

Non-choline-containing Phospholipids Solubilize Divalent Metal Ions in Organic Solvents. The Basis for a Sensitive Assay Procedure

JOSEPH W. DePIERRE * and GERD LUNDOQVIST

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

Phospholipids which do not contain choline were found to solubilize Ni(II) in the chloroform phase resulting from the Folch extraction procedure. On the basis of this phenomenon a radioactive assay for such phospholipids was developed using $^{63}\text{Ni(II)}$. This assay can be made rather sensitive by scaling down and can be performed relatively rapidly. The disadvantages are that it does not measure phosphatidyl choline or sphingomyelin and that different phospholipids bind different amounts of Ni(II). Alternatively, non-choline-containing phospholipids can be assayed colorimetrically on the basis of their ability to solubilize Cu(II) in the chloroform phase. The failure of phosphatidyl choline and sphingomyelin to bind divalent cations may be due to formation of an intramolecular salt between the negatively charged phosphate group and the positively charged nitrogen.

Free fatty acids can be quantified on the basis of their ability to form complexes with divalent metal cations and thereby render them soluble in organic solvents.¹⁻⁶ While employing such a method based on the solubilization of $^{63}\text{Ni}^{2+}$ in chloroform-heptane,⁶ we found that the presence of phospholipids resulted in a high background.⁷ We now demonstrate that phospholipids which do not contain choline can also solubilize divalent metal cations in organic solvents and that this property can be used as the basis for sensitive radiometric and colorimetric assay procedures.

MATERIALS AND METHOD

Chemicals. Several different preparations of L- α -phosphatidyl choline (P7763=purified from bovine liver; P5388=purified from egg yolk; P6263=purified from soybeans; P0763=synthetic dipalmitoyl phosphatidyl choline; P1013=synthetic dioleoyl phosphatidyl choline), sphingomyelin (S7004=purified from bovine brain), several different preparations of L- α -phosphatidyl ethanolamine (P6386=purified from egg yolk; P4513=purified from soybeans; P5138=synthetic dipalmitoyl phosphatidyl ethanolamine), L- α -phosphatidyl inositol (P0639=purified from soybeans), L- α -phosphatidyl L-serine (P6641=purified from bovine brain), L- α -phosphatidyl-DL-glycerol (P0514=from egg yolk), and L- α -phosphatidic acid (P4013=synthetic dipalmitoyl phosphatidic acid) were all purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). In addition phosphatidyl inositol (A38=purified from pig liver) and phosphatidyl serine (A37=purified from beef brain) were obtained from Serdary Research Laboratories, Inc., London, Ontario, Canada. When these phospholipid preparations were submitted to thin layer chromatography (see below), certain of them were seen to be contaminated by minor amounts of phospholipids other than the main component. However, none of them contained detectable amounts of free fatty acids. $^{63}\text{Ni(II)}$ was purchased from The Radiochemical Centre (Amersham, England). All other chemicals were of reagent grade and were obtained

* To whom correspondence should be addressed.

from common commercial sources.

Microsomes. These were prepared from the livers of 180- to 200-g male Sprague-Dawley rats by the method of Ernster *et al.*⁸

Quantification of microsomal phospholipids. Microsomal phospholipids were extracted into chloroform-methanol, 2:1, according to Folch *et al.*⁹ The phosphate content of total or individual microsomal phospholipids was determined by Bartlett's procedure¹⁰

Thin-layer chromatography. The preparation of individual microsomal phospholipids and examination of the purity of commercial phospholipid preparations (see above) was carried out on 0.25 mm thick plates of Silica Gel F₂₅₄ (Merck). The elution medium contained chloroform-methanol-acetic acid-water, 25:15:4:2 by volume.¹¹ Spots were detected by staining with I₂. Spots were scraped off the plate, extracted three times in 10 ml methanol at 60 °C for 30 min, and the methanol was then filtered and evaporated.

Measuring phospholipids with ⁶³Ni(II). Originally, we attempted to use essentially the same procedure as that developed by Ho for fatty acids⁶, which is based on the extraction of ⁶³Ni(II) into chloroform-heptane. The greatest advantage of this method is that the organic solvent phase is lighter than the aqueous phase and aliquots for scintillation counting can easily be removed from the upper phase. However, this method led to serious problems with precipitation.

The routine procedure used is as follows: stock solution containing 60–150 μCi ⁶³Ni(II) in 30 μl 0.1 N HCl, 0.97 ml 20 mM Ni(NO₃)₂ in H₂O, 0.15 ml triethanolamine, and 0.85 ml H₂O is prepared. Replacing the H₂O with another 0.85 ml triethanolamine does not affect the results. The pH of this stock solution, which is approximately 9.5, is very important (see below). 100 μl stock solution (containing approximately 1 μmol Ni²⁺ and 3–7.5 μCi ⁶³Ni(II)) is mixed with 0.1 ml H₂O (which can be replaced by a sample, *e.g.* a microsomal suspension; see RESULTS). (Increasing the amount of Ni(II) added to 2.6 μmol also increases the uptake of nickel about 7 % but also requires the use of more radioisotope to maintain the same specific radioactivity and thus, the same sensitivity.) 3.8 ml chloroform-methanol, 2:1, containing the phospholipid is then added and the mixture shaken for 2 min. Subsequently, 0.8 ml H₂O is added and the mixture shaken again

for 1 min. After separation of the phases by centrifugation aliquots of the lower organic phase are removed for scintillation counting (see below).

To scale down this procedure, 0.1 ml 20 mM Ni(NO₃)₂ and 0.87 ml H₂O are added to the stock solution instead of 0.97 ml 20 mM Ni(NO₃)₂, thereby increasing the specific radioactivity of the Ni(II) almost 10-fold. In addition all volumes are halved. Otherwise, the procedure is identical.

The standard assay can also be modified to increase sensitivity by using a stock solution containing 20 μl ⁶³Ni(II) (approximately 5 nmol and 140 μCi), 0.15 ml triethanolamine, and 1.83 ml distilled water. Otherwise, the procedure resembled the standard procedure, except that 5 ml methanol:H₂O, 1:1, was added instead of 0.8 ml H₂O to obtain two phases. 200 μl aliquots were counted.

Measuring phospholipids with Cu(II). Originally, we attempted to use essentially the same procedure as that developed by Duncombe³ for free fatty acids, but again we had serious problems with precipitation. A combination of the Duncombe procedure and the Ni(II) method described above functions well: the stock solution contains 1.0 ml 20 mM Cu(NO₃)₂, 0.15 ml 1 N acetic acid, 0.15 ml triethanolamine, and 2.7 ml H₂O. 0.3 ml stock solution is added to 5 ml chloroform-methanol, 2:1, containing the phospholipid, and the mixture is shaken for 2 min. 1.1 ml H₂O is then added and the tube is shaken again for 1 min. After separating the phases by centrifugation, 3 ml of the lower organic phase is added to 0.5 ml of Duncombe's diethyl-dithiocarbamate reagent (0.1 % diethyl-dithiocarbamate in 2-butanol). After shaking, the absorption of the sample at 440 nm is determined. A blank containing no phospholipid gives an absorption of approximately 0.2. A standard curve should be run using Cu(NO₃)₂ in order to quantitate the results.

Scintillation counting. In the nickel procedure 0.2 or 0.4 ml aliquots of the lower phase were counted in 5 or 10 ml of Aquasol or Lumagel. The counting efficiency for ⁶³Ni under these conditions is between 65 % and 45 %. After correcting for quenching, the amount of Ni(II) in the organic phase could be quantified using the specific radioactivity of the stock solution. A blank without phospholipid gave between 200 and 600 CPM in this system.

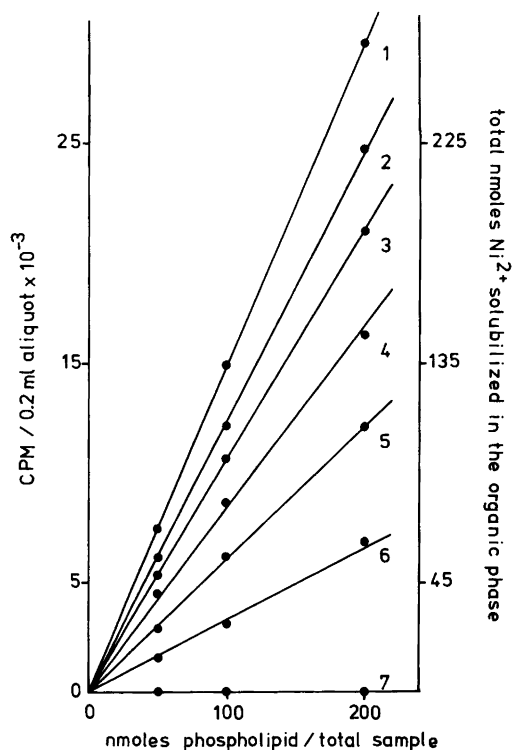


Fig. 1. Solubilization of Ni(II) in the chloroform phase by phospholipids which do not contain choline. 1=phosphatidic acid (Sigma P4013). 2=phosphatidyl inositol (Serdary A38). 3=phosphatidyl serine (average of Sigma P6641 and Serdary A37, which agreed within a few percent). 4=phosphatidyl glycerol (Sigma P0514). 5=phosphatidyl ethanolamine (average of Sigma P4513 and microsomal, which agreed within 20 %). 6=total microsomal phospholipids. 7=phosphatidyl choline (Sigma P7763, P5388, P6263, P0763, P1013, and microsomal) and sphingomyelin (Sigma S7004 and microsomal).

RESULTS AND DISCUSSION

Measuring phospholipids with $^{63}\text{Ni(II)}$.

The basic assay. As can be seen from Fig. 1, phospholipids which do not contain choline render Ni(II) soluble in the chloroform phase to an extent which is directly proportional to the amount of phospholipid present. The most probable explanation for this phenomenon is the formation of salt between Ni(II) and negatively

charged phosphate and/or carboxyl groups in the different phospholipids. Table 1 documents the stoichiometry of this phenomenon, i.e., the number of Ni(II) solubilized per phospholipid molecule. When mixtures of different phospholipids, including choline-containing phospholipids, were used in a similar experiment, the stoichiometries predicted from Table 1 were obtained, indicating that Ni(II) binds independently to different phospholipid species in such a mixture.

There are two aspects of these findings which are striking. In the first place, why is no complex formed between Ni(II) and phosphatidyl choline or sphingomyelin? One possibility is that these phospholipids already contain saturated amounts of cations which cannot be easily exchanged for Ni(II). This explanation seems unlikely. Phosphatidyl choline was dissolved in chloroform-methanol, 2:1, in the presence of a large excess of EDTA; and 0.2 volumes water was added to remove EDTA and any bound divalent cations from the organic phase. The choline-containing phospholipids cleaned up in this manner still showed no detectable ability to bind Ni(II).

A more likely explanation arises from the results illustrated in Fig. 2. The ability of phosphatidyl ethanolamine to bind Ni(II) is clearly pH-dependent. The protonation of and, therefore, the charge on the amino group in phosphatidyl ethanolamine decreases with increasing pH. These findings lead us to postulate the formation of an intramolecular salt between the phosphate group and the protonated amino group in phosphatidyl ethanolamine and between the phosphate group and the positively charged nitrogen in phosphatidyl choline. The formation of such salts would be favored by the short distance between the negatively and positively charged groups involved and perhaps by other steric factors as well. Thus, it might not be possible for Ni(II) to readily replace the positively charged group in such an intramolecular salt. An alternative hypothesis is that strong electrostatic binding may occur between two different molecules of a phospholipid containing both positive and negative charges.

However, the ability of phosphatidic acid and phosphatidyl inositol to solubilize Ni(II) in chloroform is also somewhat dependent on the pH of the stock solution (Fig. 2). This effect cannot be explained solely on the basis of

Table 1. Stoichiometries of the complexes formed between phospholipids and Ni(II) in chloroform-methanol.

Phospholipid	Ni(II) ions solubilized/ molecule phospholipid
Phosphatidyl choline	0
Phosphatidyl ethanolamine	0.54
Phosphatidyl serine	0.95
Phosphatidyl inositol	1.08
Phosphatidic acid	1.33
Phosphatidyl glycerol	0.75
Sphingomyelin	0
Total microsomal phospholipids	0.25

The values in this table are derived from the experiments illustrated in Fig. 1.

changes in the protonation of different groups in the phospholipid molecules. Solubilization by phosphatidyl serine is independent of pH in the range of 5–9.5. At pH 11 solubilization by all phospholipids increases dramatically, apparently because fatty acids are released by alkaline hydrolysis of the phospholipids.

A number of attempts were made to find conditions where phosphatidyl choline and sphingomyelin could render Ni(II) soluble in the chloroform phase, but without success. Nor do

these phospholipids form a complex with (methyl-³H)-choline.

Another striking feature of this phenomenon is the widely varying ability of different non-choline-containing phospholipids to bind Ni(II). The stoichiometries shown in Table 1 presumably result from the interplay of a number of factors, including the net negative charge on the phospholipid at pH 9.5 (the pH where the assay is performed), the steric possibilities for interaction between positive and negative charges in the

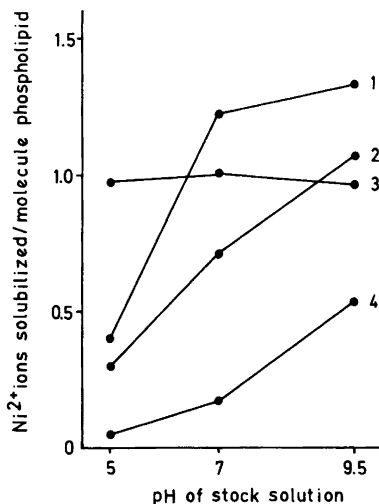


Fig. 2. Effect of pH on the solubilization of Ni(II) in the chloroform phase by phospholipids. 1=phosphatidic acid (Sigma P4013). 2=phosphatidyl inositol (Serdary A38). 3=phosphatidyl serine (Sigma P6641). 4=phosphatidyl ethanolamine (Sigma P4513).

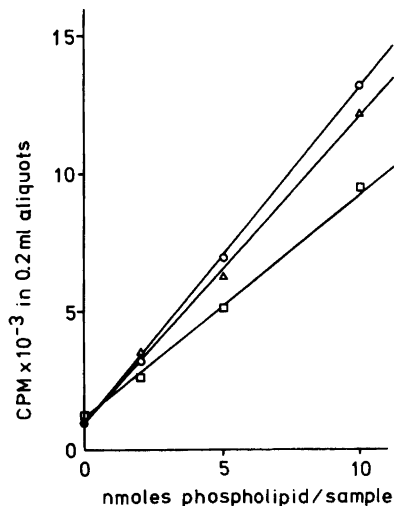


Fig. 3. Scaling down the procedure for assaying phospholipids with ⁶³Ni(II). ○=phosphatidic acid (Sigma P1013). △=phosphatidyl serine (Serdary A37). □=phosphatidyl ethanolamine (Sigma P4513).

same phospholipid molecule, and perhaps other steric features as well.

Sensitivity of the assay. As shown in Fig. 3, the assay procedure can easily be scaled down so that 2 nmoles of various phospholipids result in total CPM which are 3 times background. The assay can be modified slightly to increase its sensitivity an additional 10-fold (Fig. 4).

Interference. It is to be expected that fatty acids and at least certain negatively charged detergents can also form complexes with Ni(II) and thereby render this cation soluble in the chloroform phase. Indeed, sodium dodecyl sulfate was found to solubilize 3 Ni(II) ions in chloroform per 10 detergent molecules. On the other hand, deoxycholate does not solubilize Ni(II) in the chloroform phase.

In addition other cations may compete with Ni(II) in binding to the phosphate and carbonyl groups in various phospholipids. When 0.8 ml 100 mM or 200 mM NaCl were added instead of 0.8 ml water in the assay procedure (see Materials and Methods), the amount of Ni(II) recovered in the chloroform phase in the presence of phosphatidyl glycerol was reduced 67 and 93 %, respectively. This finding suggests that Na⁺ can also bind to phospholipids. As would be expected, addition of 0.8 ml 0.15 mM or 1.0 mM EDTA instead of 0.8 ml water in the assay procedure decreased the amount of Ni(II) recovered in the chloroform phase in the presence of phosphatidyl ethanolamine to 54 % and 0 %, respectively, of the control value.

Application to biological membranes. The inability of our assay procedure to measure choline-containing phospholipids, as well as the varying stoichiometries observed with different phospholipid species, limit the usefulness of this assay in connection with biological membranes. However, it should be possible to use a conversion factor. As shown in Fig. 5, when rat liver microsomes are added directly in the assay procedure and the 2 min shaking is replaced by homogenization, Ni(II) is recovered in the chloroform phase in amounts proportional to the amount of microsomes added. The uptake of Ni(II) ions per molecule microsomal phospholipid (as determined in the classical manner; see Materials and Methods) was found to be 0.253 ± 0.030 (mean and standard deviation of 9 determinations). Thus, using a conversion factor our assay procedure would seem to offer an

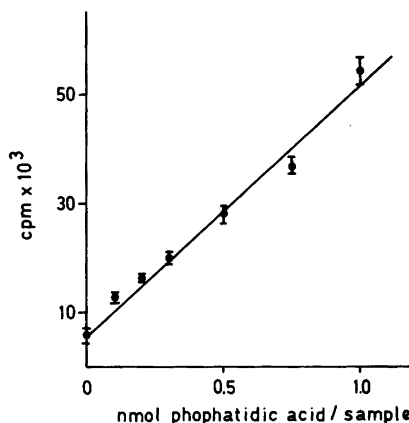


Fig. 4. Modifying the procedure for assaying phospholipids with ⁶³Ni(II) to increase sensitivity. The stock solution contained 20 μ l ⁶³Ni(II) (approximately 5 nmols and 140 μ curies), 0.15 ml triethanolamine, and 1.83 ml distilled water. Otherwise, the procedure resembled the standard procedure, except that 5 ml methanol: H₂O, 1:1, was added instead of 0.8 ml H₂O to obtain two phases. 200 μ l aliquots were counted.

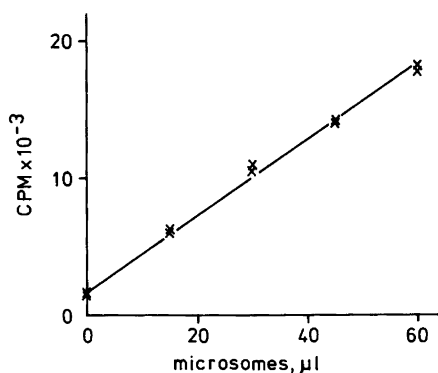


Fig. 5. Assay of microsomal phospholipids using ⁶³Ni(II). Freshly prepared liver microsomes containing very low levels of free fatty acids⁷ were added instead of water to chloroform-methanol together with the stock solution. Each μ l of microsomes contained 7 nmols of phospholipids, as measured by the classical procedure. The mixture was homogenized for 2 min instead of shaking, but the rest of the procedure was as described in Materials and Methods.

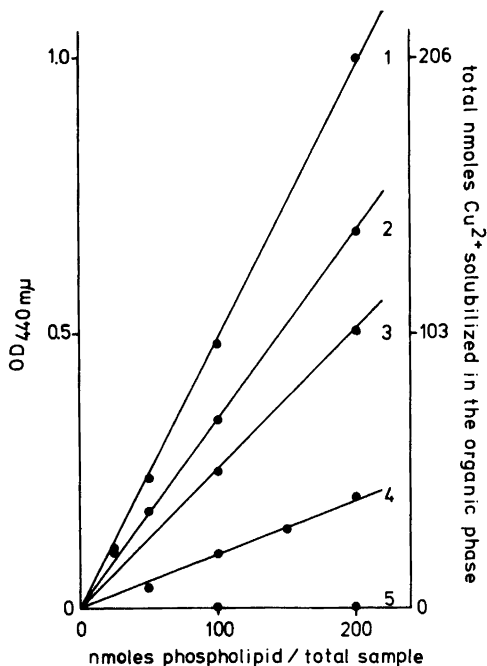


Fig. 6. Solubilization of Cu(II) in the chloroform phase by phospholipids which do not contain choline. 1=phosphatidic acid (Sigma P4013). 2=phosphatidyl serine (Serdary A37). 3=microsomal phosphatidyl ethanolamine. 4=total microsomal phospholipids. 5=phosphatidyl choline (Sigma P7763, P5388, P6263, P0763, and P1013) and sphingomyelin (Sigma S7004).

extremely rapid possibility for determining the phospholipid content of biological membranes to within 10%.

Measuring phospholipids with Cu(II). Non-choline-containing phospholipids can also render

Cu(II) soluble in organic solvents. Fig. 6 illustrates that the uptake is linearly proportional to the amount of phospholipid present and Table 2 documents the stoichiometries of the complexes formed. This procedure is less sensitive than the one employing $^{63}\text{Ni(II)}$, but avoids the use of radioactivity.

CONCLUSION

The advantages of our procedure for assaying phospholipids with $^{63}\text{Ni(II)}$ are its sensitivity (0.2 nmols phospholipid can be readily measured) and the ease with which it can be performed. Since no ashing of the phospholipids is required, the procedure takes relatively little time. In addition, phospholipids extracted from silica gel thin layer chromatography plates can be assayed using the procedure, since the blank extracted from silica gel is essentially zero.

The disadvantages of our procedure is that it does not measure choline-containing phospholipids and that standard curves should be routinely drawn, since different phospholipids give different stoichiometries. This latter property makes it difficult to use this procedure with complex mixtures of phospholipids, such as those found in biological membranes. However, most biological membranes, such as the endoplasmic reticulum, have characteristic and stable phospholipid compositions. We have found that with the use of a conversion factor the phospholipid content of microsomes can be very rapidly determined to within 10%. Another disadvantage of our procedure is that the phospholipid content of materials containing relatively high levels of free fatty acids cannot be determined, since free fatty acids also solubilize $^{63}\text{Ni(II)}$ in organic solvents.

Table 2. Stoichiometries of the complexes formed between phospholipids and Cu(II) in chloroform-methanol.

Phospholipid	Cu(II) ions solubilized/ molecule phospholipid
Phosphatidyl choline	0
Phosphatidyl ethanolamine	0.52
Phosphatidyl serine	0.70
Phosphatidic acid	1.01
Sphingomyelin	0
Total microsomal phospholipids	0.20

The values in this table are derived from the experiment illustrated in Fig. 6.

The solubilization of radioactive cations in organic solvents may turn out to be a useful tool for studying the chemical properties of different phospholipids. Such an approach might, for instance, allow determination of the relative affinities of different phospholipids for different cations. Even the simple experiments performed here have led to the suggestion that in organic solvents an intramolecular salt is formed between the negatively charged phosphate group and the positively charged nitrogen in choline-containing phospholipids.

Whether the binding of cations by phospholipids has any physiological importance remains to be seen. Since complexes between non-choline-containing phospholipids and divalent cations are soluble in organic solvents, it is not impossible that such complexes might be involved in moving divalent cations and/or phospholipids across biological membranes.¹² In addition the binding of divalent metal cations to phospholipids may partially account for the toxicity of many of these cations, including Ni(II).

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