Use of ^{31}PNMR spectroscopy to follow the time course of phosphatidylcholine chemical synthesis

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Abstract ^{31}PNMR spectroscopy is shown to be useful for studying the chemical synthesis of phosphatidylcholine from phosphatidic acid and choline. Sharp, well-resolved resonances were obtained by chelating multivalent cations, thereby enabling quantitation of reactants, products, and intermediates. The syntheses of several types of phosphatidylcholines were monitored by ^{31}PNMR spectroscopy, including perdeuterated and headgroup spin-labeled molecules. For perdeuterated phosphatidylcholines, this analysis led to reaction conditions giving much better conversion to product than conditions previously observed. In addition, a polyphosphate side-product was identified in reactions which do not produce phosphatidylcholine, implying either a polyphosphate intermediate in the reaction mechanism, or else a competing side reaction. —**Meers, P. R., and G. W. Feigenson.** Use of ^{31}PNMR spectroscopy to follow the time course of phosphatidylcholine chemical synthesis. *J. Lipid Res.* 1985. **26:** 882–888.

Supplementary key words phosphatidic acid • perdeuterated lipid

The chemical synthesis of various phospholipids has been important for the investigation of cell and model membranes. Phosphatidylcholine (PC) is the major type of phospholipid present in mammalian plasma membranes and therefore synthesis of various phosphatidylcholines is useful for the study of many biochemical problems. The synthesis of a perdeuterated PC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine-d_{72} (DMPC-d_{72}) recently has been reported (1). This molecule has proved to be quite valuable for ^{1}HNMR studies of small molecules in a perdeuterated model membrane (2, 3).

In the synthesis by Kingsley and Feigenson (1), perdeuterated 1,2-dimyristoyl-*sn*-glycero-3-phosphoric acid-d_{54} (DMPA-d_{54}) was condensed with choline-d_{13} using a modification of the method of Aneja and Chadha (4). The reported purified yield of the reaction is about 35% (1). However, using this procedure we generally obtain much lower reaction yields. Recently Harbison and Griffin (5) reported improved yields using the tetraphenylborate salt of choline. We attempted to improve the yield of the Kingsley and Feigenson (1) method by varying reaction conditions. However, monitoring the reaction using thin-layer chromatography did not lead to improvement because of overlapping spots, lack of quantitation, and changes in R_f values. Instead, ^{31}PNMR spectroscopy proved to be a precise method to assay reactants and products, and even to identify reaction intermediates.

EXPERIMENTAL

Materials

Total phospholipid was determined by a modification of the phosphate assay described by Bartlett (6) as in reference (1).

Thin-layer chromatography (TLC) plates were Adsorbisil 5-P silicic acid from Applied Science, State College, PA. Silica for medium pressure chromatography was from E. Merck. Phosphate-containing components were detected on TLC plates using a Zinzadze reagent (7). Elution solvent ratios for TLC are expressed in terms of volume.

Solvents were reagent or high performance liquid chromatography grade. Pyridine was dried by distillation over $Ba(OH)_2$ and storage over activated 4 Å molecular sieves. Water was purified using a Milli-Q Water Purification System (Millipore, Bedford, MA). D,L α-Glycerol phosphate was from Sigma, St. Louis, MO. Dowex AG 50W-X8, molecular sieves, and Chelex ion-exchange resins were from Bio-Rad, Rockville Center, NY. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoric acid (DPPA) was from Calbiochem, LaJolla, CA, or was synthesized as described below. Choline chloride and toluenesulfonyl chloride (99%), and N,N-dimethyl-4-aminopyridine were from Aldrich Chemi-

Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; DMPA, dimyristoyl phosphatidic acid; DPPA, dipalmitoyl phosphatidic acid; DMPC, dimyristoyl phosphatidylcholine; GP, glycerol phosphate; TLC, thin-layer chromatography; ^{1}HNMR, proton nuclear magnetic resonance; ^{31}PNMR, phosphorus nuclear magnetic resonance; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; DMAP, N,N-dimethyl-4-aminopyridine; TPS, triisopropylbenzene sulfonyl chloride; TosCl, toluenesulfonyl chloride.

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cal Co., Milwaukee, WI. All other materials were reagent grade or else purified as described below.

DMPA synthesis

Glycerol phosphate was synthesized as described by Kingsley and Feigenson (1), or the D,L mixture from Sigma was used. The glycerol phosphate was converted to the pyridinium form using washed ion-exchange resin in the pyridinium form. This conversion yields only about 50–60% pyridine-soluble glycerol phosphate. The insoluble material was eluted through an ion-exchange column again or discarded.

DMPA for use in test phosphatidylcholine syntheses was synthesized by a modification of the method in Kingsley and Feigenson (1) as follows. The appropriate amount (usually 0.5 mmol) of D,L-α-glycerol phosphate in the pyridinium form in wet pyridine was dried by rotary evaporation under aspirator vacuum. Then the glycerol phosphate was dried three times from dry pyridine, placing the solution in a heating mantle at 60°C between each drying and stirring for about 5 min to assure dissolution of the viscous gum. Typically, 10 ml of dry pyridine was then added and the flask was sealed and placed in a heating mantle set for 45°C and stirred for 1 hr to dissolve the contents. The appropriate amount (7.5 mmol per mmol of glycerol phosphate) of freshly synthesized myristic anhydride (8) followed by 10 ml of dry pyridine was added to a dry 100-ml three-necked flask containing a stir bar and N,N-dimethyl-4-aminopyridine (5 mmol per 7.5 mmol of anhydride). After a dry N_2 purge, the solution of glycerol phosphate in dry pyridine was added dropwise over about 20 min to the stirred anhydride solution in the flask. After glycerol phosphate addition, a continuous N_2 purge was established with an HCl trap at the outlet for pyridine vapor, and the solution was stirred for another 20 min. By adding the glycerol phosphate dropwise, a large excess of anhydride is maintained throughout most of the reaction, thereby decreasing the total amount of anhydride required.

DMPA purification

The DMPA from these procedures was purified using medium pressure silicic acid column chromatography eluting with chloroform–methanol–conc. ammonium hydroxide 65:25:5 followed by re-loading and eluting with chloroform–methanol–water 65:24:4. The yield was generally in the range of 80–90%. ^{1}HNMR and ^{31}PNMR spectra of this DMPA in chloroform showed no impurities (<1%), as was also the case with TLC in the solvent systems used for elution.

Test PC synthesis

DPPA from Calbiochem was used without further purification. D,L-DMPA and all other forms of PA were synthesized and purified as above.

Toluenesulfonyl chloride from Aldrich was purified by the method of Pelletier (9, 10). This gave toluenesulfonyl chloride which was active for months if stored over silica gel.

Choline periodide was produced by precipitation from an aqueous solution of choline chloride with the potassium iodide reagent developed by Appleton et al. (11). Tempocholine chloride was synthesized by the method of Kornberg and McConnell (12). It was converted to the periodide form before reaction, as for choline.

The appropriate amount of PA was dried from chloroform and then choline periodide was dried from pyridine in the same round-bottom flask by rotary evaporation. One of two procedures was then followed. Procedure A (reference 1): dry pyridine was added to the flask and the pyridine then removed by rotary evaporation under aspirator vacuum to remove water. This was repeated three times. In some cases, argon or nitrogen was released into the evaporation chamber and flask at the end of the last two evaporations. The indicated volume (**Table 1**) of dry pyridine was then added to the flask, which was sealed and placed in a heating mantle set for the appropriate temperature. After at least 30 min to allow for temperature equilibration, the appropriate amount of toluenesulfonyl chloride was weighed, dissolved in a volume of dry pyridine which was 10% of the total reaction volume, and immediately added to the reaction flask with swirling. The flask was sealed and then opened only to remove aliquots which were quenched by addition to D_2O. Procedure B: the choline and PA were dried once by rotary evaporation from dry pyridine and then were dried overnight in a desiccator containing phosphorus pentoxide, using a mechanical high-vacuum pump with a liquid nitrogen trap. The desiccator was filled with dry argon, opened in a dry box, and the flask was sealed with a closed stopcock. Dry pyridine was added by injection through the stopcock bore with a syringe. After temperature equilibrium as above, toluenesulfonyl chloride in dry pyridine was injected through the bore of the stopcock. Aliquots were removed by syringe through the stopcock.

Nuclear magnetic resonance spectroscopy

^{31}PNMR was performed using a Varian CFT-20 NMR spectrometer operating at 32.19 MHz or a Bruker WM-300 NMR spectrometer operating at 121.4 MHz. Spectra were proton noise-decoupled in a nuclear Overhauser suppressed mode (13). In order to test for nuclear Overhauser suppression and for saturation effects, representative spectra were recorded at increasing delay times until absolute intensities were constant. A time delay of 10 sec between spectral acquisitions proved to be adequate for all samples. Spectra were run at 50°C. Samples were prepared for NMR by taking aliquots of the reaction mixture and quenching the reaction with D_2O to give 10% D_2O in pyridine. These aliquots were added to

TABLE 1. Conditions and percentage conversion[a] for some PC synthetic reactions

Reaction #	PA[b]	Choline	Toluene-sulfonyl Chloride	Temperature	Volume	Procedure[c]	Maximum Reaction Time	Final % Conversion		
								PC	PA	11 ppm
	mmol	*mmol*	*mmol*	*°C*	*ml*		*hr*			
1	0.15	0.45	0.45	58	4.0	A	2	98	1	1
2	0.025	0.075	0.075	58	0.67	A	2	0	75	25
3	0.13[d]	0.36[e]	0.36	58	4.0	A	2	10	20	70
4	0.025	0.075	0.075	58	6.6	A	24	12	88	
5	0.025	0.075	0.075	58	6.6	B	24	100		
6	0.025[d]	0.075[e]	0.075	58	6.6	B	24	100		
7	0.025	0.75[f]	0.075	58	6.6	B	24	90		
8	0.025[g]	0.075[e]	0.075	58	6.6	B	24	100		
9	0.05[h]	0.015[f]	0.015	58	12	B	24	90		
10	0.35[i]	1.05[e]	1.05	58	50	B	24	100		

[a] Determined from ^{31}PNMR of reaction mixture.
[b] D,L-DMPA was used unless otherwise noted.
[c] Procedure B involved less exposure to moisture at all phases of reaction and work-up; see text.
[d] Deuterated glycerol moiety-DMPA.
[e] Deuterated choline.
[f] Tempocholine.
[g] Perdeuterated-DMPA.
[h] DPPA.
[i] Perdeuterated DPPA.

NMR tubes containing 0.1 g (8-mm tubes for CFT-20) or 0.3 g (10-mm tubes for WM-300) of Chelex chelating resin. Peak assignments were made by observing the chemical shifts of resonances of known phospholipids added to a reaction mix or by comparing the TLC behavior of reaction products to known compounds. Accuracy of quantitation by ^{31}PNMR, verified by comparing peak areas of samples containing known ratios of phospholipids, was ± 5% of the actual value. All percentage conversion measurements were made using peak areas. The % conversion denotes the percentage of the total signal which is PC.

RESULTS AND DISCUSSION

Previously we have used thin-layer chromatography to follow the course of PA and PC reactions (1). However, TLC has not proven reliable for this purpose. The crude PC reaction mixture gives streaking and overlapping spots even in the most useful solvent systems, perhaps because of the presence of periodide. Furthermore, in the crude reaction mix, PC spots stain more strongly with Zinzadze reagent than do PA or some other materials. Thus, TLC estimates of PC yields often appear two to four times higher than the true yields. In contrast, ^{31}PNMR spectroscopy provides high resolution and excellent quantitation and is nondestructive, although the sensitivity is much less than that of TLC.

The ^{31}PNMR resonances from the crude PC reaction mix are broad and unresolved. Dispersing the mixture in a detergent or in a different solvent was attempted, but these procedures did not dissolve the mixture completely. It was found that adding a chelator such as Chelex ion-exchange resin to the crude reaction mixture sharpened the peaks considerably, undoubtedly by complexing any multivalent cations which broaden the resonances of phosphate groups (14).

Typical ^{31}PNMR spectra of mixtures of reaction products are shown in **Fig. 1**. Different products are distinguishable (14). Using ^{31}PNMR, the time courses and final yields of many variations of reactions of interest were determined.

Test PC syntheses

We identified some of the important variables in the PC synthesis as reactant concentrations, reactant ratios, reaction scale, dryness, time of reaction, and protonated versus deuterated reactants. ^{31}PNMR spectroscopy enabled us to determine the reaction products as a function of these variables. Numerous different reactions were run, and a small sample of typical results is shown in Table 1, **Fig. 2**, and **Fig. 3**. In Figs. 2 and 3, time courses of PC production are shown. It is clear that these reactions are influenced profoundly by the temperature and by the ratios of reactants. At high toluenesulfonyl chloride concentrations, the conversion to PC first increases then decreases with time. High temperatures (Fig. 2), increase the rate of PC formation and, more importantly, the rate of breakdown. It also appears (Fig. 3) that a threshold amount of choline must be present for good conversion to PC. The best conditions appear to be toluenesulfonyl chloride–choline–PA approximately 3:3:1 at 57°C. This

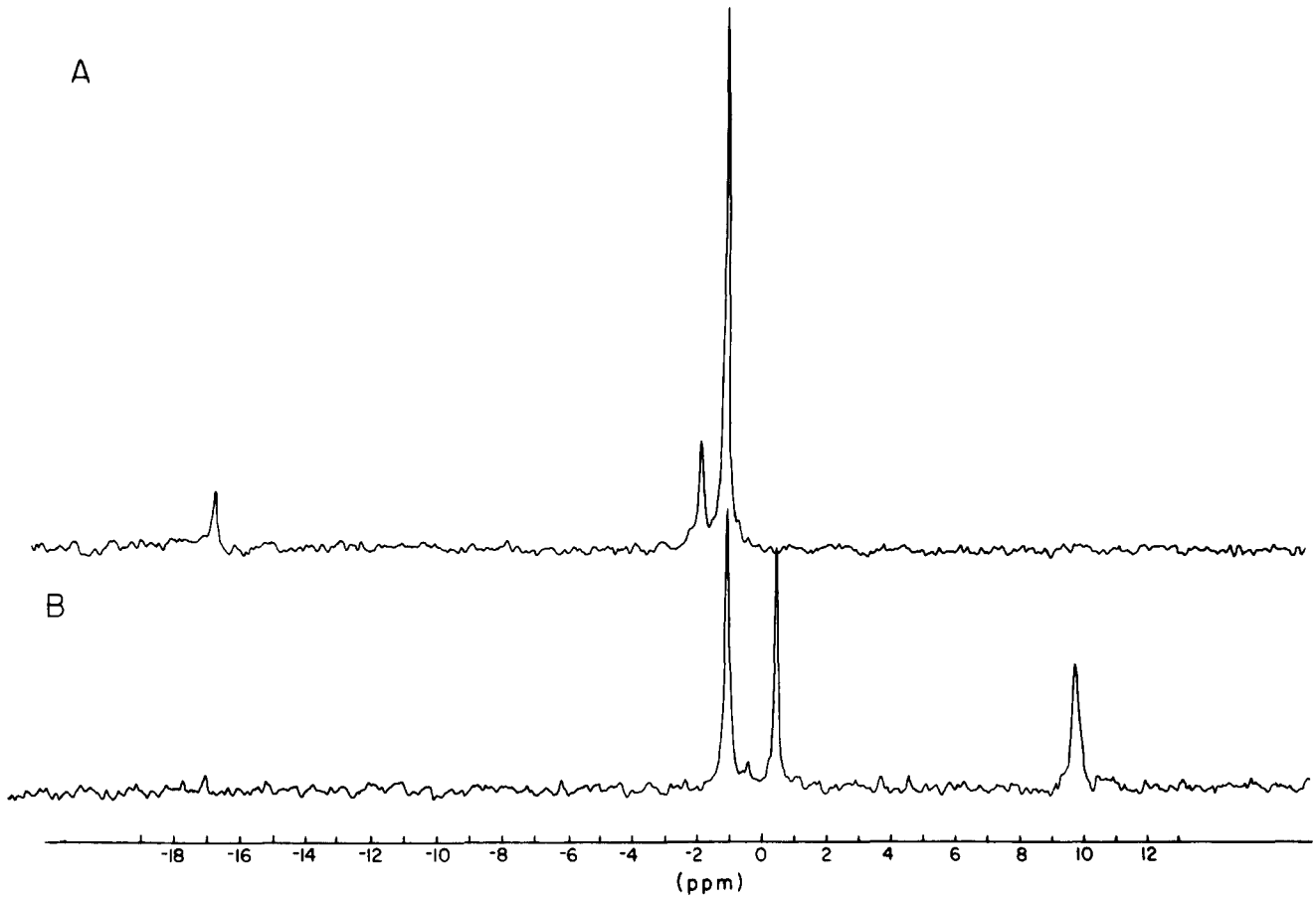

Fig. 1. ^{31}PNMR spectrum of A) a PA synthesis reaction mix showing peaks for PA, lyso-PA, and cyclic lyso-PA, and B) a PC synthesis reaction mix with peaks for PA, PC, and a product about 11 ppm upfield from PA. For spectrum A, 79 transients were collected, while for spectrum B, 465 transients were collected. Both spectra were collected with a delay time of 10 sec and a line broadening of 10 Hz was applied. Other conditions for the spectra are described in the Experimental section. Chemical shifts are measured with respect to an external capillary of 85% H_3PO_4, with correction for bulk susceptibility (17). Peak assignments are as follows: −16.9 ppm, cyclic lyso-PA; −2.0 ppm, lyso-PA; −1.2 ppm, PA; 0.4 ppm, PC; 9.7 ppm, putative polyphosphate.

contrasts with 5:2:1 (1) or 10:2:1 (P. B. Kingsley, personal communication), both at 70°C.

The reaction of perdeuterated choline and perdeuterated or partially deuterated PA using procedure A does not produce much PC as compared to the reaction of protonated species (compare reactions 1 and 3, Table 1). It is striking that reducing the scale of the reaction also dramatically lowers the % conversion to PC using procedure A (compare reactions 1 and 2, Table 1). Experiments using a Teflon flask (reactions not shown) show that the surface area exposed to glass is not important. For small scale reactions with a given mass of reactants, increasing the amount of pyridine gives a higher % conversion to PC (Table 1, reactions 2 and 4 and other data not shown). It is not clear whether this effect depends on the concentration or on the surface area-to-volume ratio of the reaction mix. However, yields are still not consistently good at small scale or with deuterated phospholipids using procedure A. The difference between protonated and deuterated reactants is important because it indicates that reaction conditions that work for protonated PC synthesis do not always work for deuterated PC synthesis.

In reactions 5–10 in Table 1, procedure B is used. The difference between protonated and deuterated reactants (reactions 5 and 8) disappears, as well as the scale dependence (compare reactions 1, 4, and 5). Under these conditions it appears that several phosphatidylcholine species can be synthesized in high yield. The rigorous drying under high vacuum of procedure B is therefore necessary for high % conversion. We note that the reactant ratios for our best conditions turn out to be very similar to those reported by Aneja and Chadha (4) for protonated PC synthesis and Harbison and Griffin (5) for deuterated PC synthesis.

Reaction mechanism of PC synthesis

Using ^{31}PNMR spectroscopy to identify reaction products and intermediates and to follow the time course of their formation and breakdown enables us to study the reaction mechanism for PC synthesis. Jacob and Khorana (15) have suggested that a cyclic polyphosphate and/or an aryl sulfonated phosphate intermediate which is easily

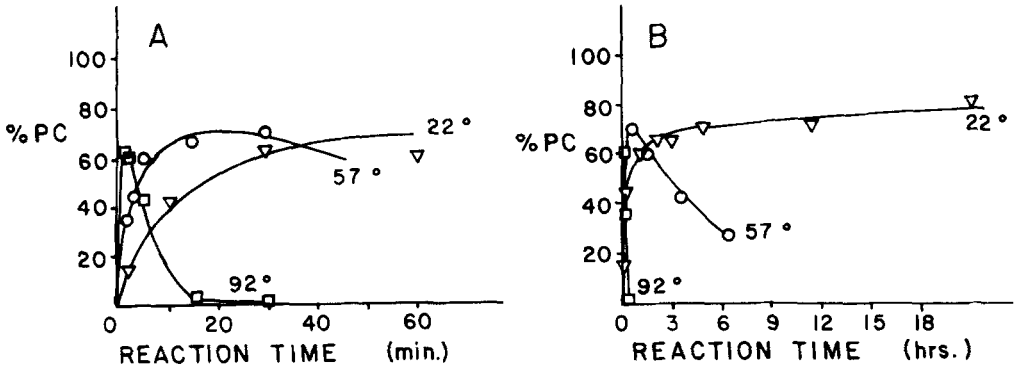

Fig. 2. Percentage of total phosphorus signal present as DMPC as a function of time at (□) 92°C, (○) 57°C, and (△) 22°C. DMPA (0.15 mmol) reacted with 0.3 mmol of choline using 1.5 mmol of toluenesulfonyl chloride, all in 4 ml of dry pyridine. Plots A and B are different time scales for the same reactions. Procedure A (Experimental) was used for these reactions.

hydrolyzed by water (16) may be formed in a reaction similar to the PC synthetic reaction. In all the reactions that failed to produce a significant yield of PC (Table 1), we observed a peak in the ^{31}PNMR spectrum at approximately 11 ppm upfield from the PA peak, which we identified as a polyphosphate. This chemical shift is very similar to that of a model compound $(-OPO(OC_2H_5)-)_n$ (13 ppm upfield from external 85% H_3PO_4 without bulk susceptibility correction (17)), which is a polyphosphate esterified to one carbon chain per phosphate (18), as would be the case for our reaction side-product. If the PC reaction mixture is examined long enough after the addition of D_2O, the ^{31}PNMR peak from the putative polyphosphate disappears and the peak from PA grows. **Table 2** shows that the ^{1}HNMR spectrum of the putative polyphosphate precipitated from the reaction (reaction 2, Table 1) is very similar to that of PA (19). Only a very small peak is seen at about −3.3 ppm characteristic of choline methyl protons. The appearance of small ^{1}HNMR peaks in the choline region and further downfield could indicate a trace of PC or toluenesulfonyl chloride incorporation into the polymer. However, since this preparation is only partially purified, these peaks could be from PC or toluenesulfonyl chloride impurities. Thus, the ^{1}HNMR spectrum of this material is very similar to that for PA; yet when hydrolysis is incomplete the ^{31}PNMR spectrum is quite different, suggesting that the molecule could be a polymer of PA joined at the phosphate groups. One possible origin of the putative polyphosphate would be an unstable cyclic polyphosphate intermediate (5) which, in reactions that fail to produce PC, is not attacked by choline but may be hydrolyzed to linear polyphosphate side-products, and eventually to PA. Alternatively, the observed side-product could result from a completely independent competing side reaction.

When PA alone (in the absence of choline) is reacted with toluenesulfonyl chloride, a ^{31}PNMR peak at approximately 11 ppm upfield from PA also appears if no water is added to the aliquot. Immediately after water is added, only a peak for PA (and a much smaller peak approx. 1 ppm upfield) appears, in contrast to the PC reactions wherein the 11-ppm peak disappears only rather slowly in the presence of D_2O, giving rise to a peak with the chemical shift of PA. We cannot explain this difference in hy-

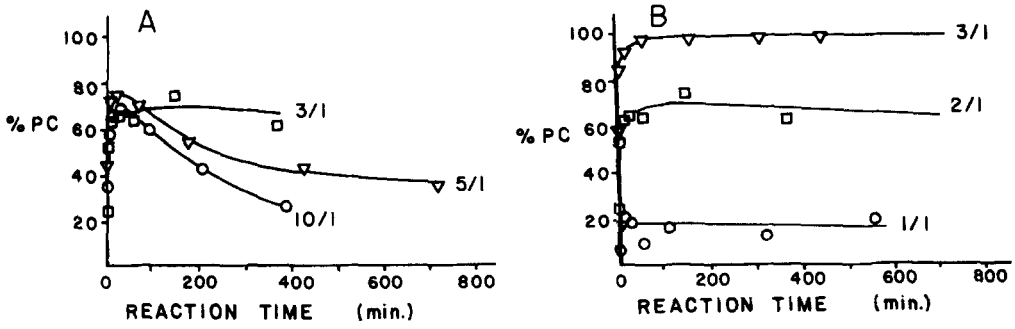

Fig. 3. Percentage of total phosphorus signal present as DMPC as a function of time at (first plot) toluenesulfonyl chloride–DMPA of (□) 3/1, (▽) 5/1, and (○) 10/1 and (second plot) choline–DMPA ratios of (▽) 3/1, (□) 2/1, and (○) 1/1. Reactions were run using procedure A (see text) with 0.15 mmol PA at 58°C in 4 ml dry pyridine. In the first plot, choline–DMPA was fixed at 2/1. In the second plot toluenesulfonyl chloride–DMPA was fixed at 3/1.

TABLE 2. ^{1}HNMR chemical shifts for major peaks of putative polyphosphate in chloroform compared to assigned peaks from Birdsall et al. (19)

	Choline			Glycerol			Fatty Acyl Methylenes			
	$(CH_3)_3N$	CH_2N	CH_2O	1-CH_2O	CHO	3-CH_2O	α	β	Rest of Chain	Methyl
DPPC	3.32	3.82	4.30	4.3	5.15	3.93	2.27, 2.30	1.58	1.27	0.88
DPPA				4.35	5.24	4.02	2.31	1.60	1.28	0.88
Major peaks of putative polyphosphate				4.14, 4.41[a]	5.23	3.92	2.29	1.57	1.26	0.88

^{1}HNMR spectra were taken at 300 MHz on a Bruker WM-300. Ten transients were taken on a solution of approx. 50 mg/ml.

[a] See peak assignments in reference 1.

drolysis rates, but we note that it implies either an inhibition of the hydrolysis by choline periodide or a chemical or physical difference in the polyphosphate formed by PA alone compared to that formed in the PC reaction. Nonetheless, this experiment shows that PA alone can be induced by toluenesulfonyl chloride to form a product with the observed chemical shift of the putative polyphosphate.

A polyphosphate intermediate or side-product might explain the drop in % conversion to PC at prolonged times and high toluenesulfonyl chloride concentrations. When too much toluenesulfonyl chloride is present, the excess toluenesulfonyl chloride might begin to incorporate already synthesized PC molecules into polymers with the remaining PA molecules. These copolymers are probably unreactive and linear, because PC has only one site for phosphoric anhydride formation, whereas pure PA polymers could be cyclic.

The H_2O sensitivity of deuterated and small scale reactions might also be explained by hydrolysis of a labile polyphosphate intermediate. Alternatively, it could be explained by hydrolysis of a small amount of toluenesulfonyl chloride followed by sulfonation of choline. In this regard, we have found that the condensing agent triisopropyl benzenesulfonyl chloride, which was reported to give less sulfonation of alcohols (20), yields essentially the same results as toluenesulfonyl chloride (as reported by Kingsley and Feigenson, reference 1).

A hypothetical sequence of reactions consistent with the data and conforming to the above explanation is shown in **Fig. 4**.

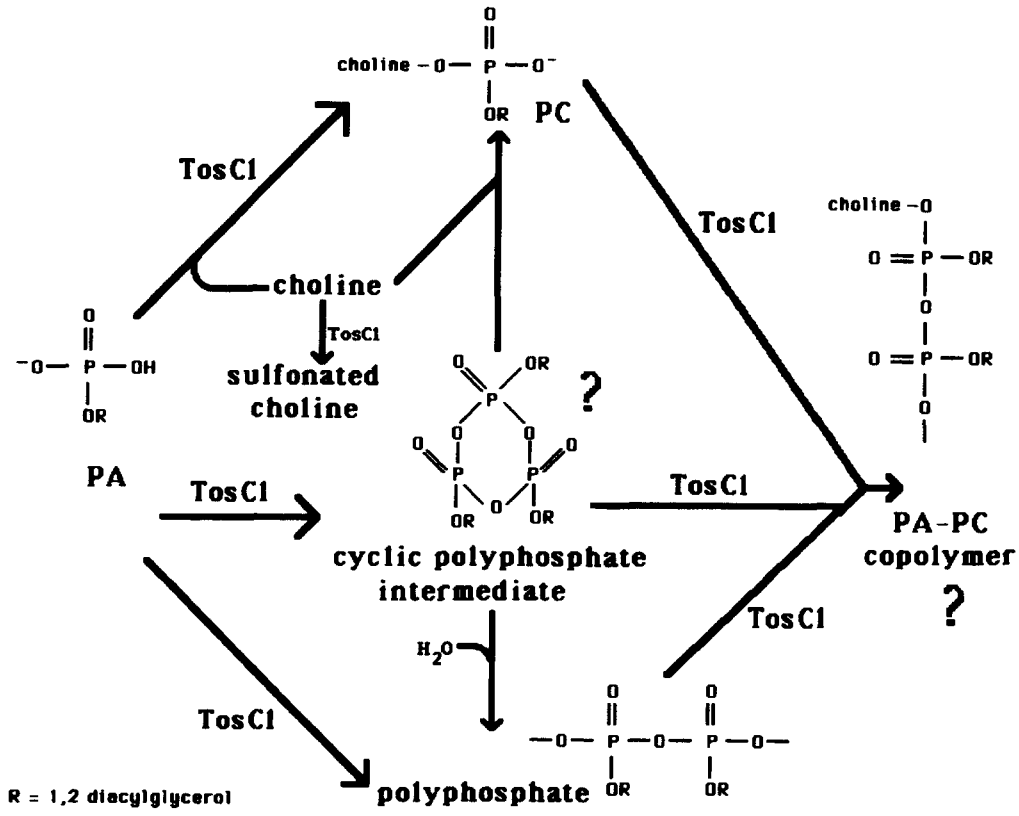

Fig. 4. Proposed reaction scheme for the PC synthesis including side reactions.

SUMMARY AND CONCLUSIONS

^{31}PNMR spectroscopy was used to monitor the products of the toluenesulfonyl chloride-induced condensation of PA and choline. A side-product was identified, in reactions which failed to produce PC, which is probably a polyphosphate. Deuterated reactants formed the side-product more easily than protonated ones. Rigorous drying of reactants and solvent either prevents an independent competing reaction from generating the side-product or else prevents hydrolysis of an intermediate that is critical for PC production.

Using ^{31}PNMR spectroscopy to follow the course of the reaction, we have been able to optimize the production of perdeuterated PA, perdeuterated PC, partially deuterated PC's, and a headgroup spin-labeled PC analog.

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