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DEPARTMENT OF THE ARMY
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PAPER-CHROMATOGRAPHIC ANALYSIS OF PLASMALOGENS

Hoppe-Seylers Zeitschrift fuer Physiologische Chemie (Hoppe-Seyler Journal for Physiological Chemistry), Vol. 315, 1959, pp. 157-162.

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Considerable progress has been made in the field of the paper-chromatographic analysis of lipids since we published our first report on this subject [1]. The sharp separation of the fractions accomplished by Marinetti and Stotz [2] on silicic acid-impregnated paper with an acetic acid-based solvent system was of especial interest. As we have established, however, two-dimensional chromatograms such as those described by these authors involve the liberation of free aldehydes and lyso-compounds: these compounds form already at 25° during the first development lasting more than 20 hours as a consequence of the high acetic acid concentration in the solvent system employed. They subsequently appear as separate fractions following development in the second dimension. This easy splittability of the aldehydes from the plasmalog by acetic acid, whereby the corresponding lysophosphatides are obtained as cleavage products, was demonstrated already by Klenk and Debuch [3,4].

In order to retain the good separating effect of these solvent systems, yet to prevent the hydrolysis of the unstable compounds insofar as possible, we developed our chromatograms within 2-5 hours at 2° in a unidimensional manner. Two-dimensional chromatograms are unreliable even under these mild conditions if the first solvent mixture contains acetic acid.

Methods

Tissue extracts: Total-lipid extracts were prepared from fresh or freeze-dried heart tissue from rats, hogs, cattle, dogs and monkeys; and also from reptiles and invertebrata with a 2:1 chloroform:methanol mixture.* The extracts from fresh tissue could be preserved especially easily, as has been shown by repeated paper chromatography after a period of up to 12 months. For comparison purposes, extracts were also made from freeze-dried material with acetone and gasoline. These extracts did not differ qualitatively from those made with a mixture of chloroform and methanol; however, they contained only one-half or one-third of the substances investigated. The presence of plasmalogen in acetone extracts is certainly an intermediate-solubility phenomenon since these substances do not move from the starting point in chromatography with acetone.

In order to identify the plasmalogens obtained in this manner, the extracts were investigated by means of paper chromatography directly and after various reactions. Kephaline and lecithine from cardiac and egg-yolk served as comparison substances after purification by chromatography on SiO_2 columns.

Reactions

All reactions were performed in test tubes and partly also directly on the paper.

a) Enzymatic hydrolysis: The following substances were used: lecithinase A (freeze-dried snake venom from Agkistrodon piscivorus received from the Ross Allen Reptile Institute in Silver Springs, Fla.); lecithinase C, prepared fresh from cabbage according to the method of Kates [7]; and lecithinase D, obtained from the toxin of Clostridium perfringens according to the method of Dr. G. H. Bornside [8]. One milligram enzyme was dissolved in one milliliter water and immediately shaken with one milliliter extract for one hour at room temperature. [See references 6-8 for enzymatic hydrolysis.]

b) Splitting with $HgCl_2$: One milliliter extract was incubated for one hour with one milliliter 0.05 M $HgCl_2$ in water at 25°.

c) Splitting with acid: One milliliter extract was incubated for 1-24 hours with one milliliter glacial acetic acid at 25°.

d) The effect of alkali was developed by adsorption on MgO [9] followed by elution with methanol or alkaline hydrolysis [10].

e) Catalytic hydrogenation: The ethanol-soluble portion of the extract was reduced with hydrogen at ambient pressure and room temperature in the presence of PtO_2.

The reactions listed under a), b), and c) were performed directly on the paper also: 0.01 µmole lipid was applied and dried. 1 µl enzyme solution or reagent was added and the paper was inserted between two Petri

* according to the method of Folch and co-workers [5]

dishes the lower one of which contained water. In order to keep the spots wet, more enzyme was added as required. At a temperature of 25°, the reaction time was 15-60 minutes; at the end of this period the material was dried and chromatographed.

Paper chromatography

Whatman paper No. 1, 14 x 14 cm, was impregnated with sodium silicate as described in [2]. The paper so treated was rinsed in ascension with a 2:1 chloroform:methanol mixture afterwards, just before use. Of the extract, 25 µl (0.01 µmole of the lipid) each was applied. The chromatograms were developed at 2° with a 40:23:3 di-isobutylketone:acetic acid:water mixture and then dried at 2° for 20 minutes.

The following papers were employed also: 1) Whatman No. 1, untreated; 2) the same, impregnated with Al_2O_3 by a modification of the method described by Bush [11]; 3) the same, impregnated with MgO by a similar method. On the papers 2) and 3), the plasmalogens migrated faster than did the corresponding diesters, in contrast to the other papers; 4) glass-fiber paper (H. Reeve Angel & Co.), impregnated with silicic acid, was employed for the identification of diglycerides [12] that formed in the hydrolysis of lecithinase D.

For the purposes of control, all tests described here were performed also at room temperature with the 2:1 chloroform:methanol solvent system. In all cases, the separation was less sharp than in the case of the di-isobutylketone:acetic acid:water system; however, these controls confirmed the results.

Stainings

1) Plasmal staining: The chromatograms were placed for 10 minutes in a freshly prepared solution of 1 ml fuchsine-sulfurous acid [1] and 1 ml 0.05 M $HgCl_2$* for each 100 ml 0.005 M H_2SO_3. The free aldehydes stained immediately to a violet color; natural plasmalogens became fully stained after 2 minutes, whereas lysoplasmalogens stained somewhat faster. Then, the chromatograms were washed three times for 10 minutes each with 0.005 M H_2SO_3, followed by either air-drying or staining with Rhodamin 6G [13] in 0.005 M H_2SO_3.

2) Amino nitrogen: The chromatograms were placed for 5 minutes in a freshly prepared 0.005 M ninhydrine solution, dried for 1 minute, and heated to 100° for 7 minutes. Tests were also conducted according to the method described in [14]. The chromatograms thus obtained could then be tested for choline and phosphorus.

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* A number of water-soluble mercury(II) compounds was investigated and found to be equally effective as $AuCl_3$ or $PtCl_2$. Mercury(I) compounds did not catalyze the reaction.

3) Choline: The chromatograms stained with ninhydrine [14] were placed for 5 minutes in 0.5% aqueous phosphormolybdenic acid, rinsed for 10 minutes under running water, and then immersed briefly in $SnCl_2$ solution (stock solution: 40% $SnCl_2$ in concentrated hydrochloric acid, and freshly diluted 1:10 before use), rinsed with water, and dried.

4) Phosphorus: The chromatograms were sprayed with freshly prepared reagent solution (2 ml H_2SO_4, 12 ml 2.5% ammonium molybdate solution, and 10 ml 0.85% NaCl), heated to 100° for 1.5 minutes, sprayed briefly (cf. 3), above) with $SnCl_2$ solution, and dried at room temperature.

5) Double bonds: The chromatograms were exposed for 10-15 minutes to OsO_4 vapors [1].

Results

A two-hour running time proved adequate for most purposes; generally, there was no evidence of plasmalogen splitting under these conditions. Wherever it appeared desirable to effect a better separation of the spots, more time was allowed; however, under such conditions the splitting products of the plasmalogens were often encountered.

The chromatography of a heart extract is shown schematically in the Figure. One may see two spots in untreated extract: according to their specific stainability, they are identified as plasmalogen-containing kephaline and plasmalogen-containing lecithine. These spots migrate only slightly slower than do kephaline and lecithine, respectively; thus, these could not be separated from the rest.

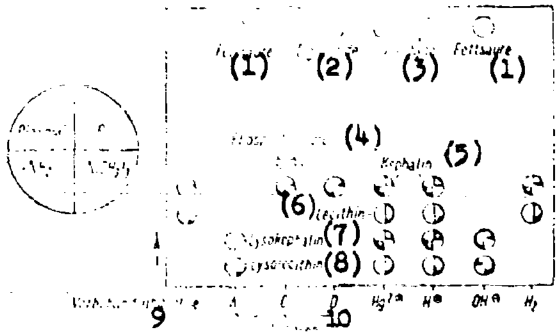
After incubation with lecithinase A, only lysokephaline and lysolecithine are identifiable in addition to fatty acids migrating at the front of the solvent. A few tests were conducted also with bee's venom: while the reaction was slower, it gave the same qualitative result. In contrast, the lecithinases C and D are specific for the choline phosphatides [15]; the kephaline fraction remained unaffected.

In cases where the enzymatic hydrolysis was performed on the paper, the reaction with lecithinases C and D was incomplete even after one hour; however, the reaction products were observable even in such cases.

The aldehydes of the plasmalogens are split off by mercury(II) chloride or acid, resulting in the formation of the corresponding lyso-compounds. These experiments also show that the extracts contain kephaline and lecithine also in addition to the plasmalogens.

As is well known, acetal phosphatides also form if alkalies are allowed to react with plasmalogen. Samples of synthetic colamine acetal phosphatide [16], kindly supplied to us by T. Malkin and C.L. Yarbo, possessed the same mobility as lysokephaline, only it reacted much slower than natural plasmalogen in plasma staining. After adsorption on MgO and elution with methanol, the plasmalogens reacted quantitatively.

Free fatty acids and the spots corresponding to the lysoplamalogens were observed as the reaction products in this case. It was more difficult to recover the reaction product of the amino nitrogen-containing plasmalogen since it exhibited a relatively strong adsorption.



Scheme of the paper-chromatographic separation of heart plasmalogenes in di-isobutylketone:acetic acid:water mixture (40:20:3) at 2° after various pretreatments. The specific stainability of the spots is indicated by the blackening of the spot diagrams according to the key shown at the left; for example, lecithine is positive for phosphorus and choline. All spots with positive plasmal staining showed also the reaction for double bond with OsO_4. 1) Fatty acid; 2) diglycerids; 3) aldehydes; 4) phosphatide acid; 5) kephaline; 6) lecithine; 7) lysokephaline; 8) lysolecithine; 9)pretreatment without; 10) lecithinases.

The hydrogenated material had to be chromatographed at room temperature since it had a low solubility at low temperatures. $HgCl_2$ effected no splitting, i.e., it gave no plasmal reaction, as has already been shown by Klenk and Debuch [3].

It should be stressed that this report describes only the main fractions of the plasmalogens, those that were present in all heart muscle extracts investigated. We consistently encountered also a third phosphorus-containing spot in the lysolecithine position. This we did not investigate further since it was present in low concentration only. In addition, there is always a weakly positive plasmal reaction in the solvent front.

The effect of lecithinase A was the same on the plasmalogens from hogs' and cattle's heart: in both cases, the corresponding lysoplasmalogens formed and only fatty acids formed in the free form. Nonetheless, we do not consider this finding as evidence that the aldehyde is attached to the glycerol in the β-position in all plasmalogens [17]; we much rather believe that the effect of this enzyme is more bond-specific than position-specific.

Analyses of human serum confirm our earlier finding [18] that the choline plasmalogen predominates there. A weak spot, that was observed by Boehm and Richarz [19] also, could be readily identified as amino nitrogen-containing plasmalogen.

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Summary

A simple and versatile chromatographic analysis, on paper impregnated with SiO_2, was described for plasmalogens and their various cleavage products. To prevent hydrolysis by the acetic acid component of the solvent system, the chromatograms were developed for 2-5 hours at 2°. The method was used for the examination of lipids from the heart tissue of various animals. The results confirm present notions on the structure of the plasmalogens.

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