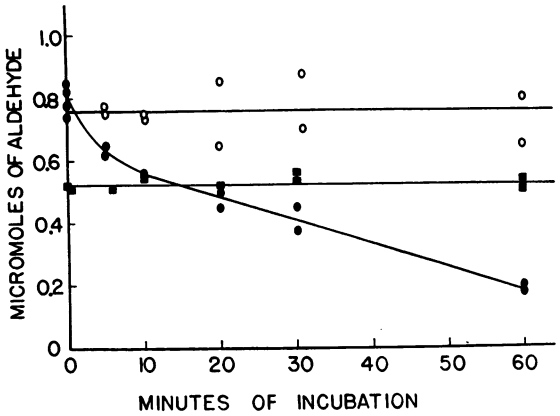


FIG. 3. HYDROLYSIS OF PLASMALOGEN BY RABBIT LIVER HOMOGENATE. Ordinate represents micromoles of aldehyde (plasmalogen) remaining in the incubation mixture. Open circles represent incubation of α′-alkenyl-glyceryl-phosphoryl ethanolamine (GPE) with boiled enzyme; closed circles, incubation of α′-alkenyl-GPE with fresh homogenate; squares, incubation of cephalin emulsion with fresh homogenate.

DISCUSSION

The physiological control of plasmalogen levels remains unclear. The circulating plasmalogen level is dependent upon diet, since plasmalogen-rich foods elevate the serum values (17) and plasmalogen deprivation lowers them (18). Serum plasmalogen levels are decreased with growth hormone (19), and increased with adrenocorticotrophic hormone and adrenalin (5). Variations in plasmalogen level may occur during different phases of the estrous cycle (20). Thiele presented an extensive review of the physiology of plasmalogen in 1956 (21). The findings in various pathologic states were summarized by Seckfort (5). It appears that plasmalogen decreases in diseases that have progressed to cachexia, but shows no consistent change in other diseases. We were unable to find in children any consistent variation in the fasting blood levels of plasmalogen related to a specific disease. Also, no elevation was found in four patients with acute myocardial infarction. The demonstration that large amounts of injected plasmalogen are removed rapidly from the blood stream of rabbits may explain the inability to find elevated serum levels in the various conditions studied. Plasmalogen is normally associated with the alpha-1 globulin fraction of serum proteins (22). Neither the amount nor the physical state of the plasmalogen injected is presumed

to be physiological. Even under these conditions, however, this work demonstrates that plasmalogen is not physiologically inert. In the blood and tissue analyses, plasmalogen level was determined by the fuchsin method. These levels may represent the sum of the injected plasmalogen and any fatty aldehyde released by the hydrolysis of plasmalogen. The aldehyde probably does not remain in either the plasma or the liver, since Feulgen, Imhäuser, and Westhues have shown that injected aldehyde cannot be detected in the blood stream (14). Also, the free aldehyde released from alkenyl-GPC in liver homogenate incubations disappears rapidly (7).

Our data show that the lungs and the erythrocytes do not accumulate injected plasmalogen. Feulgen and associates demonstrated in an *in vitro* system that defibrinogenated blood will not destroy added plasmalogen (14). Leupold and Buttner suggest that free fatty aldehydes, but not plasmalogen, will be rapidly oxidized in the lungs. These results suggest that neither tissue is involved in removing plasmalogen from the circulation.

Plasmalogen accumulated in the liver after injection. The percentage recovered there varied, however, and in one case began to decline after an initial elevation, suggesting that the plasmalogen was being metabolized. The whole homogenate of rabbit liver can hydrolyze alkenyl-GPE but apparently not the cephalin emulsion. The results are similar to those previously described for rat tissue (7), and show that the alkenyl ether cannot be hydrolyzed until the adjacent acyl group is removed. Presumably the action of a phospholipase in intact liver tissue could convert cephalin plasmalogen to alkenyl-GPE which would then be further hydrolyzed. This necessity for initial cleavage of fatty acid from the phospholipid accentuates the importance of phospholipases in plasmalogen breakdown. Phospholipase activity is present in liver tissue, but its relative activity is low in homogenates. In the liver incubations, as in the injections, the emulsion contained a ratio of diacyl-GPE to alkenyl-acyl-GPE of approximately 4:1 (see Table I). Some phospholipases hydrolyze diacyl-GPC more rapidly than alkenyl-acyl-GPC (24). Perhaps the failure to demonstrate hydrolysis of the plasmalogen in the emulsion is the result of competitive inhibition by diacyl-GPE for the enzyme. Also, in the intact cell phospholipases may be more active. These studies show that the liver may be involved in the metabolism of plasmalogen.

The possibility of plasmalogen removal by other organs, such as the spleen, or by other processes, such as excretion into the gall bladder, has not been ruled out. The rapid removal of plasmalogen from the blood makes its serum concentration of little use as a routine diagnostic or prognostic test in the assessment of degenerative diseases of the central nervous system or myocardium.

CONCLUSION

Plasmalogen emulsions were injected intravenously into adult male rabbits. Removal of 90 per cent of the injected plasmalogen occurred within 5 hours. No significant accumulation of plasmalogen in erythrocytes or the lungs was demonstrated. After injection, plasmalogen accumulates in the liver. Rabbit liver contains an enzyme that will hydrolyze the alkenyl ether linkage of α'-(1-alkenyl)-glycerylphosphoryl ethanolamine, but not that of the emulsified cephalin. The rapid removal of injected plasmalogen from the blood stream seriously limits the possible value of serum plasmalogen levels as a diagnostic or prognostic test in degenerative diseases of plasmalogen-rich tissue.

REFERENCES

1. Rapport, M. M., and Lerner, B. The structure of plasmalogens. IV. Lipids in normal and neoplastic tissue of man and in normal tissues of rabbit and rat. Biochim Biophys. Acta (Amst.) 1959, **33**, 319.
2. Debuch, H. Beitrag zur chemischen Konstitution der Acetalphosphatide und zur Frage des Vorkommens des Colamin-Kephalins im Gehirn. Hoppe-Seylers Z. physiol. Chem. 1956, **304**, 109.
3. Gray, G. M., and Macfarlane, M. G. Separation and composition of the phospholipids of ox heart. Biochem. J. 1958, **70**, 409.
4. Smith, C. H. Blood Disease of Infancy and Childhood. St. Louis, C. V. Mosby, 1960, pp. 28–29.
5. Seckfort, H. Zur Pathologie des Plasmalogen-Stoffwechsels. Verh. dtsch. Ges. inn. Med. 1953, **59**, 212.
6. Lea, C. H., and Rhodes, D. N. Phospholipids 2. Estimation of amino nitrogen in intact phospholipids. Biochem. J. 1954, **56**, 613.

7. Warner, H. R., and Lands, W. E. M. The metabolism of plasmalogen: enzymatic hydrolysis of the vinyl ether. J. biol. Chem. 1961, **236**, 2404.
8. Norton, W. T. Potentiometric iodometric determination of plasmalogen. Biochim. Biophys. Acta (Amst.) 1960, **38**, 340.
9. Lands, W. E. M. Metabolism of glycerolipides: a comparison of lecithin and triglyceride synthesis. J. biol. Chem. 1958, **231**, 883.
10. Bartlett, G. R. Phosphorus assay in column chromatography. J. biol. Chem. 1959, **234**, 466.
11. Hartree, E. F., and Mann, T. Crystalline lysoplasmalogen (lysophosphatidyl choline): preparation from heart muscle and action on erythrocytes and spermatozoa. Biochem. J. 1960, **75**, 251.
12. Warner, H. R., and Lands, W. E. M. To be published.
13. Spector, W. S. Handbook of Biological Data. Philadelphia, W. B. Saunders, 1956, 340.
14. Feulgen, R., Imhäuser, K., and Westhues, M. Zur Physiologie des Plasmalogens. I. Mitteilung: Über die Resorption des Plasmalogens und die Bedingungen für das Zustandekommen der alimentären Plasmalogenämie. Biochem. Z. 1928, **193**, 251.
15. Fishler, M. C., Entenman, C., Montgomery, M. L., and Chaikoff, I. L. The formation of phospholipid by the hepatectomized dog as measured with radioactive phosphorus. J. biol. Chem. 1943, **150**, 47.
16. Haven, F. L., and Bale, W. F. The fate of phospholipid injected intravenously into the rat. J. biol. Chem. 1939, **129**, 23.
17. Portwich, F. Über die alimentäre Plasmalogenämie beim Menschen. Dtsch. Z. Verdau.- u. Stoffwechselkr. 1954, **4**, 174.
18. Schäfer, G., and Taubert, M. Das Verhalten der Acetalphosphatide im Blutserum nach Zufuhr normaler Nahrung und bei Nahrungskarenz. Arzneimittel-Forsch. 1951, **5**, 97.
19. Haven, F. L., Bloor, W. R., Greene, A. A., and Mayer, W. D. Effect of growth hormone on lipid aldehydes. Science 1958, **128**, 1346.
20. Voit, F., and Seckfort, H. Die Serumlipoide unter besonderer Berücksichtigung des Plasmalogens. Klin. Wschr. 1955, **33**, 225.
21. Thiele, O. W. Der heutige Stand der Kenntnisse über die Acetalphosphatide. Arzneimittel-Forsch. 1956, **10**, 363.
22. Leupold, F. Über proteingebundenes Acetalphosphatid im Blut. Hoppe-Seylers Z. physiol. Chem. 1954, **296**, 55.
23. Leupold, F., and Buttner, H. Zur Physiologie der Acetalphosphatide. Verh. dtsch. Ges. inn. Med. 1953, **59**, 210.
24. Gottfried, E. L., and Rapport, M. M. The biochemistry of plasmalogens. I. Isolation and characterization of phosphatidyl choline, a pure native plasmalogen. J. biol. Chem. 1962, **237**, 329.