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The Composition of the Difficultly Extractable Soybean Phosphatides

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THE MAJORITY of the papers dealing with soybean phosphatides have been concerned with the readily extractable soybean phosphatides, *i.e.*, that group of phosphatides which under normal industrial conditions are extracted with hexane or similar nonpolar solvents. They may be divided in two subgroups, *i.e.*, the hydratable phosphatides, which constitute the commercial product lecithin, and the nonhydratable phosphatides, which remain in the oil after the hydration. It has been shown that the hydratable phosphatides consist of phosphatidylcholine, -ethanolamine, and -serine as well as phosphoinositides (15, 19). In the latter group the presence of two types of inositides has been revealed, *i.e.*, phosphatidylinositol (13, 16) and another more complex inositide which contains carbohydrate (6, 7). In addition, the presence of lysolecithin (18), acetalphosphatides (9), and a phytoglycolipide (3), which contains phytosphingosine (2, 19), has been demonstrated.

The nonhydratable soybean phosphatides consist chiefly of a mixture of phosphatidic acids and lysophosphatidic acids (12).

So far no investigations have been published on the composition of the difficultly extractable soybean phosphatides, which is taken here to mean phosphatides that cannot be extracted with hexane but only with a mixture of hexane and alcohol.

Experimental

Difficultly extractable phosphatides were isolated from flaked soybeans by extraction with a mixture of 80% hexane and 20% absolute alcohol. The greater part of the lipides was removed previously by extraction with hexane at 60°C. The water content of the flaked soybeans was about 12%, and the oil content of the hexane-extracted flakes was about 0.7%. The difficultly extractable phosphatides amounted to 0.5% of the soybeans, a little less than the percentage of phosphatides extracted with hexane.

The phosphatides were fractionated by countercurrent distribution (15). For this purpose a 100-tube glass apparatus was used. The solvent system applied was carbon tetrachloride:chloroform:methylene chloride:methanol:water, 25:15:10:40:10 (4).

The content of phosphorus was determined by means of a colorimetric semi-micro method, depending on the formation of the yellow molybdivanadophosphoric acid (12) while nitrogen was determined according to the micro-Kjeldahl method. The individual nitrogen compounds were identified and determined by paper chromatographic methods previously described (12). Phosphoric acid esters were likewise identified by paper chromatography, and the same applies to glycerol and inositols (12), which were also determined quantitatively by oxidation with periodate after elution of the paper chromatograms. The carbohydrate-containing phosphatides were isolated by chromatographic separation on a column of cellulose powder, using the solvent system

n-propanol:conc. ammonia:water, 6:3:1.

Determination of the alkoxy group was performed according to Zeisel's method, as described in (10). Fatty acid esters were determined according to the method described in (8), which is based on the formation of hydroxamic acids.

Carbohydrates were determined according to the colorimetric method devised by Somogyi (17) and Nelson (11), following hydrolysis performed by refluxing for 7 hrs. with 0.6 N sulfuric acid.

Results and Discussion

Figure 1 shows the different distribution curves plotted by means of the above-mentioned analytical methods while Figure 2 illustrates the distribution of the nitrogen compounds.

The weight-distribution curve has four peaks and thus indicates the presence of four different groups of compounds.

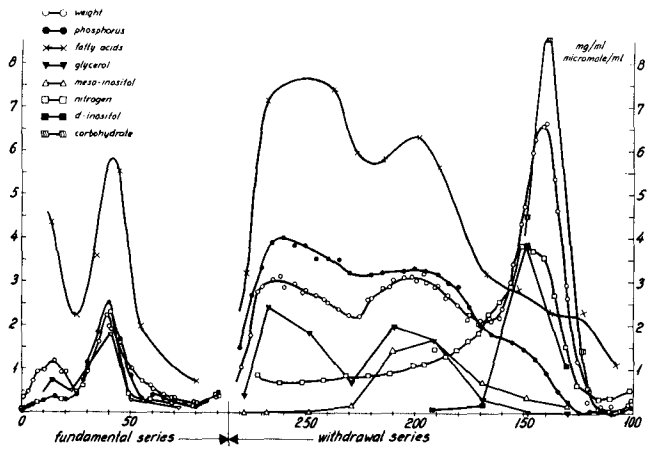

FIG. 1. Distribution curves for the difficultly extractable soybean phosphatides.

If we consider the group of compounds represented by the peak to the extreme right, *i.e.*, the most polar group, (transfers Nos. 130–150), it will be seen that the weight-curve peak to some extent coincides with the peaks of some of the other curves. The carbohydrate curve, in particular, displays an enormous peak within this range. As this peak is not reflected in any corresponding peak of the phosphorus curve, the linking of the carbohydrates to the phosphatides should thus, in accordance with the view generally held, be fairly weak. It is of greater interest however that the d-inositol curve also displays a peak. By paper chromatography of hydrolysates (6 N HCl for 48 hrs.) it was found that, in addition to the polyhydric alcohols, *i.e.*, glycerol and meso-inositol, which the phosphatides have hitherto been found to contain, the presence of a third polyol was revealed. Development of a paper chromatogram with the solvent system 75% acetone, 25% water, visualized by spraying with ammoniacal silver nitrate, resulted in the formation of two spots with the R_F values 0.38 (meso-inositol) and 0.45.

When a corresponding chromatogram was treated as described in (12), it was found that the periodate oxidation resulted in the formation of formic acid at $R_F = 0.38$ as well as at $R_F = 0.45$. Visualization of the chromatogram according to the method described in (12) shows that both substances produce the red color which is characteristic of inositols. From an HI-hydrolysate, deionized by means of two ion exchange columns (Amberlite IR 120 and Nalcite WBR), it proved possible to isolate a sufficiently large quantity of the substance with $R_F = 0.45$ to allow for a determination of the specific rotation, and this was found to be +70°. These results seem to indicate that the substance concerned is d-inositol. However this substance is present in combined form in the phosphatides since direct paper chromatography of the substances represented by the peak around transfer No. 145 does not disclose the presence of any substance with $R_F = 0.45$. It appears however that in the range around $R_F = 0.55$ there is, in addition to carbohydrates, a compound which cannot be fermented by means of *Saccharomyces cerevisiae* and neither yields the reactions characteristic of reducing carbohydrates nor those of nonreducing carbohydrates. Elution of this compound from the paper chromatogram and refluxing for 48 hrs. with 6 N HCl results in the formation of a compound with $R_F = 0.45$. After removal of carbohydrates by fermentation with *S. cerevisiae* and deionization by means of the above-mentioned ion exchangers, chromatographic separation on a cellulose column (solvent system 75% acetone, 25% water) of the nonhydrolyzed mixture in the peak at about transfer No. 145 resulted in the isolation of the substance with the R_F value 0.55. In equivalent quantities of a solution of the substance isolated in this way the two following determinations were made: a) the amount of d-inositol released after boiling for 48 hrs. with 6 N HCl, assuming that the quantities of formic acid resulting from the oxidation with periodate are the same for meso-inositol and d-inositol, b) the content of alkoxy groups. The molar ratio of d-inositol to alkoxy groups (calculated as $-OCH_3$) was 1.00:0.91. These results suggest that we have to do with an alkyl ether of d-inositol, probably the methyl ether called pinitol. The presence of this substance in soybean phosphatides has not been observed previously, but it has recently been found in the form of traces in the leaves of the soybean plant (14). As the peak of the distribution curve is not reflected in the phosphorus curve, the linkage of d-inositol to the phosphatides must, like that of the carbohydrates, be fairly weak.

The third curve to show a pronounced peak within this range is the nitrogen curve. Paper chromatographic separation applied to a hydrochloric hydrolysate (6 N HCl for 48 hrs.) demonstrates the presence of a large number of amino compounds. By adsorption on a strongly acid ion exchanger (Amberlite IR 120) and selective elution with dilute pyridine and dilute ammonia the amino compounds were divided up into acid, neutral, and alkaline compounds. By two-dimensional paper chromatography, using the solvent systems n-propanol:conc. ammonia:water, 6:3:1, and n-butanol:acetic acid:water, 4:1:1, it proved possible to identify, in addition to serine and ethanolamine, alanine, lysine, and glutamic acid. The nature of the linkages between these compounds and the phosphatides was not studied.

The other peak of the weight curve (around transfer No. 200) is reflected in the phosphorus curve and in the glycerol and meso-inositol curves but not in the nitrogen curve. The molar ratio glycerol:meso-inositol:phosphorus:fatty acid is about 1:1:2:4. After a brief acid hydrolysis (2 N HCl for 30 min.) it was found to be possible by paper chromatography to demonstrate the presence of inositolmonophosphoric

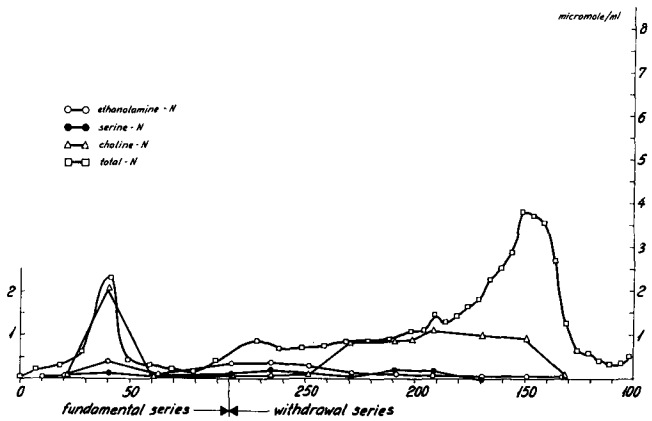

FIG. 2. Distribution curves for the nitrogen-containing compounds.

acid and glycerophosphoric acid. The compound concerned is evidently a glyceroinositophosphatidic acid, which is perhaps identical with the one previously found in soybean phosphatides (13).

The third weight-curve peak, around transfer No. 250, is like the last-mentioned peak, reflected both in the phosphorus and in the glycerol curve but neither in the nitrogen nor in the meso-inositol curve. As the molar ratio phosphorus:fatty acid is about 1:2 and since glycerophosphoric acid is the only phosphoric acid ester occurring in an acid hydrolysate, it would be reasonable to assume that the compound concerned was an ordinary glycerophosphatidic acid. The position of the peak does not however correspond to the position it should have if this were true, and electrometric titration shows it to be a monobasic acid with an equivalent weight of about 2,700. After being washed with acid for a short period, it may be titrated as a dibasic acid with the same equivalent weight. These facts suggest the presence of a polyglycerophosphatidic acid.

The fourth weight-curve peak occurs at about tube No. 40. It is reflected in the glycerol, phosphorus, and nitrogen curves. The molar ratio between these compounds is very nearly equal to 1. Moreover this peak coincides with a peak of the fatty acid curve. The molar ratio of fatty acid:phosphorus is about 2:1. Paper chromatographic separation of the substances released by acid hydrolysis (6 N HCl for 24 hrs.) shows that the only substances present are choline, ethanolamine, and serine. Therefore it is a reasonable assumption that what we are dealing with is a mixture of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine.

Summary

The object of the present work has been to study those soybean phosphatides which cannot be extracted by means of nonpolar solvents but only by means of mixtures of nonpolar and polar solvents, for instance hexane and ethanol. These phosphatides were fractionated by the countercurrent distribution technique, and the following groups of substances were found: a) carbohydrates with d-inositol in the form of the methyl ether called pinitol; b) a number of nitrogen-containing substances, the nature of which is not as yet fully elucidated but which is perhaps merely decomposition products of proteins; c) a glyceroinositophosphatidic acid which contains equimolar quantities of glycerophosphoric acid and inositolmonophosphoric acid and phosphorus and fatty acids at a ratio of about 1 to 2; d) a high-molecular phosphatidic acid; and e) a mixture of the three glycerophosphatides: phosphatidylcholine, -ethanolamine, and -serine.

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An Interlaboratory Study of Test Methods

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AN INTERLABORATORY STUDY of the accuracy and precision of test methods is one of the activities of the Smalley subcommittee on glycerin of the American Oil Chemists' Society. In this paper the statistical methods used to analyze the results of the 1957–58 study are described. The scoring system used to select the two laboratories awarded certificates of merit is also explained.

In addition to fulfilling Smalley Committee objectives, an interlaboratory test study could shed light on the following questions:

Do any of the laboratories have a constant error for the test?
What degree of variation can be expected when the test is used
a) by the same analyst on the same day?
b) over a period of several months within the same laboratory?
c) in different laboratories over a period of several months?
Can the variation of the test be considered the same
a) from month to month within the same laboratory?
b) from month to month within different laboratories?

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To the individual participating laboratory, the first question is probably the most important. For companies using the services of referee laboratories the degree of laboratory-to-laboratory variation for the test method is also an important consideration.

Discussion and Calculations

To realize the full potential information in an interlaboratory study it is necessary to have the results reported in the same uniform way by all participating laboratories. It is desirable to have each laboratory run and report the same number of determinations per sample. If laboratories run from one to 10 determinations per sample and report only the average value, not only is a great deal of information lost but the results are almost impossible to interpret statistically.

Twenty-five laboratories participated in the glycerin subcommittee program. Five samples were distributed at monthly intervals. The series included