Fractionation of Linseed Phosphatides[1]

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VERY little has been published on the composition of linseed phosphatides, and the recent papers have dealt only with the solubility in alcohol (1) and the fatty acid composition (2). Discovery of inositol in soy phosphatides by Klenk and Sakai (3) and the later studies by Woolley (4), Folch (5), and Scholfield *et al.* (6, 7) demonstrate the complexity of the soy and corn phosphatides. By analogy, linseed phosphatides might be expected to exhibit similar complexity.

This paper presents some analyses of the alcohol-soluble and alcohol-insoluble phosphatides from linseed oil and of the sub-fractions obtained from each portion by countercurrent extraction in the Craig apparatus. Although no pure compounds were isolated or shown to be present, several relationships are pointed out which should be of value in guiding future research.

Analytical Methods

The colorimetric method of Truog and Meyer (8) was used to determine the phosphorus content of samples which had been ashed with added magnesium nitrate. Nitrogen was determined by the micro-Kjeldahl method. Choline was determined as the reineckate by Glick's method (9). Sugar was determined by the method of Somogyi (10) on solutions prepared by hydrolyzing the samples for seven hours on a steam bath with 0.6 *N* sulfuric acid. Sugar was calculated as galactose although other sugars are known to be present also. Inositol was estimated by a modification of the Atkin, Schultz, Williams, and Frey (11) microbiological method for pyridoxin; solutions used had been autoclaved with 20% hydrochloric acid at 120° C. for 15 hours, filtered, and neutralized by passage through an ion-exchange column. Another portion of the sample was hydrolyzed for 15 hours at 120°C. with 2 *N* sulfuric acid for the determination of amino nitrogen by the Van Slyke method (12) and of "ethanolamine" nitrogen by the periodate oxidation method of Burmaster (13). The oxidation is not specific for ethanolamine, and the results include any other amino nitrogen adjacent to a keto or hydroxyl group as, for example, in serine.

Preparation of Fractions

The material was treated as were the soybean and corn phosphatides reported in previous papers (6, 7). Crude gums from commercial degumming of extracted linseed oil [3] were dissolved in ether and centrifuged to remove a small amount of ether-insoluble material. Part of the ether was removed and the phosphatides were precipitated by acetone. The precipitate was extracted twice with acetone, dissolved in ether, and reprecipitated with acetone. After three further extractions with acetone, the last two with the aid of a Waring Blendor, the crude phosphatides were dried and analyzed. The data are recorded in Table I.

The phosphatides were separated into an alcohol-soluble and an alcohol-insoluble fraction by repeated extraction with absolute alcohol in a Waring Blendor. The soluble fraction was evaporated to a pasty condition and redissolved in a small volume of absolute alcohol, resulting in a major portion soluble in alcohol (Fraction I) and a small amount which did not redissolve (Fraction II). The crude alcohol-insoluble fraction was treated twice with absolute alcohol at 50°C. in the Waring Blendor. That portion which dissolved was called Fraction III and the insoluble portion, Fraction IV.

All fractions were dried to constant weight in a vacuum desiccator over Drierite. They were protected from oxidation by keeping them under nitrogen as much as possible during their preparation and by storing them at 0°F. under vacuum.

Analytical results on these fractions are given in Table I. The figures for choline and inositol show that the removal of Fractions II and III resulted in some purification of Fractions I and IV.

Countercurrent Distribution

Fractions I and IV were subjected to countercurrent distribution in the preparative model of the Craig apparatus, used previously on corn phosphatides (7). As before, the solvents used for Fraction I were hexane and 90% methanol. For Fraction IV however 90% ethanol was used with hexane since the emulsions separated more readily. To minimize emulsion troubles the first three distributions of each fraction were made in separatory funnels. The solutions were then transferred to the Craig apparatus and the distribution completed. The volume of the bottom layer from each tube was measured to demonstrate that there had been no significant shift of the level of the interface. After the solvents from each tube had been evaporated, the residue was dried to constant weight, as described for the primary fractions. The residues were dissolved and made to volume, usually in chloroform. Small amounts of alcohol and water were added where necessary to cause solution, and aliquots were taken for analysis. The results are plotted in Figures 1 and 3.

Discussion

The analyses for choline and inositol shown in Table I indicate that essentially all the choline compounds, presumably lecithin, occur in the alcohol-soluble portion of the phosphatides and that the inositol compounds occur in the alcohol-insoluble portion. This is in agreement with results on corn and soy phosphatides although the separation obtained in this work is not as complete as was obtained on the other materials.

The sugar content is higher than in the corn and soy phosphatides and, in contrast to them, is evenly distributed among the fractions.

Values for amino nitrogen (Van Slyke) and ethanolamine nitrogen (periodate method) are in good agreement in Fraction I, but not in Fraction IV. This situation has been reported previously (6, 7, 13) and undoubtedly indicates the presence of amino

[1] This paper is based on work submitted by T. A. McGuire in partial fulfillment of the requirements for the M.S. degree at Bradley University, Peoria, Ill.

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TABLE I

Analysis of Phosphatide Preparations

	Proportion	Nitrogen	Phosphorus	Molar ratio	Sugar	Inositol	Choline nitrogen	Van Slyke nitrogen	Burmaster nitrogen
	%	%	%	*P/N*	%	%	%	%	%
Crude phosphatides (Acetone Insoluble)	100	1.44	3.70	1.16	10.8	5.7	0.24	.....	.....
Fraction I (Alcohol Soluble)	17.1	1.37	2.63	.87	10.4	.78	.96	0.16	0.12
Fraction II	1.7	.62	1.90	1.38	11.8	4.5	.33	.....	.....
Fraction III	2.8	1.05	2.60	1.12	11.2	3.5	.66	.....	.....
Fraction IV (Alcohol Insoluble)	78.5	1.39	3.97	1.29	10.2	7.1	.04	.94	.23

nitrogen of a type not attacked by periodate. It is possible that some compound could be interfering with the oxidation reaction, but as yet no interfering substance has been found to cause a low result when an adequate supply of periodate is present. Inositol, glycerol, dextrose, and choline do not interfere.

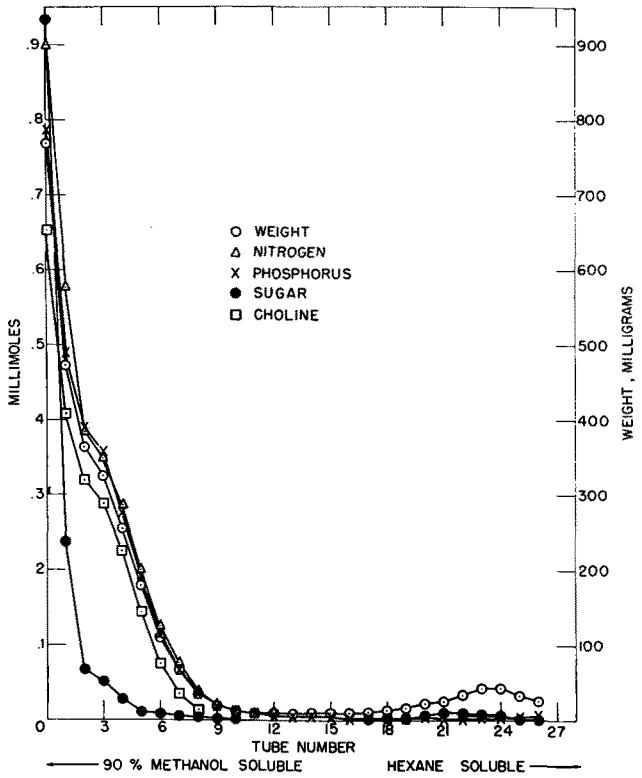

FIG. 1. Distribution of Fraction I.

The weight distribution curve for the alcohol-soluble phosphatides (Fig. 1) shows very little fractionation of the major constituents. Essentially, all the material is much more soluble in 90% methanol than in hexane. The small peak in the weight curve at Tubes 23 and 24, which also appears in the curves for corn and soy phosphatides, cannot represent phosphatides because the phosphorus and nitrogen curves show the amount of phosphatide to be no greater in these than in the adjacent tubes. The sugar curve has a slight maximum in this region but accounts for very little of the weight in these tubes. The saponification equivalent of the material in Tubes 23 and 24 (composite) was 520. This figure must be accepted with reservation because some insoluble substance remained after the saponification reaction. Since Fraction I saponified apparently completely, giving a saponification equivalent of 330, we conclude that the material causing the peak contains some unsaponifiable or difficulty saponifiable compound.

The difference between the slope of the sugar curve and the slopes of the phosphorus and nitrogen curves for the first three tubes suggests that there is no relation between sugar and the major phosphatide constituent in these tubes. Combination with a minor constituent is not ruled out, but it seems probable that much of the sugar in these tubes exists as free sugar.

The similarity of the curves for total weight, total nitrogen, phosphorus, and choline suggests that the phosphorus and nitrogen occur in a 1:1 molar ratio in most of the material and that much of the material contains phosphorus and choline in 1:1 molar ratio.

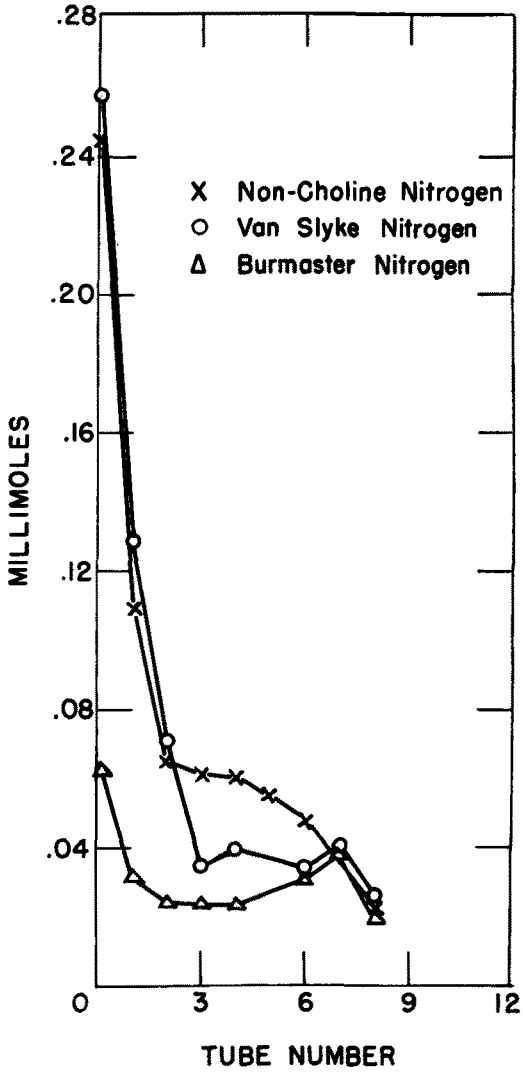

FIG. 2. Nitrogen distribution in Fraction I.

Since the preponderance of the choline compounds tends to obscure differences in the minor components, Figure 2 is presented to show the distribution of non-choline nitrogen (total nitrogen minus choline nitrogen), amino nitrogen, and ethanolamine nitrogen. Comparison of the curves for non-choline and Van Slyke nitrogen suggests that essentially all the non-choline is present as primary amine. The difference between nitrogen curves determined by the Van Slyke and the Burmaster methods shows the presence of some amino compound which does not give ammonia when oxidized by periodate. The Burmaster curve, with peaks at Tube 0 and Tube 7, shows two different nitrogen compounds reacting with periodate or, perhaps, one nitrogen compound combined with two different materials. This division of the Burmaster nitrogen precludes the calculation of the amount of cephalin, which in any event would be a decidedly minor constituent of the alcohol-soluble phosphatides.

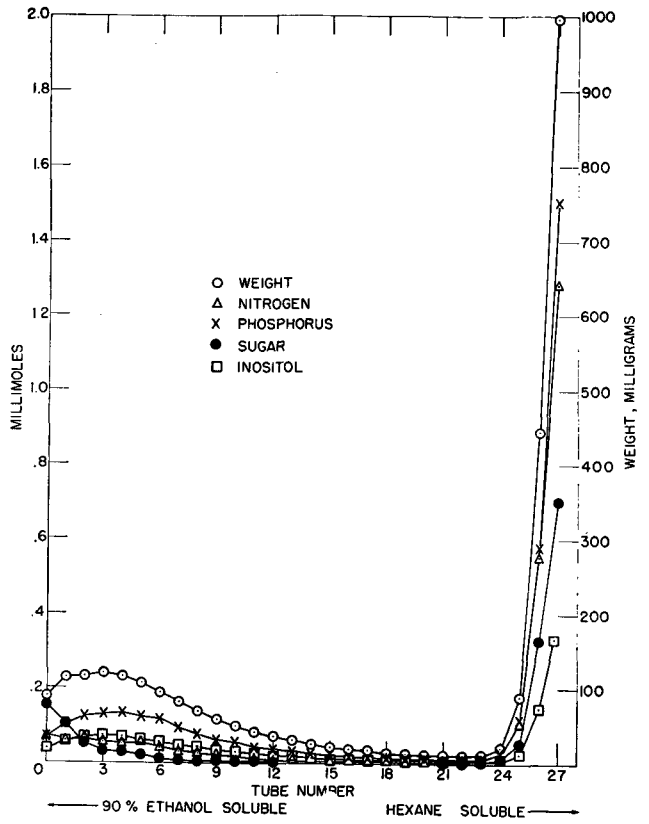

FIG. 3. Distribution of Fraction IV.

The distribution curves for the alcohol-insoluble fraction (Figure 3) show more separation than was obtained with the soluble portion. Approximately half the material is concentrated in the last four tubes whereas the other half is distributed through the first 24 tubes. The breadth of the peak, with maximum at Tube 3, indicates that the material is a mixture of several compounds. Calculation of molecular ratios leads to interesting speculations. The similarity of the phosphorus, nitrogen, and inositol curves at the left suggests that the group of compounds in this region contains these constituents in the ratio of 2:1:1. This suggestion is given some

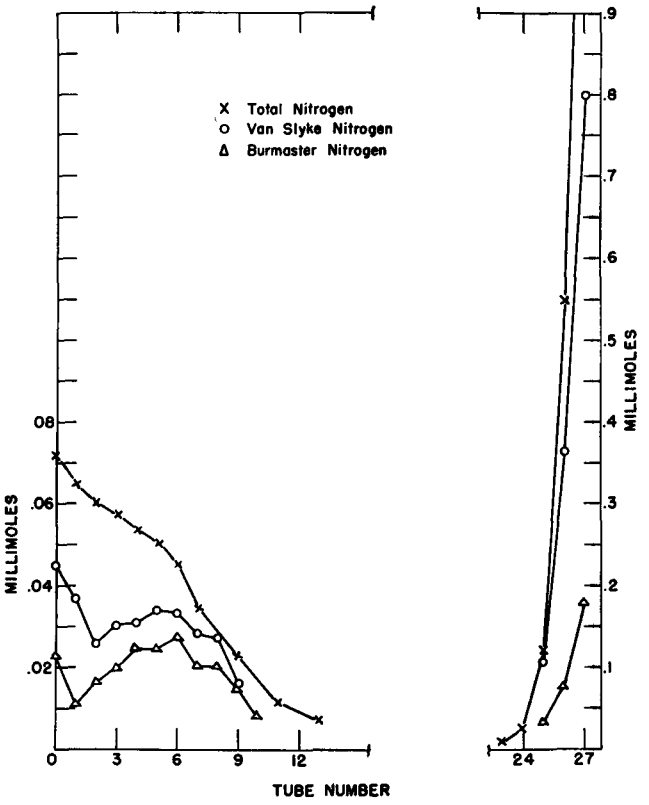

FIG. 4. Nitrogen distribution in Fraction IV.

support by the occurrence of these constituents in the same ratio in the corresponding tubes from the fractionation of corn and soy phosphatides. It may well be however that the appearance is fortuitous and results from the average composition of several different compounds. The sugar curve in this region shows no relation to the other curves, indicating that the sugar probably is not combined with any of the constituents plotted.

The steepness of the curve for Tubes 25, 26, and 27 probably means only that these compounds are so soluble in hexane that no fractionation is obtained with this solvent pair. The molecular ratio of 4:4:1 for phosphorus : nitrogen : inositol in Tube 27 is not an impossible combination, but we believe it more probable that the inositol is combined with phosphorus and nitrogen in some simpler ratio such as 1:1:1 or 2:2:1 and that the remainder of the phosphorus and nitrogen occur in other compounds. The presence of the large amount of sugar in the hexane-soluble fraction suggests that it is combined with the phosphorus and nitrogen. Further separations must be made before these speculations can be resolved.

The nitrogen distribution curves for the alcohol-insoluble fraction are shown in Figure 4. In the first seven tubes the difference between the total nitrogen and Van Slyke nitrogen demonstrates the presence of a nitrogen compound which does not react with nitrous acid under the Van Slyke conditions. Most of the amino nitrogen in the first 10 tubes is accounted for by the periodate oxidation. In Tube 27 the amino nitrogen is again about two-thirds of the total nitrogen, but the Burmaster nitrogen is quite low, only one-fourth of the amino nitrogen. The proportions of Van Slyke and Burmaster nitrogen in Tube 27 agree well with the results on Fraction IV in Table I.

Although the phosphatides of linseed, corn, and soybean oil show many similarities, they also exhibit many differences. All can be separated into alcohol-soluble and alcohol-insoluble fractions, but the proportions are widely different. Seventeen per cent of the linseed phosphatides appear in Fraction I (alcohol-soluble) and 78% in Fraction IV. Corresponding values for these fractions of soy phosphatides (6) are 51% and 38%. Besides having the smallest alcohol-soluble fraction, linseed also has the lowest concentration of phosphorus (2.63%) in this fraction. It is interesting that the large alcohol-insoluble fraction of linseed also contains the highest concentration of phosphorus (3.97%). Amino nitrogen occurs in both Fractions I and IV; the concentration is the same in the two fractions of soy phosphatides, but widely different in linseed.

Countercurrent distribution between hexane and 90% methanol shows that the alcohol-soluble fraction of the phosphatides from soybean, corn, and linseed oils consists of a group of compounds of similar solubility, more soluble in alcohol than in hexane. Distribution of the alcohol-insoluble fractions of the phosphatides from the three sources shows two groups, one more soluble in alcohol and the other more soluble in hexane. In the soy fraction the two groups are about equal in amount; in corn, the hexane-soluble portion is somewhat larger; and in linseed, it is much larger.

The differences in composition are great enough to suggest that linseed phosphatides might be of outstanding value in applications where the desired effect is caused primarily by the alcohol-insoluble fraction.

Summary

Alcohol-soluble and alcohol-insoluble fractions of linseed phosphatides were subjected to countercurrent distribution in the Craig apparatus. The soluble portion, which was shown by analysis to contain essentially all of the choline and very little of the inositol, gave little further fractionation on distribution between hexane and 90% methanol. Distribution of the alcohol-insoluble phosphatides between hexane and 90% ethanol showed two types of phosphoinositides to be present. Those concentrated in the alcohol phase had a phosphorus : nitrogen : inositol ratio of approximately 2:1:1 whereas those more soluble in hexane had a ratio of approximately 4:4:1.

Analysis for "ethanolamine" by the periodate method failed to show as much amino nitrogen as analysis by the Van Slyke method. This difference, considered with the shape of the ethanolamine nitrogen curves, prevents a satisfactory calculation of the amount of cephalin.

Sugar occurs also in all fractions, but its mode of combination, if any, was not demonstrated.

Acknowledgment

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Report of the Spectroscopy Committee, 1951

IN 1948 this committee recommended to the Society the adoption of a detailed ultraviolet spectrophotometric method for the determination of polyunsaturated acids. The method was essentially that of Beadle and Kraybill (1), Brice and Swain (2), Lemon (3), and others as modified for use in determining the polymerization index of soap used in synthetic rubber manufacture. The method as recommended and subsequently adopted by the Society was tested by the Spectroscopy Committee and reported upon in previous committee reports. Since the last report further cooperative work has been done to establish the validity of the method and to investigate suggested changes. This year's work reported herein was pointed toward:

a) The use of a new set of constants for calculating the tetraene, triene, and diene constituents of fats and oils. The new constants were reported on at the 1948 Fall Meeting in New York by Brice *et al.*, following work done on natural acids prepared at the Eastern Regional Research Laboratory.

b) The use of a 45-minute instead of a 25-minute isomerization time.

c) Substitution of the equation $k_2 = k_{233} - k_o$ for $k_2 = k_{233} - 0.029 - 0.052$ P. In the calculation k_o is 0.07 for esters and 0.03 for soaps and fatty acids. P was the estimated decimal fraction of oleic acid content of the sample being examined. Brice and Swain (2) showed that the absorption of methyl stearate and methyl oleate was identical and proposed in their report the use of the correction k_o above.

d) Simplification of the method by elimination of all measurements in the tetraenoic region when vegetable oils were being analyzed.

Four samples of vegetable oils, two cottonseed and two soybean, were submitted to six collaborating laboratories for analysis. Detailed instructions were given for analyzing the oils and for calculating the fatty acid contents. Five laboratories report the analysis of the samples by the prescribed spectrophotometric methods. Two laboratories also reported saturated acids by the Bertram oxidation procedure, two