

AN EVALUATION OF MICROMETHODS FOR PHOSPHOLIPID

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A number of analytical methods for phospholipid are in routine use by various workers in the field of lipid chemistry. Only limited data are available to show the extent of agreement or disagreement among the several methods, or to point out the most reliable means of comparing data obtained by one method with phospholipid values obtained by an entirely different analytical procedure. Some of the difficulties or discrepancies encountered in comparing data from two laboratories using even slightly different phospholipid determinations have been pointed out by Bloor (4) and Ellis and Maynard (5).

During the course of an investigation of the lipids of the developing pig fetus, data were collected on the phospholipid content of the tissues as measured by the three principal methods in common use, each being focused on either a different part of the phospholipid molecule, *viz.* the phosphorus and the fatty acids, or on the intact lipid. A comparison of the analytical results by the several methods is reported in the present paper.

EXPERIMENTAL

Extracts—Lipid extracts were prepared by exhaustively extracting the ground fetal tissue with hot alcohol-ether (3:1). The combined extracts were concentrated under a vacuum at 50° to a small volume. The concentrate was then exhaustively extracted with redistilled petroleum ether. Suitable aliquots of the clear petroleum ether solution were taken for the various analyses.

Lipid Phosphorus—An aliquot containing 2 to 4 mg. of phospholipid was evaporated to dryness, then digested with 3 ml. of 10 N H₂SO₄ and several drops of phosphorus-free 30 per cent hydrogen peroxide (superoxal) until water-clear. Excess peroxide was removed by adding 10 ml. of water and boiling down to the appearance of dense white fumes. The liquid was transferred to a 50 ml. volumetric flask, with 20 ml. of water, and an additional 3 ml. of 10 N H₂SO₄ were added.¹ The phosphorus was

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¹ This solution thus contained 6 ml. of 10 N acid as against 5 ml. in the flask containing the phosphorus standard. Insufficient acid, such as from loss of SO₃ fumes, gave erroneously low P values. A slight excess of acid, such as the above procedure assured, merely slowed the rate of color development. Loss of SO₃ fumes during digestion need not be avoided in this modification.

then determined by the colorimetric procedure of Kuttner and Lichtenstein (7), 20 to 30 minutes being allowed for full color development prior to reading. In the conversion factor, phospholipid = lipid phosphorus $\times 24.0$, an "average phospholipid" of molecular weight 744 (*i.e.*, an equimolecular mixture of oleopalmityl lecithin and cephalin) is assumed.

Phospholipid Fatty Acids—The acetone-insoluble lipids were prepared by the technique described by Bloor (4).² The phospholipid fatty acids were then prepared and assayed by the oxidative method of Bloor (2). The oxidation factor of 3.60 was used to calculate fatty acids. Conversion to phospholipid was based on numerous observations that approximately 63 per cent of the weight of the phospholipid is recoverable as fatty acid, which confirms the findings of Bloor (3), Sinclair (8), Bierich and Lang (1), Haven (6), and others, who report recovering less than two-thirds of the weight of the phospholipid as fatty acid.

Phospholipid—The phospholipid determination of Bloor (4)² was used on aliquots of the petroleum ether solution of lipids. The oxidation factor of 3.0 was used.

Experimental Data—The data obtained with three different methods for determining phospholipid on thirty-four samples of the tissue lipids of the fetal pig are summarized in Table I.

DISCUSSION

Considerably better agreement among replicate analyses was obtained from lipid phosphorus determinations than from either of the two oxidative methods for phospholipid. Thus, the standard error averaged less than ± 0.8 per cent (the range was 0.0 to 2.0 per cent) of the mean value for the phosphorus method, whereas the standard error averaged ± 2.1 per cent of the mean of the phospholipid oxidation values and ± 2.2 per cent of the fatty acid oxidation values, ranging from 0.3 to 6.3 per cent in the former and from 0.9 to 4.9 for the latter procedure.

Aside from the reduced scattering of replicate analyses in the phosphorus method, there was little choice for one method over another. The average of the thirty-four mean values for phospholipid based on the phosphorus was 593 mg. per 100 gm. of tissue; the average of the means was 594 for both of the oxidative procedures. This is additional confirmation of the validity of the conversion factors used for the several methods. The data indicate that for the lipids of animal tissues phospholipid values obtained by the method of Bloor (4) will be comparable to values calculated from the phosphorus content when an average molecular weight of

² The petroleum ether solution was concentrated to a smaller volume than that used by Bloor prior to the addition of acetone, and the precipitated lipid was twice washed with acetone.

TABLE I

*Mean Values for Phospholipid As Determined by Oxidation of Intact Molecule,
Oxidation of Fatty Acids, and Determination of Lipid Phosphorus*

The values ($\pm$ the standard error of the mean) are expressed as mg. per 100 gm. of fresh tissue. The figures in parentheses are the number of replicate determinations.

Sample No.	Lipid phosphorus $\times 24.0$	Oxidation of phospholipid	Phospholipid fatty acids 0.63
38x	248 $\pm$ 0.6 (3)	205 $\pm$ 2.6 (3)	265 $\pm$ 10.7 (3)
170	371 $\pm$ 2.0 (3)	350 $\pm$ 4.5 (3)	424 $\pm$ 16.0 (3)
162	413 (2)	421 $\pm$ 10.5 (4)	387 (2)
196	492 $\pm$ 9.1 (3)	495 $\pm$ 10.5 (4)	480 $\pm$ 10.1 (3)
189	540 $\pm$ 1.7 (3)	553 $\pm$ 11.9 (4)	534 $\pm$ 8.0 (3)
65	548 (2)	544 $\pm$ 4.0 (4)	550 $\pm$ 5.0 (3)
22	550 (2)	553 $\pm$ 11.7 (3)	614 $\pm$ 10.3 (3)
28	552 $\pm$ 1.0 (3)	555 $\pm$ 4.4 (4)	626 $\pm$ 11.5 (3)
200	553 $\pm$ 8.3 (3)	619 $\pm$ 6.6 (4)	619 $\pm$ 6.1 (3)
254	562 $\pm$ 11.4 (3)	608 $\pm$ 1.7 (4)	632 $\pm$ 7.5 (3)
187	564 $\pm$ 9.9 (3)	586 $\pm$ 7.7 (4)	558 $\pm$ 22.5 (3)
103	575 $\pm$ 8.6 (3)	658 $\pm$ 17.4 (3)	610 $\pm$ 13.8 (3)
118	592 (2)	533 $\pm$ 9.2 (4)	505 $\pm$ 8.2 (4)
135	601 $\pm$ 10.2 (3)	583 $\pm$ 4.2 (3)	635 $\pm$ 16.0 (3)
260	610 $\pm$ 1.0 (3)	611 $\pm$ 9.2 (4)	633 $\pm$ 6.0 (3)
95	611 $\pm$ 0.0 (3)	656 $\pm$ 11.9 (4)	616 $\pm$ 17.8 (3)
17	612 $\pm$ 2.0 (3)	671 $\pm$ 8.5 (4)	517 $\pm$ 13.9 (3)
72	614 $\pm$ 0.0 (3)	617 $\pm$ 5.7 (4)	581 $\pm$ 7.8 (3)
180	616 $\pm$ 0.0 (3)	593 $\pm$ 5.0 (3)	616 $\pm$ 12.0 (3)
230	617 $\pm$ 6.3 (3)	621 $\pm$ 14.5 (4)	622 (2)
107	627 $\pm$ 1.0 (3)	672 $\pm$ 7.6 (4)	754 $\pm$ 17.0 (3)
240	636 (2)	689 $\pm$ 43.1 (3)	627 $\pm$ 15.7 (3)
55	641 $\pm$ 0.0 (3)	593 $\pm$ 5.6 (4)	646 $\pm$ 10.4 (4)
29	643 $\pm$ 10.9 (3)	667 $\pm$ 25.7 (4)	675 $\pm$ 11.9 (3)
85	644 (2)	630 $\pm$ 7.4 (4)	598 $\pm$ 20.8 (3)
23	646 $\pm$ 10.3 (3)	657 $\pm$ 7.5 (4)	700 (2)
114	649 $\pm$ 10.0 (3)	554 $\pm$ 16.7 (3)	585 $\pm$ 12.4 (3)
34	658 (2)	630 $\pm$ 16.3 (4)	700 $\pm$ 12.5 (3)
105	662 $\pm$ 5.8 (3)	617 $\pm$ 13.9 (4)	533 (2)
38	681 $\pm$ 4.0 (3)	696 $\pm$ 9.1 (4)	629 $\pm$ 24.9 (3)
66	690 $\pm$ 0.0 (3)	686 $\pm$ 24.4 (4)	692 $\pm$ 6.0 (4)
21	714 $\pm$ 14.0 (3)	673 $\pm$ 14.5 (4)	674 $\pm$ 5.6 (3)
56	719 $\pm$ 2.0 (3)	751 $\pm$ 16.3 (4)	687 $\pm$ 34.0 (3)
39	726 $\pm$ 3.9 (3)	664 $\pm$ 50.8 (6)	661 $\pm$ 21.2 (3)

744 is assumed for the mixed phospholipids, and to values calculated from the phospholipid fatty acids when an average recovery of 63 per cent of the weight of the phospholipid is assumed.

It should be emphasized that any conversion factor is subject to error

when a mixture of lecithin, cephalin, and sphingomyelin is to be analyzed, since the relative distribution of the three fractions as well as the molecular weights of the fatty acids present affect the conversion factor. Close agreement among the three phospholipid methods will not be found when the composition of the sample varies significantly from the conditions assumed in making the calculations. Thus, it was not surprising to note that, although there was generally satisfactory agreement among the several methods, in certain instances one procedure gave results quite different from the other two. For example, there was excellent agreement between the two oxidation methods in the case of Samples 200 and 21, but the phospholipid content as determined from the phosphorus analysis was much lower in the former, and in the latter sample was considerably higher than the content as determined oxidatively. Similarly, in Samples 28 and 72 the lipid phosphorus values agreed closely with those obtained from oxidation of the intact phospholipid, while the contents indicated by the fatty acid determination differed significantly, in one case being higher and in the other, lower.

The data confirm the findings of others that although a conversion factor may prove fairly reliable in the "average" lipid sample, it may lead to erroneously low or high values for phospholipid in some instances. The unpredictability of these cases in which the factor is incorrect is the unavoidable, inherent fault of any micromethod for phospholipid. The lipid phosphorus analysis, however, will give a fairly true picture of the *molar concentration* if not of the *weight* of the phospholipid sample.

SUMMARY

Thirty-four lipid extracts of animal tissue have been analyzed for phospholipid by lipid phosphorus determination, by oxidation of the fatty acids from the acetone-insoluble lipids, and by direct oxidation of the intact phospholipid. When a phospholipid to phosphorus ratio of 24 was used, and when a fatty acid recovery of 63 per cent of the weight of the saponified phospholipid was assumed, the three methods gave comparable results. Considerably better agreement among replicate samples was obtained by phosphorus analyses than by either of the two oxidative procedures studied.

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