SEPARATIONS OF SOY BEAN INOSITIDE FRACTIONS OF
LOW PARTITION COEFFICIENT*

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The inositol-containing phosphatides of soy beans have been the sub-
ject of a number of investigations (1–7) since 1939 when Klenk and Sakai
(8) first reported the presence of inositol in soy bean phosphatides. Pre-
vious work at the Northern Regional Research Laboratory (3) has shown
that the alcohol-insoluble portion of soy bean phosphatides can be sepa-
rated into two inositol-containing fractions by counter-current distribution
between hexane and 95 per cent methanol. If free sugars have previously
been removed by extracting the phosphatides with 55 per cent alcohol,
then that inositol-containing fraction of lower partition coefficient (the
one more soluble in methanol) is almost sugar-free (9). We have repeated
this work on a larger scale with essentially the same results, which are
shown graphically in Fig. 1. In the present paper we wish to report on the
further fractionation of the material of lower partition coefficient in Tubes
7 through 13 (Fraction A) and in Tubes 14 through 17 (Fraction B).
These fractions were chosen for further work because they are low in sugar-
containing impurities and because the nitrogen, phosphorus, and inositol
contents of the tubes combined were quite comparable. In our fractiona-
tion studies we employed two methods: (1) precipitation from chloroform
solution with methanol, and (2) precipitation from methanol with lead
acetate. By means of these procedures, it has been possible to separate
Fractions A and B into a fraction rich in phosphatidyl ethanolamine and
another rich in nitrogen-free phosphoinositides. Although inositol phos-
phatidic acids have been reported from other sources (10–12), the present
communication constitutes the first description of them in soy bean phos-
phatides. Similarly phosphatidyl ethanolamine has been prepared in com-
paratively pure state from brain lipides (13) and was, in fact, obtained from
soy bean phosphatides in 1925 by Levene and Rolf (14) in a concentrate
containing 25 per cent lecithin. However, the present isolation from the
fraction of low partition coefficient by the use of lead acetate yields phos-
phatidyl ethanolamine in 90 to 95 per cent purity and provides a potential
preparative method.

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461
EXPERIMENTAL

Analytical Methods—Phosphatides were analyzed by methods similar to those used in earlier work (3, 9). Phosphorus was determined on samples ashed with magnesium nitrate by the method of Truog and Meyer (15). Total nitrogen was determined by a micro-Kjeldahl procedure. Samples for nitrogen determination by the Burmaster periodate method (16) were hydrolyzed by refluxing for 24 hours with 2 n H₂SO₄. For use in the inositol assay (17) samples were hydrolyzed with 20 per cent HCl at 120° for 16 hours. Hydrolysates of samples which had been treated with lead were passed through a cation exchange resin column before inositol assay.

In the determination of sugar on samples from which free sugars have been extracted, higher and probably more correct values are obtained on samples refluxed with 2 n H₂SO₄ than on samples hydrolyzed under the milder conditions described in a previous paper (9). Sugar values are presented in certain instances which have been obtained by both type of hydrolysis; those obtained by refluxing with 2 n H₂SO₄ are so indicated.

Lead was measured approximately by ashing with H₂SO₄ and weighing as PbSO₄. It was assumed that other cations had been removed by the previous fractionation.

Glycerol was estimated by the procedure of Bradbury (18). Tripalmitin, used as a reference compound, produced a value 14 per cent too high. Appropriate corrections have therefore been applied for the results presented in Tables I to IV.

Preparation of Phosphatide Fractions—The phosphatides were prepared in a way similar to that previously described (9). Commercial soy bean lecithin was first stirred with acetone. The insoluble material was dissolved in ether and poured into acetone, and the insoluble material from this operation was extracted four times with acetone in a Waring blendor. Residual acetone in the precipitate was removed under a vacuum.

In order to remove free sugar components, the acetone-insoluble phosphatides were dissolved in hexane and extracted four times with 50 per cent alcohol. In some cases it was necessary to centrifuge in order to break emulsions. The hexane was removed by evaporation under a vacuum at about 50°; the residue dissolved in ether was again poured into acetone, and the precipitated phosphatides were again extracted three times with acetone in the Waring blendor. Residual acetone was again removed under a vacuum. From 2185 gm. of crude lecithin, 1045 gm. of oil-free, low sugar phosphatides were obtained.

The absolute alcohol-insoluble fraction was prepared by shaking 927 gm. of these phosphatides with absolute alcohol. Insoluble material was then extracted five times in a Waring blendor with absolute alcohol at room temperature and six times with absolute alcohol warmed to 50°. The insoluble
material was then extracted once more in the Waring blender with acetone and the residual solvent again removed under a vacuum. The weight of the absolute alcohol-insoluble fraction was 337 gm. Analytical data on this fraction are shown in Table I.

A twenty-five tube counter-current distribution of 301 gm. of this alcohol-insoluble material was carried out in 5 liter separatory funnels with 2 liters each of mutually saturated hexane and 95 per cent methanol in each funnel. The weight curve and analytical data in the fractions obtained are presented in Fig. 1.

It may be pointed out that the amount of material in the most hexane-soluble fraction, Tube 25, is much greater than in previous work (9). This is due to the variation of the partition coefficient of the more hexane-soluble fraction with concentration. In this fraction the partition coefficient is found to increase greatly with increasing concentration. Olley (19) has previously reported this same behavior.

It is observed in most of the fractions that the same approximate molar ratios of phosphorus to nitrogen to inositol of 2:1:1, found in previous work (3), are obtained. The shapes of the curves give no evidence of the separation of pure compounds. For further study, Tubes 7 through 13 were combined as Fraction A, and Tubes 14 through 17 as Fraction B. Data on these samples appear in Table I. Amino nitrogen in both fractions was shown by paper chromatography to be predominantly ethanolamine. In Fraction B, two very weak spots, one above and one below ethanolamine, were also found.

Fractionation by Treatment with Methanol Chloroform—Woolley (1) and Folch (2, 13) isolated inositol phosphatides from soy bean and brain by precipitation from chloroform solution with alcohols. Similar procedures

### Table I

**Composition of Phosphatide Fractions**

<table>
<thead>
<tr>
<th></th>
<th>P per cent</th>
<th>N per cent</th>
<th>Choline N per cent</th>
<th>Inositol per cent</th>
<th>Glycerol per cent</th>
<th>Sugar per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol-insoluble fraction</td>
<td>3.50</td>
<td>0.72</td>
<td>0.014</td>
<td>9.75</td>
<td>2.11</td>
<td>3.23*</td>
</tr>
<tr>
<td>Fraction A†</td>
<td>3.63</td>
<td>0.63</td>
<td></td>
<td>9.17</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>&quot; B†</td>
<td>3.46</td>
<td>0.72</td>
<td></td>
<td>7.78</td>
<td>9.0</td>
<td>1.43*</td>
</tr>
</tbody>
</table>

* Refluxed for 20 hours with 2 N H₂SO₄.
† Fraction A is a combination of Tubes 7 through 13 from the counter-current distribution of the alcohol-insoluble fraction; Fraction B is a combination of Tubes 14 through 17.
were found of value in this work, and one experiment is described in detail as follows: To 34.57 gm. of Fraction A in 200 ml. of chloroform, 1 liter of methanol was added. A precipitate formed and was allowed to stand overnight at \(-18^\circ\). The supernatant solution was decanted and evaporated to give Sample A1. The precipitate was dissolved in 100 ml. of chloroform. 1 liter of methanol was added, and the solution was again allowed to stand overnight at \(-18^\circ\). The supernatant solution was decanted and the precipitate dissolved in 50 ml. of chloroform. 500 ml. of methanol were added, and the solution was allowed to stand at 0\(^\circ\) overnight. The supernatant solution was decanted and the precipitate dissolved in 40 ml. of chloroform. 500 ml. of methanol were again added, and the supernatant solution was decanted from the material insoluble at room temperature. The supernatant liquids from the last three precipitations were combined and evaporated under a vacuum to give an intermediate fraction, No. A2. The insoluble material from the last precipitation is Fraction A3.

Analytical data on these fractions appear in Table II. As can be seen, the material is separated into a high nitrogen, low inositol fraction, No. A1,
and a low nitrogen, high inositol fraction, No. A3. It is possible that improved separation could be obtained after further study of the conditions of precipitation. However, more promising results were obtained by treatment with lead acetate, and the study was concentrated on this procedure.

Fractionation by Treatment with Lead Acetate—Previous workers (20-22) have used basic lead acetate for the purpose of separating α- and β-cephalins before the complex nature of the cephalin fraction was fully recognized. Chibnall and Channon (23) prepared the lead salt of phosphatidic acids as a step in their purification. In a preliminary experiment 1.336 gm. of Fraction A2 were dissolved in 10 ml. of chloroform and 515 ml. of 95 per cent ethanol. Addition of 30 per cent lead acetate gave a precipitate. In a similar way, with 1.346 gm. of Fraction A2, a precipitate was obtained upon addition of 50 per cent cadmium chloride. Both samples were cen-

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Weight</th>
<th>P</th>
<th>N</th>
<th>Inositol</th>
<th>Glycerol</th>
<th>Sugar</th>
<th>Fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>19.5</td>
<td>3.70</td>
<td>0.90</td>
<td>2.3</td>
<td>10.8</td>
<td>0.39</td>
<td>72.4</td>
</tr>
<tr>
<td>A2</td>
<td>14.1</td>
<td>3.41</td>
<td>0.52</td>
<td>12.8</td>
<td></td>
<td>0.90*</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>2.1</td>
<td>3.39</td>
<td>0.23</td>
<td>21.6</td>
<td>8.9</td>
<td>1.58</td>
<td>55.9</td>
</tr>
</tbody>
</table>

* Refluxed for 20 hours with 2 N H$_2$SO$_4$.

trifuged. The supernatant solutions were evaporated to dryness and both the soluble and insoluble fractions taken up in chloroform. The chloroform solutions were washed with 10 per cent acetic acid to remove lead and cadmium and then with 30 per cent alcohol to remove residual acetic acid. Chloroform was removed by evaporation to give the samples in Table III. Since lead acetate produced a much better separation on the basis of nitrogen content and since the emulsions formed on washing broke more readily than in the cadmium chloride-precipitated sample, lead acetate was used in further investigations.

In several subsequent fractionations difficulty was encountered in removing all of the lead from the insoluble fraction. This lead was most easily detected as lead sulfate in samples digested for Kjeldahl nitrogen or as lead oxide in solutions of ash used for phosphorus determination. Because of this difficulty, in later preparations excess lead acetate was removed by washing a chloroform solution of the product with 30 per cent alcohol until a negative lead test was obtained with H$_2$SO$_4$, and the insol-
uble fraction was isolated as a lead salt or addition compound. One such experiment is described as follows:

A solution of 10.99 gm. of Fraction B in 40 ml. of chloroform was added to 865 ml. of methanol and warmed gently to aid solution of the phosphatide in the methanol. This solution was decanted from a trace of insoluble material into 250 ml. centrifuge tubes, and 60 ml. of 30 per cent lead acetate were added. The precipitate was removed by centrifuging and washed with 80 ml. of methanol-wash solution (100 ml. of methanol plus 3 ml. of

### Table III

Subfractions Obtained by Treatment of Fraction A2 with Cadmium Chloride and Lead Acetate

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight (gm.)</th>
<th>P (per cent)</th>
<th>N (per cent)</th>
<th>Inositol (per cent)</th>
<th>Sugar (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium chloride-soluble</td>
<td>0.549</td>
<td>0.67</td>
<td></td>
<td>1.30</td>
<td></td>
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<tr>
<td>( \text{_}\text{_} )</td>
<td>0.584</td>
<td>0.30</td>
<td></td>
<td>0.46</td>
<td></td>
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<tr>
<td>Lead acetate-soluble</td>
<td>0.455</td>
<td>3.31</td>
<td>1.04</td>
<td>7.2</td>
<td>1.48</td>
</tr>
<tr>
<td>( \text{_}\text{_} )</td>
<td>0.806</td>
<td>3.14</td>
<td>0.12</td>
<td>16.2</td>
<td>0.63</td>
</tr>
</tbody>
</table>

### Table IV

Subfractions Obtained by Treatment of Fraction B with Lead Acetate

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight (gm.)</th>
<th>P (per cent)</th>
<th>N (per cent)</th>
<th>N (Bur-master)</th>
<th>Inositol (per cent)</th>
<th>Glycerol (per cent)</th>
<th>Lead (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1, insoluble fraction</td>
<td>0.91</td>
<td>2.16</td>
<td>0.06</td>
<td></td>
<td>6.05</td>
<td>5.6</td>
<td>32.6</td>
</tr>
<tr>
<td>B2, original soluble fraction</td>
<td>2.41</td>
<td>3.82</td>
<td>1.80</td>
<td>1.65</td>
<td>&lt;0.2</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>B3, intermediate soluble fraction</td>
<td>0.76</td>
<td>3.38</td>
<td>1.44</td>
<td></td>
<td>&lt;0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4, ( \text{_}\text{_} )</td>
<td>0.24</td>
<td>2.44</td>
<td>0.84</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B5, ( \text{_}\text{_} )</td>
<td>0.13</td>
<td>0.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6, ( \text{_}\text{_} )</td>
<td>0.11</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7, ( \text{_}\text{_} )</td>
<td>0.09</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

30 per cent lead acetate). The wash liquid and the soluble fraction were combined as the first soluble fraction. The precipitate was dissolved in 100 ml. of chloroform and added to 1600 ml. of methanol-wash solution. After centrifuging, the precipitate was redissolved in 80 ml. of chloroform and added to 1600 ml. of methanol-wash solution. The precipitate which formed was removed by centrifuging and precipitated in the same way a third, fourth, and fifth time. After this treatment, the insoluble fraction was dissolved in 250 ml. of chloroform and washed fourteen times with 30 per cent alcohol to remove excess lead acetate. The solution was filtered to remove a small amount of insoluble material. Chloroform was removed under a vacuum. This sample is designated as Fraction B1 in Table IV.
The soluble fractions were evaporated to dryness under a vacuum, and the residues were dissolved in chloroform. These chloroform solutions were washed three times with 30 per cent alcohol, then three times with 10 per cent acetic acid, and five times again with 30 per cent alcohol. In all cases the last acetic acid wash gave a negative lead test with dithizone. The solutions were evaporated under a vacuum and the residues listed as Fractions B2 through B7 in Table IV.

DISCUSSION

As has been shown by previous work, the alcohol-insoluble fraction of soy bean phosphatides is separated into two inositol-containing fractions by counter-current distribution between hexane and 95 per cent methanol (3). Neither of these fractions gives the binomial type of distribution curve expected for pure compounds. This is probably due to association between the various components as well as to the fact that both fractions appear to be complex mixtures. The composition of both of these fractions is still largely unknown. This paper discusses two procedures which have proved of value for further fractionation of the more methanol-soluble fraction.

The principal advantage of the precipitation by organic solvents is that the insoluble fraction is recovered in its original state rather than as a lead salt. The fact that similar, although less complete, separations are obtained also furnishes a confirmation for conclusions based upon the lead acetate separation.

In our work fractionation by treatment with lead acetate was much more effective. The soluble fraction, No. B2, is undoubtedly mostly phosphatidyl ethanolamine. The phosphorus to nitrogen ratio of 0.96 and the phosphorus to glycerol ratio of 1.03 are both in the correct range. Only a very small amount of inositol is present. Qualitative paper chromatograms together with the Burmaster nitrogen analysis indicate that nearly all of the nitrogen is present as ethanolamine. Impurities which produce low phosphorus and nitrogen values in the alcohol-soluble fraction are largely absent. Based upon the analyses for total phosphorus and total nitrogen, the sample may be calculated to contain 90 to 95 per cent phosphatidyl ethanolamine.

The very low nitrogen content of the material precipitated by lead acetate makes it almost certain that a nitrogen-free inositol phosphatide is present. Such a compound has not previously been reported in soy bean phosphatides. However, brain diphosphoinositide prepared by Folch (10) appears to be nitrogen-free and Macheboeuf and Faure (11) reported the presence of complex inositol-containing phosphatic acids in lipides of the tubercle bacillus. Also Faure and Morelec-Coulon have (12) recently isolated a glyceroinositophosphatidic acid from wheat germ by precipitation
as a barium salt. The molar ratios of Fraction B1 (P-inositol 1.81, P-glycerol 1.15, Pb-P 2.26) differ too much from small whole numbers to permit reliable assumptions to be made concerning the composition of the inositol phosphatide and suggest that it has not yet been obtained in a pure form. However, it probably is an inositol-containing phosphatidic acid occurring as calcium, magnesium, or potassium salts, since these metals have been reported in soy bean inositol phosphatides (1, 2). The phosphatidic acid is precipitated as an insoluble lead salt by treatment with lead acetate. The reason for the difficulty in removing lead is not known. It may be mentioned that Welch (21) also experienced difficulty in removing lead from cephalin fractions.

SUMMARY

It has previously been shown that the alcohol-insoluble portion of soy bean phosphatides can be separated into two inositol-containing fractions by counter-current distribution between hexane and 95 per cent methanol. It has now been shown that the fraction of low partition coefficient contains phosphatidyl ethanolamine and a nitrogen-free, inositol-containing phosphatide. These can be separated by precipitation of the inositide from chloroform solution with methanol or more efficiently by precipitation of the inositide from a dilute methanol solution with lead acetate.

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