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Colorimetric Determination of Phospholipids by Use of Molybdophosphate, and Its Application to Amniotic Fluid

Howard G. J. Worth and Dennis J. Wright

We studied the reaction between molybdophosphoric acid and lecithin. The resulting complex was isolated and shown to contain molybdophosphate and lecithin in the molar ratio 1/3. It is insoluble in water but soluble in chloroform, and the reaction is specific for molecules containing electrophilic nitrogen, indicating good specificity in biological fluids for nitrogen-containing phospholipids. In solution the complex may be reduced, yielding molybdenum blue. We applied the reaction to the measurement of phospholipid concentrations in amniotic fluid. As compared with procedures involving either digestion or the determination of the lecithin/sphingomyelin ratio, the present technique is faster and its analytical precision exceeds that of the lecithin/sphingomyelin ratio determination. Although results by the three methods do not correlate well, the predictive value of the present method appears to be comparable with the other two.

Additional Keyphrases: *fetal status · lecithin/sphingomyelin (L/S) densitometric ratio · amniotic fluid · molybdophosphate/lecithin complex · intermethod comparison*

The clinical potential of the routine measurement of phospholipids in biological fluids has not been fully exploited, largely because of the lack of a quick and reliable assay. The work of Gluck (*1*) and his co-workers on fetal lung maturation and the respiratory distress syndrome in the neonate has resulted in a considerable interest in phospholipid measurement in amniotic fluid. Despite this, however, routine analytical methodology has not been developed and in most centers thin-layer chromatography is used to separate phospholipids, followed by a quantitation procedure (*2, 3*). This approach gives poor precision in all but skilled and specialized hands (*4*). The alternative is to produce a wet ash after extraction with organic solvent, with final measurement of inorganic phosphate (*5*). Although analytically acceptable, this is too time consuming to be suitable for routine analysis.

In the present study we describe a colorimetric assay based on a chromatographic spray reagent used by Levine and Chargaff (*6*). The method is sensitive to most, but not all, phospholipids; studies with pure lecithin indicate that a complex is formed between the phospholipid and molybdophosphoric acid, which in the presence of a suitable reducing agent will produce a molybdenum blue color. The lecithin/molybdophosphate complex is soluble in chloroform and may therefore be separated from excess molybdophosphoric acid, which is not chloroform soluble. The intensity of the molybdenum blue color is proportional to the amount of lecithin present.

Materials and Methods

Reagents

Dipalmitoyl phosphatidyl choline (synthetic, 98% pure), Sigma Chemical Co., Kingston, U.K.

Dodeca-molybdophosphoric acid, BDH Chemicals Ltd., Poole, Dorset, U.K.

Chloroform, analytical grade.

Methanol, analytical grade.

Anhydrous sodium sulfate, analytical grade.

p-*Methylaminophenol sulfate* (*"Metol"*), BDH Chemicals Ltd., Poole, Dorset, U.K.

Preparation of the Lecithin Molybdophosphate Complex

Lecithin (250 mg) was dissolved in chloroform (25 ml) and shaken with 100 ml of 5 mmol/liter molybdophosphoric acid in a 250-ml separating funnel. The chloroform (lower) layer was collected and the aqueous layer re-extracted three times with 25-ml portions of chlo-

Division of Clinical Chemistry, Northwick Park Hospital and Clinical Research Centre, Watford Road, Harrow AA1 3UJ, U.K.
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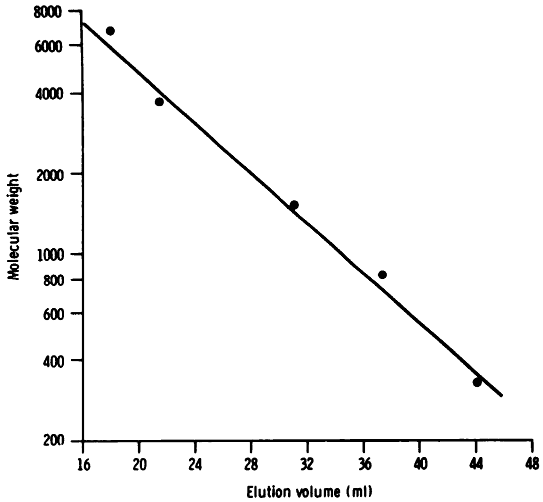


Fig. 1. Molecular weight vs. elution volume

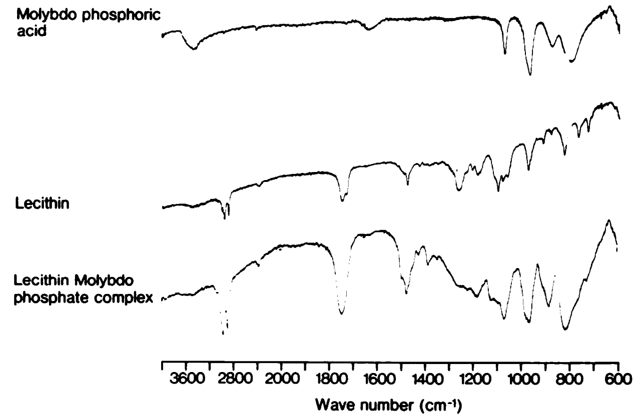


Fig. 2. Infrared spectrum of molybdophosphoric acid, lecithin, and lecithin molybdophosphate complex

roform. These extracts were pooled, dehydrated with anhydrous sodium sulfate (15 g), and the volume reduced to 5 ml in a rotary evaporator (Buchi Laboratory Techniques Ltd., Flarvick, Switzerland) on a water bath at 55 °C. Portions (1 ml) were purified by passing them through a 23.5 × 2.4-cm gel-filtration column of Bio-beads S-X1 (Bio-Rad Laboratories, Richmond, Calif.) with chloroform as eluent. The column was prepared by using beads that had been swollen by allowing them to stand over degassed chloroform for 4 h. [Sand (5 g) that had been washed in chloroform was placed on top of the column to prevent the beads from floating in the solvent.] Because of its color, we could see when the lecithin molybdophosphate complex was being eluted, and this fraction was collected and evaporated under nitrogen at 55 °C. The resulting solid was dried under decreased pressure overnight in the dark. Elemental analysis of this solid yielded: hydrogen 6.62%, nitrogen 1.07%, carbon 39.06%, phosphorus 3.53%, molybdenum 28.70%, and oxygen (by difference) 21.02%. When we tried to determine a melting-point, there was no visible change in appearance on heating to 300 °C.

Determination of Molecular Weight of the Complex

A column of Bio-beads was prepared as above. One-milliliter portions of 10 g/liter solutions of malachite green (mol wt, 330), Coomassie Blue (mol wt 854), Polyethylene Glycol 1500 (mean mol wt, 1500) Polyethylene Glycol 4000 (mean mol wt, 3650), and Polyethylene Glycol 6000 (mean mol wt, 6750) were loaded onto the column separately and eluted with a constant head of chloroform. The column eluent was collected at 1-min intervals and the time noted at which the compounds just began to emerge; from this the elution volumes were calculated. The polyethylene glycols were made visible by spotting samples from each fraction onto thin-layer chromatographic plates (ITLC-SG, Gelman Instruments Co., Ann Arbor, Mich.), spraying with ammonium sulfate (50 g/liter), and heating at 300 °C. The elution volume was plotted vs. molecular weight on semilogarithmic paper (Figure 1). A sample of the purified lecithin molybdophosphate complex was passed through the column in a similar fashion and gave an elution volume of 20.7 ml, which corresponds to a molecular weight of approximately 4300.

Infrared Spectroscopy

Samples of lecithin, molybdophosphoric acid, and solid lecithin/molybdophosphate complex were dried for three days over silica gel under reduced pressure. The infrared spectrum of each compound in the form of a KBr disc was obtained from 600 to 4000 cm^{-1} with a Hilger & Watts Infrascan spectrophotometer. The spectra are compared in Figure 2.

Determination of Lecithin as the Molybdophosphate Complex

Methanol (1 ml) was added to 10 × 1 cm glass tubes containing 2 ml of solutions of lecithin in chloroform. Then, after 1 ml of an aqueous solution of molybdophosphoric acid (10 mmol/liter) was added, the tube contents were mixed for 15 s on a vortex-type mixer, and were centrifuged at 1000 × *g* for 10 min. The aqueous (upper) layer was aspirated and the chloroform layer was dehydrated by adding 100 mg of anhydrous sodium sulfate. The absorbance of the chloroform layer was measured in cells of 1-cm pathlength and 1-ml capacity at 410 nm vs. a chloroform blank, in a Pye Unicam SP 1800 spectrophotometer. The absorbance/concentration response was linear, and a 1 mmol/liter solution gave an absorbance of 0.308.

The assay was repeated with the 1 mmol/liter lecithin solution and various concentrations of molybdophosphoric acid. Figure 3 shows that the molybdophosphate has little effect on the final absorbance when its concentration exceeds 2 mmol/liter.

Determination of Lecithin by Reduction of Its Molybdophosphate Complex

The lecithin/molybdophosphate complex was prepared in chloroform as previously described, with use of molybdophosphoric acid at a concentration of 5 mmol/liter and mechanically mixing for 15 s with 1 ml

Table 1. Effect of Some Reducing Agents on the Lecithin/Molybdophosphate Complex (Concentration of Lecithin Reagent, 450 μmol/liter)

Reducing agent (aqueous solution)	Absorbance at 680 nm (ref.)
Stannous chloride[a] (6 g/liter)	0.710 (*7*)
Metol[b] (10 g/liter)	0.210
Sodium metabisulfite (30 g/liter)	zero
Metol (10 g/liter)	(*8*)
Sodium metabisulfite (30 g/liter)	0.210
1,2,4-Aminonaphtholsulfonic acid (2.5 g/liter)	zero (*9*)
Ferrous ammonium sulfate hexahydrate (30 g/liter)	
Thiourea (10 g/liter)	0.140
Trichloroacetic acid (100 g/liter)	(*10*)
Ferrous ammonium sulfate hexahydrate (30 g/liter)	0.154
Thiourea (10 g/liter)	
Hydrazine sulfate (2 g/liter)	
Stannous chloride (0.2 g/liter)	zero
Sulfuric acid (28 ml/liter)	(*11*)

[a] Beer's law not always obeyed.
[b] Metol is unstable in solution unless sodium metabisulfite, is added.

of an aqueous reducing solution (see Table 1), followed by centrifugation at 1000 $\times$ *g*. The aqueous (upper) layer was discarded, the chloroform layer was dehydrated by adding 100 mg of anhydrous Na_2SO_4, and the absorbance measured at 680 nm vs. a blank containing no lecithin, as before. Table 1 shows the absorbances obtained with a lecithin solution (450 μmol/liter) and different reducing agents. A mixture of Metol and bisulfate was found to be the most satisfactory and was used in future experiments. A linear relationship was demonstrated between absorbance and lecithin concentration. A 1 mmol/liter solution of lecithin gave an absorbance of 0.495.

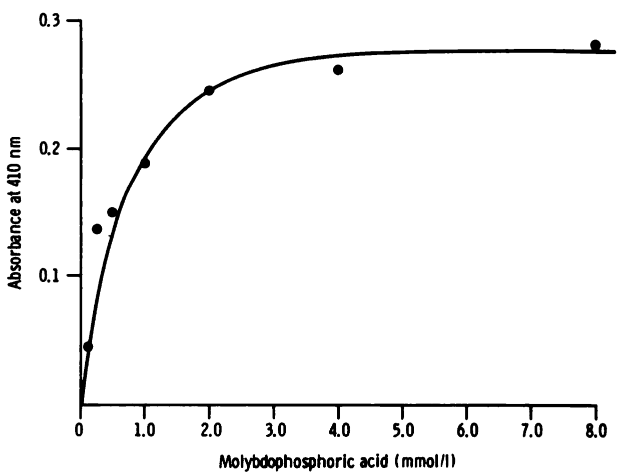


Fig. 3. Effect of molybdophosphoric acid concentration on color formation

Table 2. Formation of Molybdenum Blue with Different Reactants

Reactant	% Absorbance compared with lecithin
Tridodecylamine	57
Didodecylamine	100
Monododecylamine	46
Trioctylamine hydrochloride	65
Trioctylpropyl ammonium idodide	66
Aniline	11
Diphenylamine	2
N,N,N′,N′-Tetramethylethylene diamine	1
N,N-Dimethylaniline	3
Piperidine	<1
p-Phenylamine diamine	<1
Palmitic acid	<1
Tripalmitin	1
Dipalmitin	1
Monopalmitin	1
Linoleic acid	<1
Cholesterol	<1
Cholesteryl oleate	1
Cholesteryl stearate	<1
Sphingomyelin	183
Lysolecithin	30
Cephalin	14
Phosphatidyl serine	14
Phosphatidyl inositol	2

Specificity of the Reaction

We prepared 600 μmol/liter solutions of many compounds related to lecithin either chemically or physiologically (see Table 2), in methanol or chloroform. Samples of these were treated with molybdophosphoric acid followed by reduction with Metol as previously described. The absorbance at 680 nm was recorded and expressed as a percentage of the absorbance observed with lecithin treated similarly at the same molar concentration (Table 2).

Measurement of Phospholipid in Amniotic Fluid by the Molybdophosphate Method

Amniotic fluid (1 ml) was extracted with a mixture of 2 ml of chloroform and 1 ml of methanol by mixing for 15 s on a mechanical mixer and centrifuging for 10 min at 1000 $\times$ *g*. The aqueous phase was aspirated, 1 ml of molybdophosphoric acid was added, and the mixture was centrifuged for 10 min at 1000 $\times$ *g*. The aqueous phase was again discarded and 1 ml of reducing agent was added (10 g/liter Metol and 30 g/liter sodium bisulfate, see Table 1). Mixing, centrifugation, and removal of aqueous phase was repeated. Final traces of water were removed by adding 100 mg of solid anhydrous sodium sulfate. The absorbance was measured at 680 nm vs. a blank containing water instead of amniotic fluid. The method was calibrated with a lecithin solu-

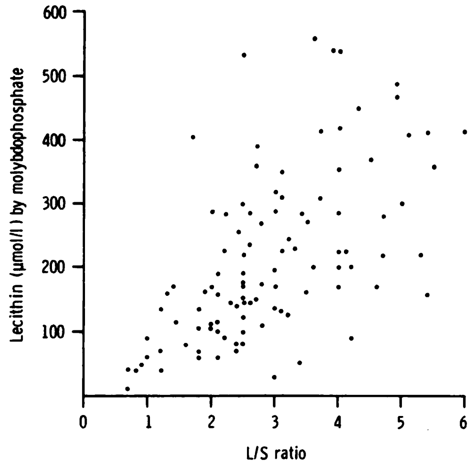


Fig. 4. Amniotic fluid analysis: Comparison of lecithin concentration as measured by molybdophosphate and L/S ratio

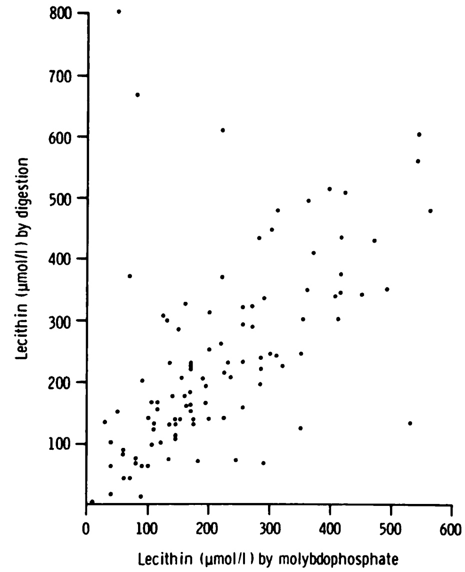


Fig. 5. Amniotic fluid analysis: Comparison of lecithin concentration as measured by digestion and molybdophosphate

tion in chloroform. Samples of a "control" amniotic fluid were analyzed in duplicate with each batch. This "control" consisted of samples of an amniotic fluid collected at the induction of a mature pregnancy and stored at −20 °C.

Measurement of Phospholipid in Amniotic Fluid by a Technique Involving Digestion

Amniotic fluid (1 ml) was extracted, digested, and assayed by using ammonium vanadate according to a method previously described (*4*). Inorganic phosphate, used for calibration, was also subjected to the digestion procedure. The "control" amniotic fluid was assayed in duplicate with each batch.

Determination of Lecithin/Sphingomyelin (L/S) Ratio

Amniotic fluid (2 ml) was extracted with a mixture of 2 ml of methanol and 4 ml of chloroform by mixing on a mechanical mixer for 30 s and centrifuging for 5 min at 1000 × *g*. The organic phase was evaporated under nitrogen on a water bath at 40 °C, the tube was transferred to an ice bath, and to the residue was added a mixture of 5 µl of chloroform and 400 µl of acetone. The tube was re-centrifuged, the solvent decanted, and the residue was then dissolved in 30 µl of chloroform. Portions of 10 µl were chromatographed on a thin-layer chromatographic plate (ITLC-SG; Gelman Instruments Co.) using for development chloroform/methanol/ammonium hydroxide (170/20/3 by vol); the spots were made visible by spraying with ammonium sulfate (50 g/liter) and heating the plates in an oven at 300 °C for 10 min. The product of the length and width of the spots was used to calculate the lecithin/sphingomyelin ratio.

Results

Precision

For duplicate measurements done by the present method on 18 occasions on the "control" amniotic fluid, results averaged 388 µmol/liter (CV 5.7%). The CV between duplicates was 1.3%. The results of 15 similarly duplicated measurements by the digestion method gave a mean of 274 µmol/liter (CV 4.5%).

Analysis of Amniotic Fluid

The three methods described were used to analyze 105 specimens, and the results are compared in Figures 4–6. The mean values for all specimens were: for the molybdophosphate method, 215 µmol/liter; the digestion method, 241 µmol/liter; and the L/S ratio determination, 2.9. Each method correlated with the other two as follows: molybdophosphate and digestion, $r = 0.57$; molybdophosphate and L/S ratio, $r = 0.59$; L/S ratio and digestion, $r = 0.52$.

Discussion

Phospholipid concentration in biological fluids has been mainly measured in research investigations rather than in the routine laboratory because of the lack of a method suited to the many specimens required to be assayed on a routine basis. The problems of phospholipid analysis are those common to all lipid fractions. Apart from the well-documented importance of the assessment of fetal lung maturity by the analysis of phospholipids in amniotic fluid, the possible significance of such analyses in other biological fluids is uninvestigated. The method described is reliable and sensitive and we think it makes analysis of large numbers of specimens feasible for the first time.

The principle of the analysis is the same as that used in one of the methods for the gravimetric analysis of phosphorus. Phosphate reacts with molybdic acid to give molybdophosphoric acid, which may be precipitated as an insoluble ammonium or quarternary ammonium salt (*12*). Lecithin, through its choline moiety, is a quarternary ammonium salt and forms a complex that is water-insoluble. Because of the lipophilic nature of the rest of the lecithin molecule the complex is chloroform-soluble and this property allows it to be sepa-

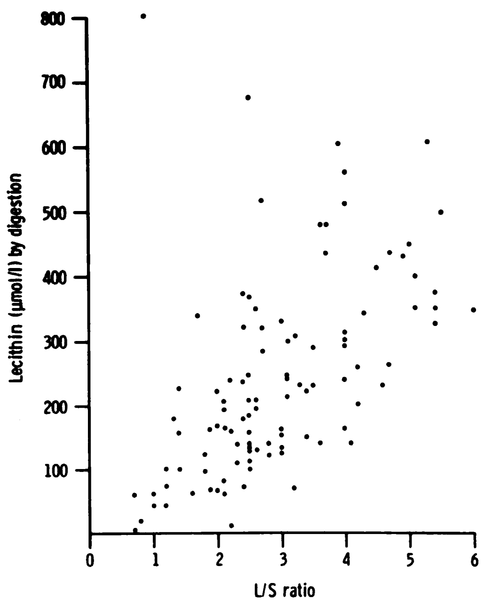
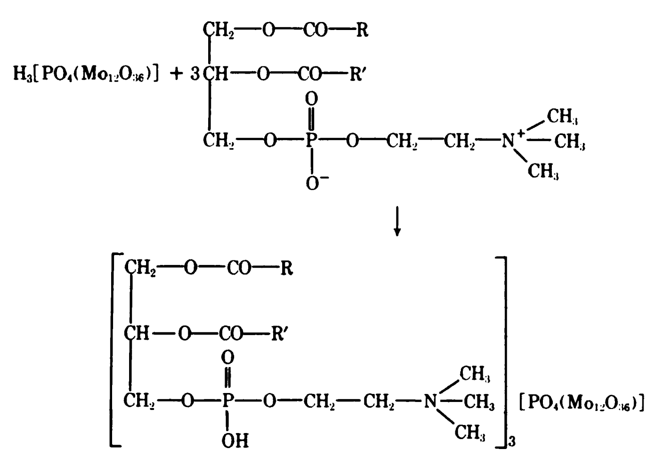


Fig. 6. Amniotic fluid analysis: Comparison of lecithin as measured by digestion and L/S ratio

rated from any unreacted molybdophosphoric acid. The reaction with ammonium salts shows the molybdophosphate is tribasic, viz.:

$$H_3[PO_4(Mo_{12}O_{36})] + 3\ NH_4^+ \rightarrow (NH_4)_3[PO_4(Mo_{12}O_{36})] + 3\ H^+$$

We therefore suggest that the analogous reaction with lecithin is thus:



The analogy was justified by preparing a complex with dipalmitoyl phosphatidyl choline. The empirical formulae for the tri-, di-, and mono-lecithin complexes are then, respectively $C_{120}H_{243}O_{64}Mo_{12}P_4N_3$, $C_{80}H_{163}O_{56}Mo_{12}P_3N_2$, and $C_{40}H_{83}O_{48}Mo_{12}P_2N$. The most sensitive changes in elemental ratio are C to Mo and C to P, these being C/Mo 10 → 7.5 → 3.3 (tri → di → mono) and C/P 30 → 26.6 → 20 (tri → di → mono). Our elemental analysis gave an empirical formula of $C_{120}H_{260}O_{52}Mo_{12}P_4N_3$ with C/Mo = 10.7 and C/P = 32. The theoretical molecular weights of the tri-, di-, and mono-lecithin derivatives are 4025, 3292, and 2559, respectively. Although the results of the elemental analysis do not agree absolutely with the theoretical value for tri-lecithin molybdophosphate, they are close. The discrepancy in the carbon and hydrogen analyses could be explained by the presence of small quantities of unreacted lecithin. The molecular weight found is close to the theoretical value for the tri-lecithin derivative, considering the effect of molecular shape on molecular weight determination by gel filtration.

Infra-red spectroscopy supports the formation of a lecithin molybdophosphate complex, as the strong absorption bands of lecithin (2960, 2880, 1745, 1475, and 1180 cm^{-1}) and molybdophosphoric acid (1070, 965, and 880 cm^{-1}) are seen in the spectrum of this complex (Figure 2). The peak at 1100 cm^{-1} for lecithin is indicative of P—O^-(13); this disappears in the case of the complex, supporting its conversion to P—OH.

The specificity of the reaction was investigated by measuring the color developed with other amines and their derivatives, and other lipids. Absorbances were compared with that for lecithin on an equimolar basis, and from the results shown in Table 2 it may be concluded that a positive reaction occurs when certain criteria are satisfied. The molecule must contain a nitrogen that is sufficiently electrophilic to enable it to react with molybdophosphoric acid, but the molecule as a whole must be sufficiently nonpolar to remain in the organic phase. These are opposing criteria in the sense that electrophilic compounds usually are not soluble in organic solvents such as chloroform, and consequently many compounds give a negative or weak reaction. The most electrophilic amino nitrogen, represented by the quarternary ammonium salt trioctylpropylammonium iodide, gives a reaction, but to a lesser extent than for lecithin, presumably because of its greater solubility in the aqueous phase. By the same argument the responses from lysolecithin, cephalin, and phosphatidyl serine are less than that for lecithin. The enhanced reaction with sphingomyelin possibly is attributable to the presence of an amide nitrogen as well as the amine. Phosphatidyl inositol and the neutral lipids investigated give no reaction because they contain no nitrogen. Similarly, no reaction is observed with less-electrophilic amines such as aromatic and heterocyclic compounds. From the data in Table 2 it seems likely that lecithin, sphingomyelin, lysolecithin, cephalin, and phosphatidyl serine are the only compounds encountered in biological fluids that give a positive reaction. Further proof of the accuracy of the technique will have to await the development of definitive and reference methods for phospholipid measurement.

Application of the primary reaction to measurements in amniotic fluid showed that the sensitivity was too low, and so the reduction step was included. Metol stabilized with metabisulfite was chosen as the most suitable reductant, considering sensitivity and stability (Table 1).

Application of the method, including reduction, to amniotic fluid analysis showed that repeatability was acceptable. The method has similar precision to the digestion procedure and is more precise than the determination of the L/S ratio, for which a CV of 20% was

Table 3. Specimens with One Determination at Variance with the Other Two

Specimen no.	Molybdophosphate method	Digestion method	L/S ratio
	Phospholipid, μmol/liter		
1	70*[a]	373	2.4
2	90	12*	2.2
3	100	64*	2.1
4	290	69*	2.0
5	245	72*	3.2
6	30*	136	3.0
7	135*	75	1.2
8	70	183*	1.8
9	160	180	1.3*
10	50*	154	3.4
11	80	74	2.4*
12	115	157	1.4*
13	105*	98	1.8
14	170	229	1.4*
15	405	340	1.7*
16	50	804*	0.9

[a] * denotes determination at variance.

obtained in a previous study (*4*). It is quicker than either; as many as 20 specimens can be processed within half a day by one worker, and single assays can be done within 1 h. The three techniques were used on 105 specimens of amniotic fluid and the results are compared in Figures 4–6. Although there is some correlation, the results on many specimens do not compare well by the three methods. This includes the results obtained with the control fluid, the results from the molybdophosphate method being higher than those from the digestion method. However, these results are within the scatter of the two methods (Figure 5).

In any event, as different analytes are being measured in each case this situation is not surprising and it is more important to compare prediction. Fetal lung maturity is assumed by Gluck et al. (*2*) if the L/S ratio is greater than 2.0, and similarly a phospholipid concentration of greater than 100 μmol/liter by the digestion method (*4*). A decision level of 88 μmol/liter was derived for the molybdophosphate method by multiplying the ratio of the mean phospholipid concentration for all specimens obtained by the molybdophosphate method and the digestion method by 100 (the value of the decision level derived for the digestion method). By these criteria, 22 of 105 specimens were found to have one prediction at variance with the other two. By excluding those specimens where the determinant at variance was so close to the action value as to be within the limits of the precision of the method, the number was reduced to 16 (Table 3). Of these, the molybdophosphate method was at variance in five cases, the digestion method in six, and the L/S ratio in five. In only five cases were the specimens taken sufficiently close to delivery (within two days), for the value to be considered predictive of the actual outcome. These were specimens No. 1, 3, 5, 10, and 16. In all of these except No. 16, the outrider predicted immaturity, whereas the infant when born showed no respiratory distress. The results on specimen No. 16 are curious, because the determinant at variance gave the correct prediction; also, the difference between the variants was much greater than we observed for any other patient. The value of 804 μmol/liter was in fact the highest recorded with the digestion method. If it were not for the fact that this value fitted with the clinical outcome we would suggest that it was a spurious result due to contamination of the specimen.

Clearly there is need for a further investigation into the predictive aspect of this assay by using only specimens collected at delivery where the outcome of pregnancy can then be directly related to the value obtained.

The technique is useful because of its relative simplicity, speed, and ease of application to large numbers if necessary.

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