A SIMPLIFIED PURIFICATION OF LECITHIN

BY MARY C. PANGBORN

(From the Division of Laboratories and Research, New York State Department of Health, Albany, New York)

(Received for publication, August 20, 1950)

A practical and easily reproducible method for preparing lecithin of a high degree of purity is urgently needed in connection with the use of lecithin in cardiolipin antigens (1, 2). Methods devised for this purpose have been published (3, 4), but the procedures, based on the classic studies of Levene and his coworkers (5, 6), were somewhat tedious and the lecithins so prepared have not always been satisfactory. Continued effort has therefore been devoted to simplifying and improving the available methods. The procedure here described has been adopted after numerous experiments in which many combinations of solvents were tried.

The details are presented by describing a typical preparation of egg lecithin. The purification of lecithin occurring in such complex mixtures as tissue extracts is more difficult, and each source material can be expected to present special problems. An example is beef heart, which was actually used for most of the experiments by which the present method was developed, and the modifications found necessary for this particular case are discussed. Since egg lecithin can be purified so much more easily, it is hoped that this product can be universally substituted for beef heart lecithin in cardiolipin antigens. Comparative studies of the two lecithins from this point of view will be reported elsewhere.

EXPERIMENTAL

For the chemical analyses reported in this paper the author is indebted to Mr. Milton B. Allen. Phosphorus was determined essentially according to the method of Elek (7). Iodine numbers were determined by the method of Yasuda (8). To determine amino nitrogen the samples were hydrolyzed by boiling with 10 per cent HCl, and aliquots of the filtered and neutralized aqueous solutions were analyzed by the manometric method of Van Slyke (9), with certain modifications suggested by Kendrick and Hanke (10).

Egg Lecithin—Twelve fresh eggs were separated and the yolks stirred for a few seconds in a Waring blendor; 200 ml. of acetone were added and the mixture was again stirred for about half a minute, then transferred to a beaker, and treated with 400 ml. of more acetone. This mixture was stirred thoroughly and filtered by suction. Repeated extraction with
Purification of lecithin

Acetone was carried out by returning the filter cake to the blender with 200 ml. portions of acetone and mixing briefly before refiltering. After five such extractions the yolk powder was creamy white, and the fifth acetone filtrate was only faintly yellow. Use of the blender made it possible to carry out the acetone treatment very rapidly and with minimum volumes of solvent.

The yolk powder was next extracted with 800 ml. of 95 per cent alcohol by shaking for \( \frac{1}{2} \) hour in a glass-stoppered bottle on a mechanical shaker. The alcoholic extract was filtered by suction and the filter cake discarded, since in a previous experiment it had been found that a second alcohol extraction yielded only very small additional amounts of lecithin. The alcoholic extract was precipitated with a slight excess of 50 per cent aqueous CdCl\(_2\), about 15 ml. being required. The mixture was refrigerated about 1 hour and then filtered by suction, and the precipitate was washed twice with acetone on the filter.

The precipitate was dissolved in 100 ml. of chloroform, yielding a slightly cloudy and faintly brown solution. This was poured with constant mixing into 700 ml. of alcohol to which 10 ml. of 50 per cent aqueous CdCl\(_2\) were added. The addition of CdCl\(_2\) is necessary to prevent dissociation of the lecithin salt and consequent loss of free lecithin into the filtrate. After the mixture had stood about 10 minutes at room temperature with frequent shaking, the precipitate flocculated well, leaving a clear supernatant; the mixture was filtered by suction. The precipitate was redissolved in 100 ml. of chloroform and the colorless solution poured into alcohol containing CdCl\(_2\) exactly as before. This time the precipitate was at first rather fine and tended to run through the filter; hence it was allowed to stand at room temperature for \( \frac{1}{2} \) hour with frequent shaking before filtration. One more chloroform-alcohol precipitation was performed in exactly the same way.

After three precipitations the cadmium salt was next suspended in about 150 ml. of petroleum ether, and to this were added 500 ml. of 80 per cent alcohol previously saturated with petroleum ether and containing 0.1 per cent of CdCl\(_2\) \((3, 4)\); this solution is referred to for convenience as the 80 per cent alcohol mixture. The suspension was shaken vigorously in a separatory funnel until solution was complete; the alcoholic layer was drawn off, and the amount of material remaining in the petroleum ether layer was roughly estimated by evaporating a small aliquot to dryness and drying it for about \( \frac{1}{2} \) hour in a vacuum desiccator before weighing. There were about 3 gm. of material in the petroleum layer. It had been found in previous trials that the use of 100 ml. of 80 per cent alcohol mixture per gm. of lecithin would give essentially complete extraction. The petroleum layer was therefore reextracted once
with 200 ml. and once with 100 ml. of the 80 per cent alcohol mixture. Less than 0.1 gm. of material now remained in the petroleum layer. The 80 per cent alcohol extracts were combined and concentrated in vacuo to approximately two-thirds of the original volume; this is usually sufficient to insure the removal of petroleum ether and the consequent separation of a fine flocculent precipitate of lecithin-CdCl₂. The concentrated mixture was refrigerated at about -5° overnight.

The lecithin-CdCl₂ which had separated from the chilled 80 per cent alcohol solution was filtered by suction and freed from Cd as follows: The precipitate was dissolved in about 150 ml. of chloroform and the solution was shaken vigorously for about 5 minutes with an equal volume of 30 per cent alcohol. Under these conditions the lecithin double salt dissociates, and the CdCl₂ is washed out in the dilute alcohol. This is readily demonstrated by testing the aqueous-alcoholic layer with a drop of 5 per cent AgNO₃. If the aqueous layer is cloudy owing to suspended droplets of chloroform, a sample may be clarified for the chloride test by warming it slightly.

The extraction with 30 per cent alcohol was repeated until the test for chloride was negative; this required four extractions. One more extraction with 30 per cent alcohol was then made to insure complete removal of cadmium. The final mixture emulsified somewhat but was easily separated by centrifugation. In numerous trials of this method, it has been found that the first extractions usually separate quickly with no emulsification. The process is therefore a very simple and rapid one, and it has been found much more convenient than the former methods in which Cd was removed by treatment with ammonia.

The cadmium-free chloroform solution was evaporated to dryness by vacuum distillation. In order to remove residual alcohol and chloroform as completely as possible, the lecithin was shaken vigorously with a little acetone, the acetone was decanted, and the residue was dried for a few minutes in vacuo. The lecithin was then dissolved in 100 ml. of anhydrous ether and to the clear water-white solution were added 20 ml. of acetone. This mixture was placed at 3-6° overnight. A finely flocculent, dense precipitate separated slowly in the cold and this was readily removed by rapid filtration on a small Büchner funnel. The ether-insoluble matter weighed 0.5 gm. The clear ether-acetone filtrate was evaporated to dryness in vacuo, and the purified lecithin was dissolved in absolute alcohol.

1 The addition of a little acetone to the ether solution greatly facilitates the removal of the ether-insoluble impurities, but in the case of egg lecithin, with its relatively low iodine number, there may also be a slow precipitation of the lecithin itself. Longer refrigeration or lower temperatures than those specified are therefore not desirable.
The yield was 7 gm.; P 4.03 per cent, N 1.98 per cent, NH₂-N 0.02 per cent, N:P 1.06:1.

Secondary Fractions—The several fractions removed during the course of purification were recovered for analysis.

The three chloroform-alcohol filtrates were separately evaporated nearly to dryness and the residues dissolved in ether. The ethereal solutions were repeatedly extracted with 0.5 saturated NaCl; at each extraction alcohol was added equal to one-tenth the volume of the ether (4). This treatment converts the Cd salts of acidic phosphatides, such as cephalin, to Na salts, and also washes out the CdCl₂ from any lecithin salt that may be present. The ethereal solutions thus freed from Cd were evaporated to dryness and the residues dissolved in methyl alcohol for analysis. Only traces of the material failed to dissolve.

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions Separated from Egg Lecithin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total weight</th>
<th>Lecithin*</th>
<th>NH₂-N per cent of total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl₃-alcohol Filtrate 1</td>
<td>2.6</td>
<td>0.79</td>
<td>64.5</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot; 2</td>
<td>1.3</td>
<td>46.5</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot; 3</td>
<td>0.71</td>
<td>12.2</td>
</tr>
<tr>
<td>80% alcohol filtrate†</td>
<td>0.67</td>
<td>0.60</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Estimated from the amount of non-amino nitrogen.
† This fraction was hemolytic. An alcoholic solution containing 13 mg. per ml. was diluted with 9 volumes of 0.85 per cent NaCl and the resulting suspension tested against sheep erythrocytes.

The 80 per cent alcohol filtrate from the lecithin-Cd salt was concentrated in vacuo to about 200 ml., and the suspension was shaken with about 100 ml. of chloroform and 100 ml. of 0.5 saturated NaCl. The chloroform layer was separated and washed four times with 30 per cent alcohol, then evaporated to dryness, and dissolved for analysis in absolute alcohol, in which it was completely soluble.

The properties of these fractions are summarized in Table I. From these data it is evident that the chloroform-alcohol precipitation gives a rapid and efficient removal of cephalin with relatively slight losses of lecithin. In another preparation, four instead of three chloroform-alcohol precipitations were carried out, and this yielded a lecithin containing no detectable amino nitrogen and having P 3.94 per cent, N 1.80 per cent, N:P 1.01:1. The iodine number was 54.8. The procedure including four chloroform-alcohol precipitations was therefore adopted for routine use.
Beef Heart Lecithin—The crude CdCl₂ precipitate obtained from the methanol extract of 6.2 kilos of minced beef heart (wet weight), after removal of the fraction precipitable by BaCl₂ (4), was precipitated three times from chloroform solution into alcohol exactly as described above. For each precipitation 200 ml. of chloroform and 1400 ml. of alcohol were used. The CdCl₂ was then removed, the washed chloroform solution was evaporated to dryness, and the lecithin was dissolved in 180 ml. of anhydrous ether. To the cloudy solution were added 36 ml. of acetone, when a rather bulky flocculent precipitate formed. The amount of this precipitate increased somewhat when the mixture was refrigerated overnight; in preliminary experiments, there did not seem to be any advantage in removing the first crop of precipitate before chilling the mixture.

In marked contrast to the precipitates that form at this point in ether alone, which are separable only with difficulty in a refrigerated centrifuge, the precipitate from the cold ether-acetone mixture was readily removed by suction filtration on a chilled Büchner funnel. The total weight of ether-insoluble matter was 4.4 gm.

The ether-acetone solution was evaporated to dryness and the lecithin was dissolved in alcohol. A little brownish, insoluble matter was present and the solution was slightly yellow. The partly purified lecithin weighed about 18 gm. and contained 0.25 per cent amino nitrogen.

For further purification the alcoholic solution was made alkaline to phenolphthalein with 10 ml. of saturated aqueous Ba(OH)₂; the mixture was immediately neutralized with CO₂ and 2 ml. of saturated NaCl were added with vigorous shaking to flocculate the precipitate. This procedure aids purification in two ways: traces of acidic phospholipides are precipitated on neutralizing with Ba(OH)₂, and the voluminous BaCO₃ precipitate adsorbs most of the pigmented impurities.

The precipitate was filtered by gravity, and the clear, nearly colorless filtrate was reprecipitated with CdCl₂. The Cd salt was twice precipitated by pouring the chloroform solution into alcohol as before and was then fractionated once by the petroleum ether-80 per cent alcohol method as described for egg lecithin. Cadmium was removed and the lecithin dissolved in ether to which 20 per cent of its volume of acetone was added. When refrigerated at -5° overnight, a very small precipitate formed which was filtered by gravity in the cold room. The ether-acetone solution was evaporated to dryness, and the lecithin was dissolved in absolute alcohol. The material removed in the last chloroform-alcohol fractionation was recovered for analysis. It weighed 0.7 gm. and contained 19 per cent of its nitrogen as amino nitrogen.

The yield of lecithin was 10.5 gm.; found, P 4.21 per cent, N 1.94 per cent, N:P 1.01; iodine number 83.7; NH₂-N < 0.01 per cent.
Since the analysis indicated the presence of some impurity with a higher P content than lecithin, a sample of this product was reprecipitated with CdCl₂ and purified once more by the petroleum ether-80 per cent alcohol method, but this treatment did not alter the composition, as on repeated analysis for P 4.21 per cent was found. In repeated preparations of beef heart lecithin the tendency for the phosphorus values to be higher than the theoretical has been consistently observed.

SUMMARY

A simple and rapid method is described for the purification of lecithin from fresh egg yolks.

By suitable modification, with a slightly larger number of steps, the method can be used for purifying lecithin from more complex source material such as beef heart.

BIBLIOGRAPHY

A SIMPLIFIED PURIFICATION OF LECITHIN
Mary C. Pangborn