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DISCLAIMER

MAGNETIC RESONANCE STUDIES ON MEMBRANE AND MODEL MEMBRANE SYSTEMS: II. PHOSPHORUS SPECTRA AND RELAXATION RATES IN DISPERSIONS OF LECITHIN

by

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SUMMARY

We present phosphorus magnetic resonance (PhMR) spectra, relaxation rates, and chemical shifts for unsonicated and sonicated lecithins in aqueous dispersions and for egg lecithin in chloroform and methanol. Aqueous lecithin dispersions are characterized by long values for T_1 and considerably shorter values for T_2. Both of these values as well as the value of the linewidth change with sonication. Lecithin dispersions in methanol and chloroform have relaxation rates shorter than those seen for sonicated lecithin. We do not, at this time, present a detailed interpretation of these results. On an empirical level, however, since the relaxation rates are sensitive to the type of dispersion and possibly to the solvent, we are optomistic that they will be sensitive to structural changes involving the head group region.

Phospholipids are a major constituent of most biological membranes. Intrinsic to these molecules is a phosphodiester which links the head-group to the glycerol backbone. Phosphorus-31 nuclear magnetic resonance (PhMR) studies of these molecules have the potential of providing information concerning the structure, environment, and dynamics of the head-group region (and in some instances the entire molecule) in membrane and model membrane systems. Since evidence is accumulating that many membranes contain regions of phospholipids arranged in a bilayer (1-3), aqueous dispersions of phospholipid bilayers provide a suitable model system for initiating such work (4-6). In this paper we present our initial observations of the PhMR spectra and relaxation rates for sonicated and unsonicated aqueous lecithin dispersions, and a preliminary observation of a PhMR spectrum from unsonicated membranes from E. coli.

MATERIALS AND METHODS

Lecithin, prepared from hen egg yolks according to the method of Singleton et al. (7), was further purified by silicic acid chromatography. The column was eluted with the following solvents: $CHCl_3$, $CHCl_3$:CH_3OH (7:2), and $CHCl_3$:CH_3OH (7:3). Traces of column material were removed by diluting the lipids into absolute ethanol and centrifuging at 5,000 x g for 15 min. The lecithin was stored in absolute ethanol under argon at -22°C. The lecithin was determined to be pure by proton magnetic resonance (PMR) spectroscopy at 220 MHz and by silica gel thin-layer chromatography using $CHCl_3$:CH_3OH:H_2O (65:25:4) as the solvent (7). The lipids were visualized with iodine and identified by comparison to a set of standards. The extent of oxidation was low as determined by the oxidation index of Klein (8). The lipid concentration was determined by the phosphate procedure (scaled down by 10) of McClair (9).

Unsonicated egg yolk lecithin dispersions were prepared by drying 100-250 μmoles of lipid onto the walls of a round bottom flask, adding 2 ml of a buffer containing 0.15 M KCl, 10^{-4} M EDTA, and 0.1 M tris-HCl at pH = 8.5 and agitating with two glass beads for 5 min on a vortex mixer (10). The samples were prepared under argon and stored on ice; prior to use the samples were agitated for an additional minute on a vortex mixer.

Sonicated lecithin dispersions (11,12) were made by sonicating the unsonicated dispersions prepared as described above (with the exception that 30-100 μmoles of lipid were used and that no EDTA was added). The sonication was performed for 15 min on 2 ml samples, on ice and under argon, using the 1/2 inch tip on a Branson 185E sonicator at a power setting of 4-5 (68 watts). The sonicated lipids were then centrifuged at 17,000 x g for 30 min at 4°C. The supernatant was poured off and made 10^{-4} M in EDTA. Dispersions of dimyristoyl L-α lecithin were prepared in a similar manner with the exception that the lipids were prepared and stored above their transition temperature, 23°C (13).

Merck-acid washed alumina, suitable for chromatographic analysis, was used for alumina column chromatography. Silicic acid chromatography was performed using Mallinkrodt SilicAR CC-4, 100-200 mesh. Dimyristoyl and dipalmitoyl L-α lecithin were obtained from Calbiochem and from K and K, and the cadmium chloride salt of L-α glycerophosphorylcholine was obtained from Sigma. These chemicals were used without further purification. Phospholipid standards and Absorbsil 5-P for thin-layer chromatography were purchased from Applied Science. Sonicated dispersions of didihydrostreculoyl lecithin were a gift of Dr. R. D. Kornberg (14).

PhMR measurements were made on a sample volume of ∿1 ml contained in glass spheres with a diameter of ∿13 mm. Spectra of the solid samples were taken on 1 g of material dried by evacuation or by storage over P_2O_5 and sealed in 15 mm sample tubes. The ambient probe temperature was 31°C. Spectra were taken on a Fourier transform NMR spectrometer operating at a frequency of 24.3 MHz (15). A 180° pulse was 80 μsec. Linewidths were determined from the non-exponentially filtered Fourier transform spectra (15). The spectra were integrated using internal pyrophosphate as a standard. The spin-lattice relaxation times, T_1, were measured by the method described by Vold et al. (16). Transverse relaxation times, T_2, were determined by a spin-echo Fourier transform method to be described elsewhere (17).

RESULTS

Estimates of motional parameters from magnetic resonance linewidths require a knowledge of the second moment, $\Delta\omega_o^2$ (the square root of which is close; in value, to the linewidth), in the absence of any motion (18). The linewidth of the cadmium chloride salt of L-α glycerophosphorylcholine was measured to provide at least a lower limit for $\Delta\omega_o^2$. The result of this measurement is shown in Table I. Using the coordinates from the crystal structure for this compound (19), we have estimated the contribution to the second moment from only the 4 protons on the two methylene carbon carbon atoms adjacent to the phosphorus. Although the remaining protons in the molecule will contribute to the linewidth, they were not considered in our calculations because their distances from the phosphorus are difficult to determine. The result of this estimate, also given in Table I,

is in reasonable agreement with the linewidth measurement, suggesting, therefore, that the dipolar interactions with neighboring protons are the primary source contributing to the linewidth in this solid structure. The symmetric appearance and gaussian shape of the resonance also support this interpretation (see Fig. 1).

Also presented in Table I is the value of the linewidth for solid dipalmitoyl L-α lecithin. This value is similar to, but somewhat smaller than, that for L-α-glycerophosphorylcholine, suggesting again that dipolar interactions with neighboring protons determine the linewidth. The smaller linewidth may reflect some motion of the headgroup in lecithin precluded by the presence of the salt in the glycerophosphorylcholine sample or, alternately, may reflect different conformations of the headgroup.

The Fourier transform spectrum of unsonicated egg yolk lecithin is shown in Figure 2. The chemical shift of the phospholipid phosphorus is 127 Hz (5.3 ppm) to higher frequency than the phosphorus in pyrophosphate, at pH = 8.9,* used as an internal standard. The corresponding free induction decay shown in Fig. 3 reveals two components whose relaxation times and fractional intensities are given in Table II. Therefore the resonance shown in Figure 2 a superposition of the resonances corresponding to two different classes of phosphorus with different relaxation times. The respective origins of these two components must at this time be speculative, but we suggest three possibilities:

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*At this pH the variation in chemical shift with pH is minimal (21). The data in Table III give the chemical shift with respect to the external structure, 50% H_3PO_4.

1) The two components may arise from the distribution of particle sizes characteristic of these unsonicated vesicles (6). In this interpretation one class of sizes would give rise to one relaxation time while another class of sizes would give rise to the other relaxation time. 2) Alternatively, the two components may arise from the differing mobilities of the headgroup resulting from two classes of sizes within each vesicle (6,21). 3) And, finally, each headgroup may exist in two conformations.

The effect of sonication on unsonicated lecithin dispersions is to produce vesicles which give rise to relatively narrow proton (22) and carbon-13 resonances (23). This behavior is paralleled with PhMR. With increasing sonication time a relatively narrow resonance with the same chemical shift emerges from the broad resonance that is characteristic of unsonicated lecithin (vide supra). This time course is shown in Figure 4. Integration using pyrophosphate as an internal standard revealed that $90\pm10\%$ of the phospholipid contributes to this narrow resonance. A spectrum of egg yolk lecithin is shown in Figure 5.

The relaxation data for lecithin in water are summarized in Table II. It is evident that the effect of sonication on the linewidth (as described above) is also reflected in the values of T_2 and, to a lesser extent, T_1. That the T_1 value increases with sonication suggests but by no means establishes that the short correlation time regime for T_1 relaxation is applicable. The data in Table II show that the values of the spin-lattice relaxation times of the two synthetic lecithins are nearly identical, while that for egg yolk lecithin is substantially longer. We do not have, at this time, an explanation for this difference.

For sonicated lecithin dispersions the value of T_2 determined from a spin-echo experiment is significantly longer than that estimated from the linewidth, suggesting a finite non-dipolar contribution to the linewidth. This presumably non-dipolar contribution has also been seen in proton NMR (17,25). The value of T_2 is independent of the echo spacing, thus no substantial contribution to the linewidth arises from diffusion through magnetic field gradients. Since sonicated lecithin dispersions are quite small, ~250 Å in diameter, it is important to consider the contribution of particle tumbling to the dipolar linewidth. Using the values of the second moment given in Table I and a value of 10^{-6} sec for the reorientation time of the entire vesicle (25), the residual dipolar linewidth will be of the order of 100 Hz (18). The observed linewidths and transverse relaxation times, T_2, determined by spin-echo experiments, are clearly indicative of significantly shorter correlation times for the molecular motions.

The data in Table III show the effect of different solvents on the chemical shifts and relaxation rates in lecithin. In no case does $T_1 = T_2$, a pattern observed in this laboratory for many other phosphorus compounds. In both methanol and in chloroform the spin-lattice relaxation times are shorter than those in water with the value in the latter solvent an order of magnitude shorter than in the former. By contrast, the value of the transverse relaxation decreases and is nearly the same in both solvents. The chloroform results were not substantially altered by drying the lipids from D_2O and resuspending them into $CDCl_3$, indicating that the solvent protons per se are not primarily responsible for the thermal relaxation. The relaxation rate in these solvents may be reflecting the type of "micellar" structure(s) in each particular solvent.

Figure 6 shows a spectrum obtained from unsonicated E. coli membranes prepared by the method of Kaback (26). A qualitatively similar resonance was seen in membranes prepared by sonicating whole cells of E. coli. Tentatively we assign this phosphorus resonance to the E. coli phospholipids. The chemical shift of this resonance is 100 Hz to lower frequency with respect to pyrophosphate at pH = 10.5 used as an external standard. It is interesting that the E. coli resonance is 227 Hz lower than that of lecithin in water. The phospholipid composition of E. coli membranes is reported to be 70% phosphotidylethanolamine, 20% phosphotidylglycerol, and 5% diphosphotidylglycerol (28). Assuming that the resonance is indeed from the phospholipids rather than from nucleotides or nucleic acids, this difference may reflect the chemical shifts of these phospholipids in membranes as contrasted to lecithin in water. Estimates suggest that there is at least an order of magnitude more phospholipid phosphorus than other phosphorus containing molecules in these membrane preparations.

DISCUSSION

The main features of the PhMR spectra presented in this note are the simple (one line), relatively narrow resonances. This feature, as well as the general applicability of PhMR to all phospholipids and its adequate sensitivity, renders PhMR an attractive approach to the study of membrane systems. The spectral simplicity can be an advantage in experiments with phospholipid dispersions, but its major advantage may be in membrane studies in which overlapping resonances and ambiguous assignments have been of major concern in PMR. Although the sensitivity of PhMR is less than that of PMR, we obtain signal-to-noise ratios >7 on a 1 ml sample of

30 mM dimyristoyl lecithin with 100 pulses (at 24.3 MHz). For relaxation studies where one must wait 5 T_1's between pulses, about 12 min of data accumulation are required.

An incomplete understanding of the relaxation mechanisms in simple phosphorus containing compounds as well as in phospholipid molecules clearly limits the interpretability of relaxation data at present. Work by ourselves and others will lead to clarifications. Since the PhMR spectra and relaxation times are sensitive to the type of dispersion and possibly to the solvent, it is likely that these parameters will also be sensitive to other changes in the headgroup region of these molecules.

In conclusion, PhMR of phospholipids has the potential of providing useful structural and dynamic information about the headgroup region of phospholipid molecules. Recently, for example, PhMR in conjunction with PMR was used to determine the rate of two-dimensional diffusion in sonicated lecithin (14). With sufficiently sophisticated instrumentation this rate could be measured by pulsed gradient techniques (29). In subsequent communications we hope to develop the use of PhMR as a general probe of the inter- and intramolecular structure of the headgroup region in phospholipids.

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Table I. Second Moment Calculations and Linewidth Estimates of L-α glycerophosphorylcholine and Dipalmitoyl L-α Lecithin.

	Glycerophosphorylcholine†	Dipalmitoyl lecithin
$\Delta\nu$ observed	4.5×10^3 Hz	2.0×10^3 Hz
$\Delta\nu$ calculated*	4.8×10^3 Hz	--

† Cadmium chloride salt; the second moment calculation used a value of 2.9 Å for the phosphorus-proton distances.

* Calculated value of the linewidth at half maximum using the Van Vleck moment analysis and assuming a gaussian line shape. Contributions from only the 4 neighboring methylene protons were considered (see text) (30).

Table II. Phosphorus Magnetic Resonance Linewidths and Relaxation Rates from Lecithin Dispersions.

	T_1	T_2	$\Delta\nu$*	$\Delta\nu$
Unsonicated Egg Yolk Lec.	0.9 ± 0.2	0.07 sec		590 Hz 73.5%*** 72 Hz 26.5%***
Sonicated Egg Yolk Lec.	8.3 ± 0.5	0.11** sec 0.13† sec	2.8 Hz	19.5 ± 1 Hz
Sonicated Dimyrist. Lec.††	1.4 ± 0.1	-	-	20.0 ± 1 Hz
Sonicated Didihydrostrec.††	1.4 ± 0.1	-	-	19.0 ± 1 Hz

*The linewidth estimated from the spin-echo T_2 by the relation $\Delta\nu = 1/\pi\, T_2$.

**The value of T_2 using a spin-echo spacing of 0.006 sec.

†The value of T_2 using a spin-echo spacing of 0.015 sec.

††These samples were in D_2O; the other samples were in H_2O.

***Linewidth estimated from the free-induction decay.

Table III. Phosphorus Magnetic Resonance Relaxation Rates, Chemical Shifts, and Linewidth Estimates for Egg Yolk Lecithin in Different Solvents.

	T_1	T_2*	$\Delta\nu$†	$\Delta\nu$	δ††
Water	8.3 ± 0.5 sec	0.12 sec	2.8 Hz	19.2 ± 1 Hz	-1.0 ± 0.2
Methanol	1.2 ± 1 sec	0.009 sec	34 Hz	46 ± 10 Hz	-1.5 ± 0.2
Chloroform	0.12 ± 0.02 sec	0.010 sec	32 Hz	58 ± 10 Hz	-1.2 ± 0.2

*Value of T_2 measured by collecting only the first echo (varying the echo spacing).

†Estimate of the linewidth from the spin-echo T_2 using the relationship $\Delta\nu = 1/\pi T_2$.

††Shifts are referred to 50% H_3PO_4 used as an external reference.

FIGURE CAPTIONS

Figure 1. P^{31} Fourier transform spectrum of L-α glycerophosphorylcholine. 1872 pulses were accumulated with a 30 sec wait between pulses.

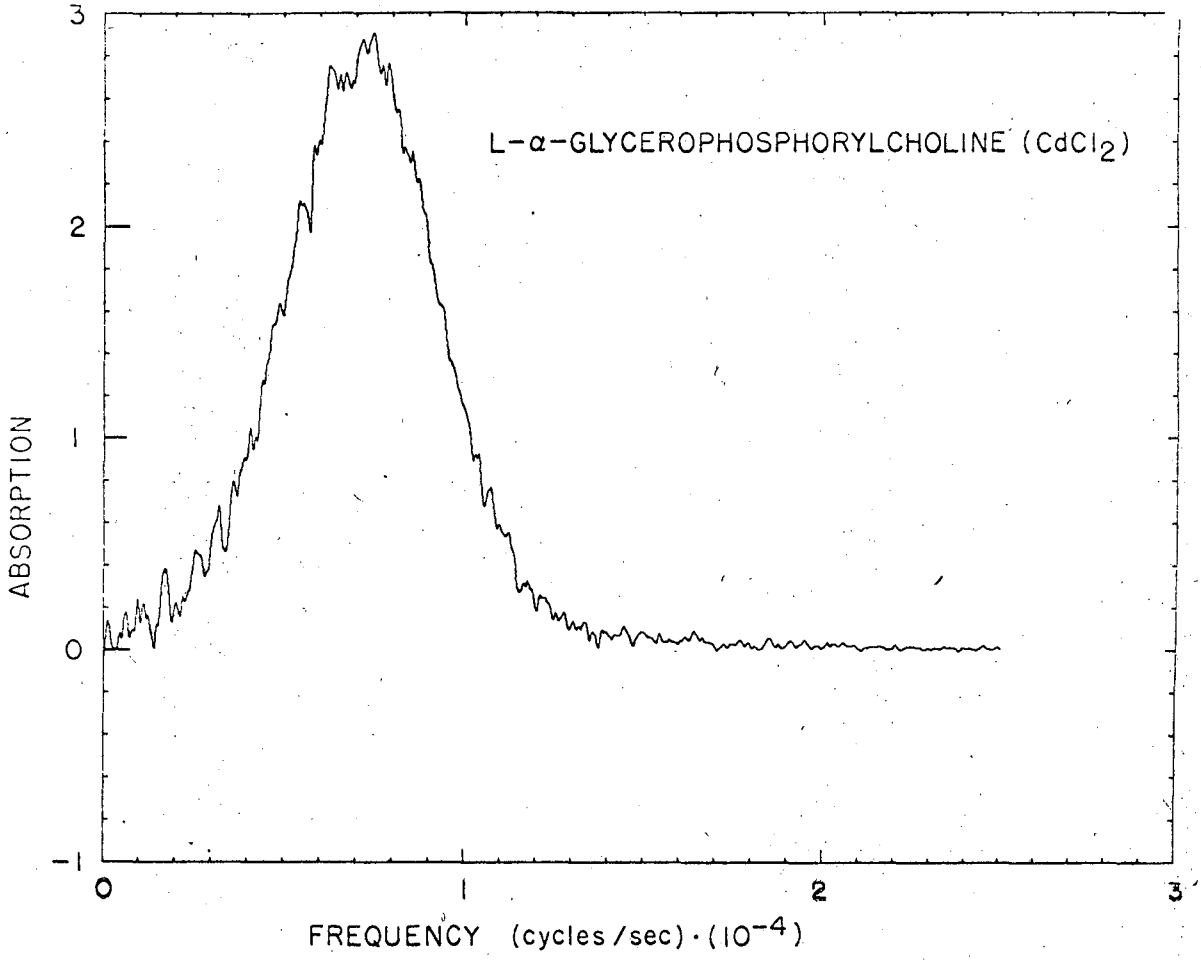
Figure 2. P^{31} Fourier transform spectrum of ~90 mM unsonicated egg yolk lecithin in 0.1 M tris-HCl, 0.15 M KCl, pH = 8.5. 400 pulses were accumulated with 6 sec between each pulse.

Figure 3. Plot of the free induction decay envelope, corresponding to the spectrum in Figure 2. o = negative envelope; ● = positive envelope. The apparent scatter of the later time points results from modulation between spectral components.

Figure 4. The effect of sonication on the phosphorus magnetic resonance spectrum of egg yolk lecithin. Separate samples were sonicated for the specified length of time and were not centrifuged; all other conditions were similar to those described in the Methods section.

Figure 5. P^{31} Fourier transform spectrum of (a) 90 mM pyrophosphate and (b) 15 mM sonicated egg lecithin in 0.1 M tris-HCl, 0.15 M KCl, pH = 8.5. 500 pulses were accumulated with 7 sec between pulses.

Figure 6. P^{31} Fourier transform spectrum from E. coli membranes. A 3 ml sample containing 20 mg/ml protein in 0.05 M tris-HCl, 0.15 M KCl, at pH = 7.5, was pulsed 1052 times.



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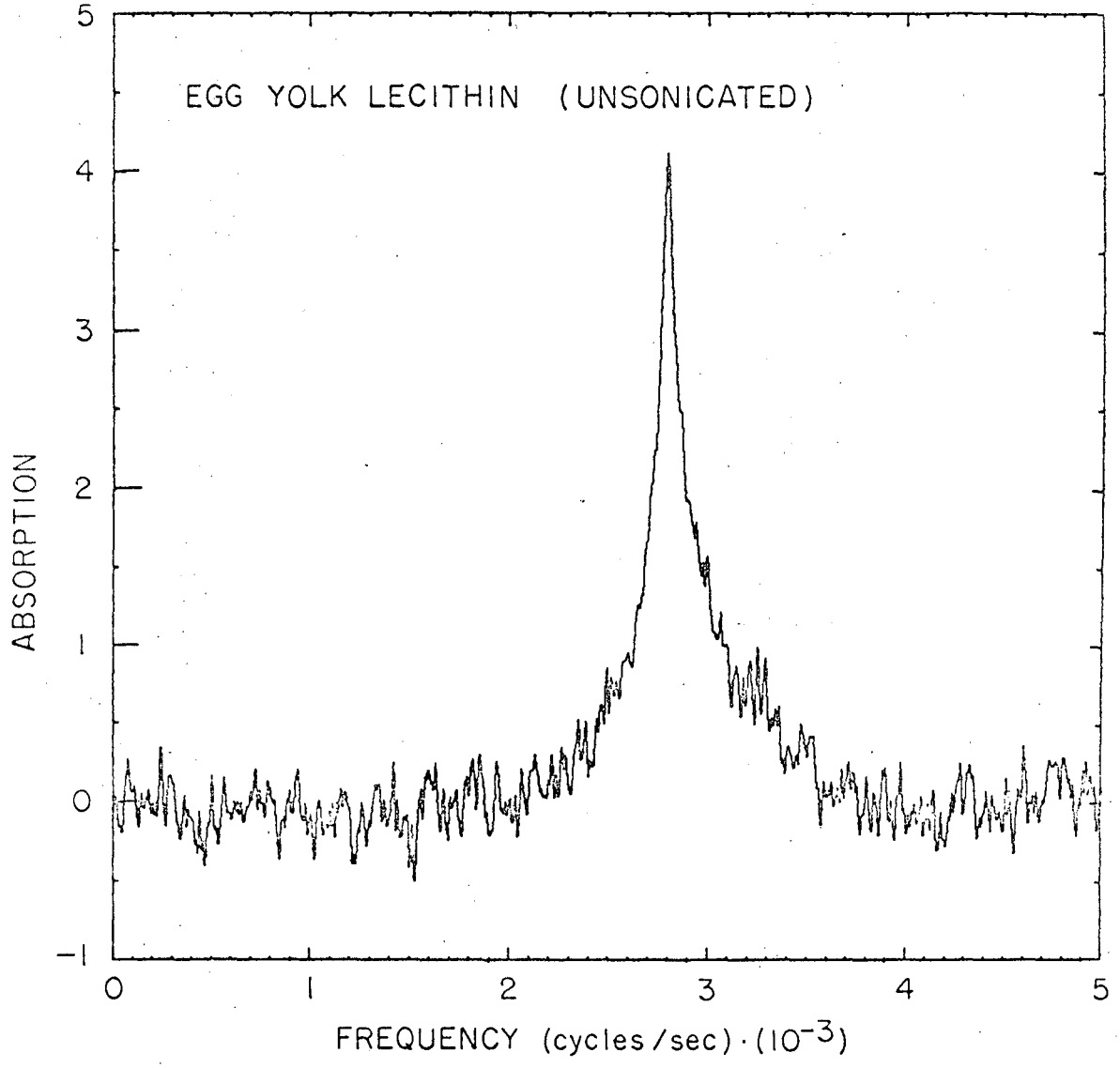

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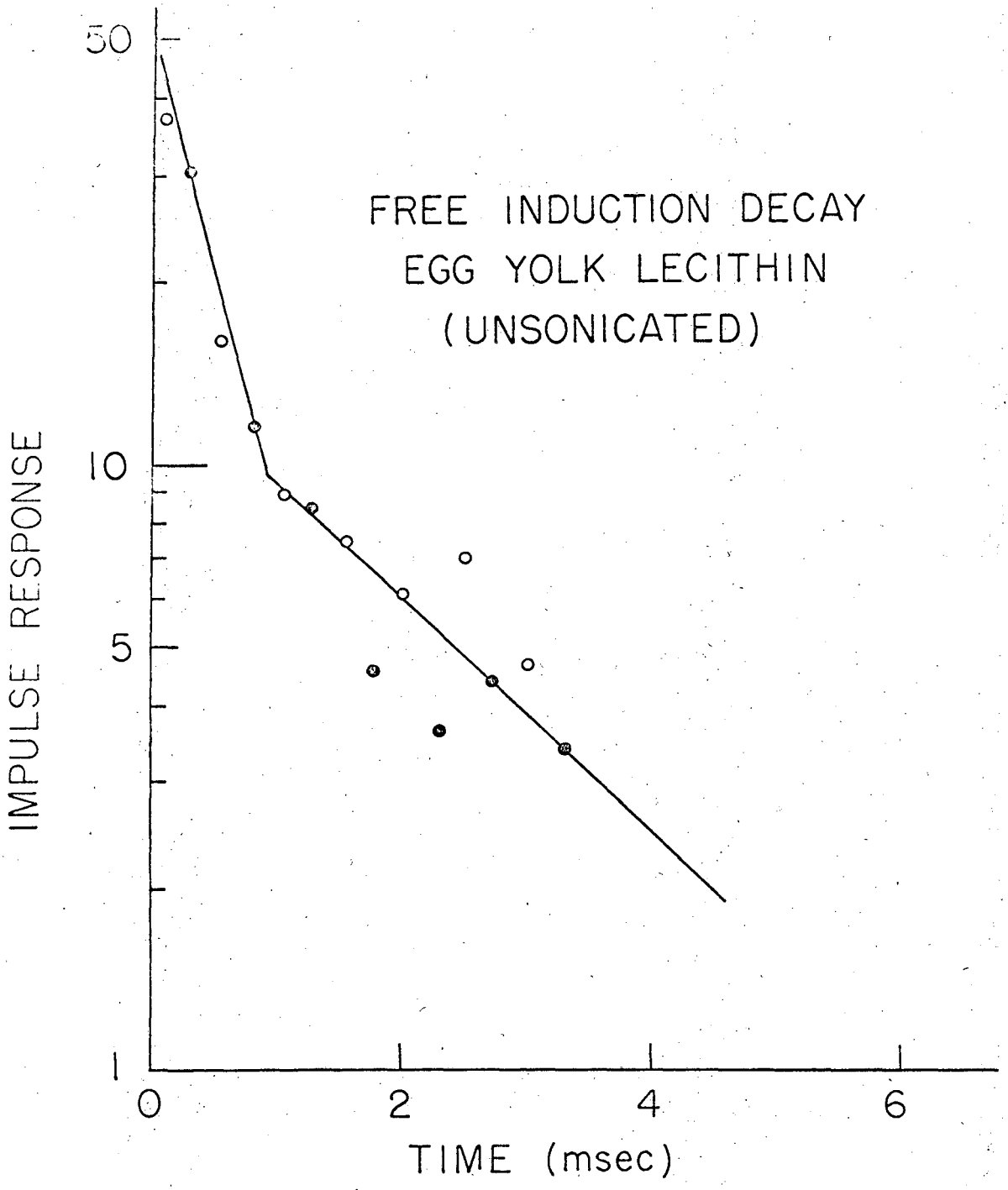

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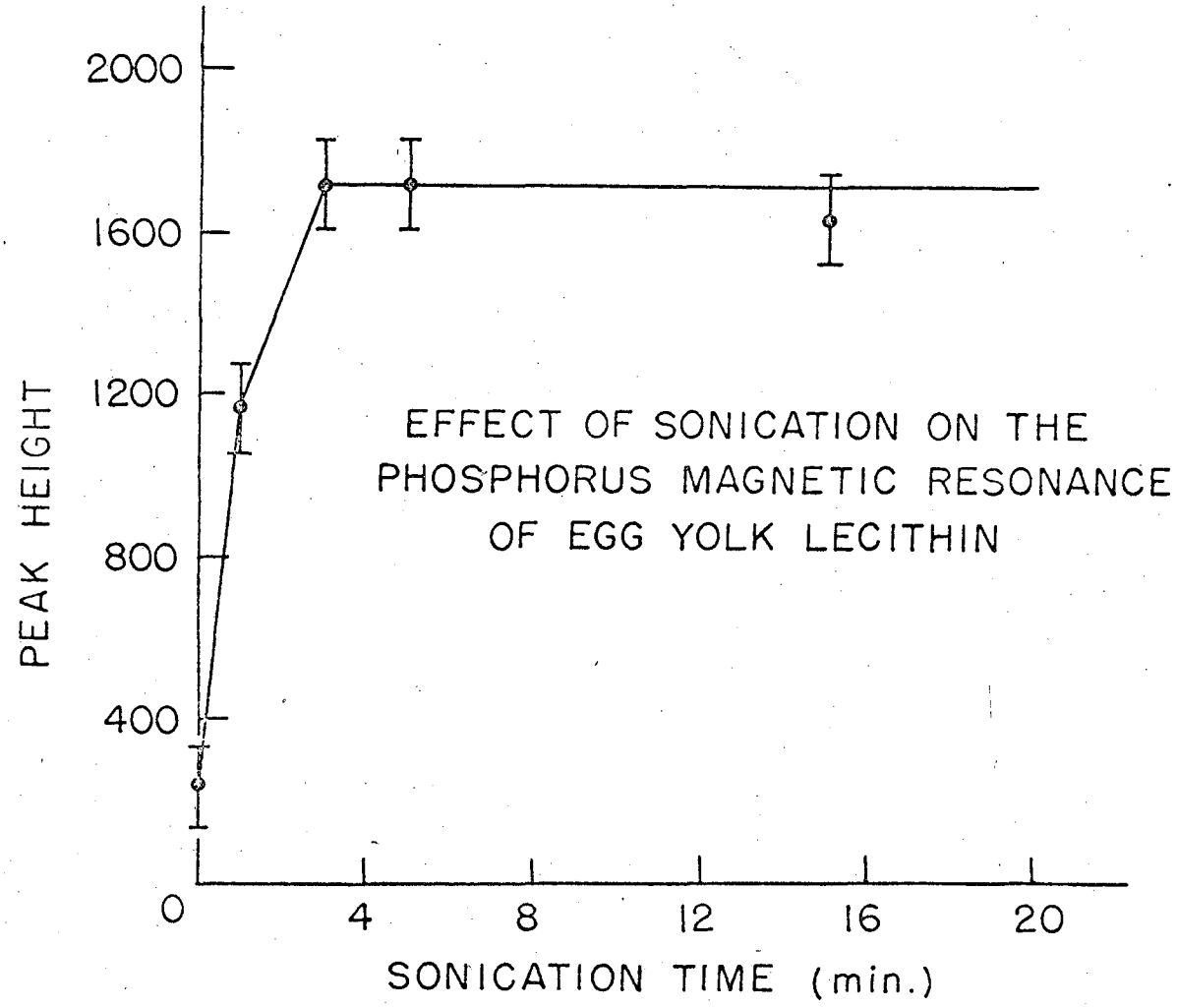

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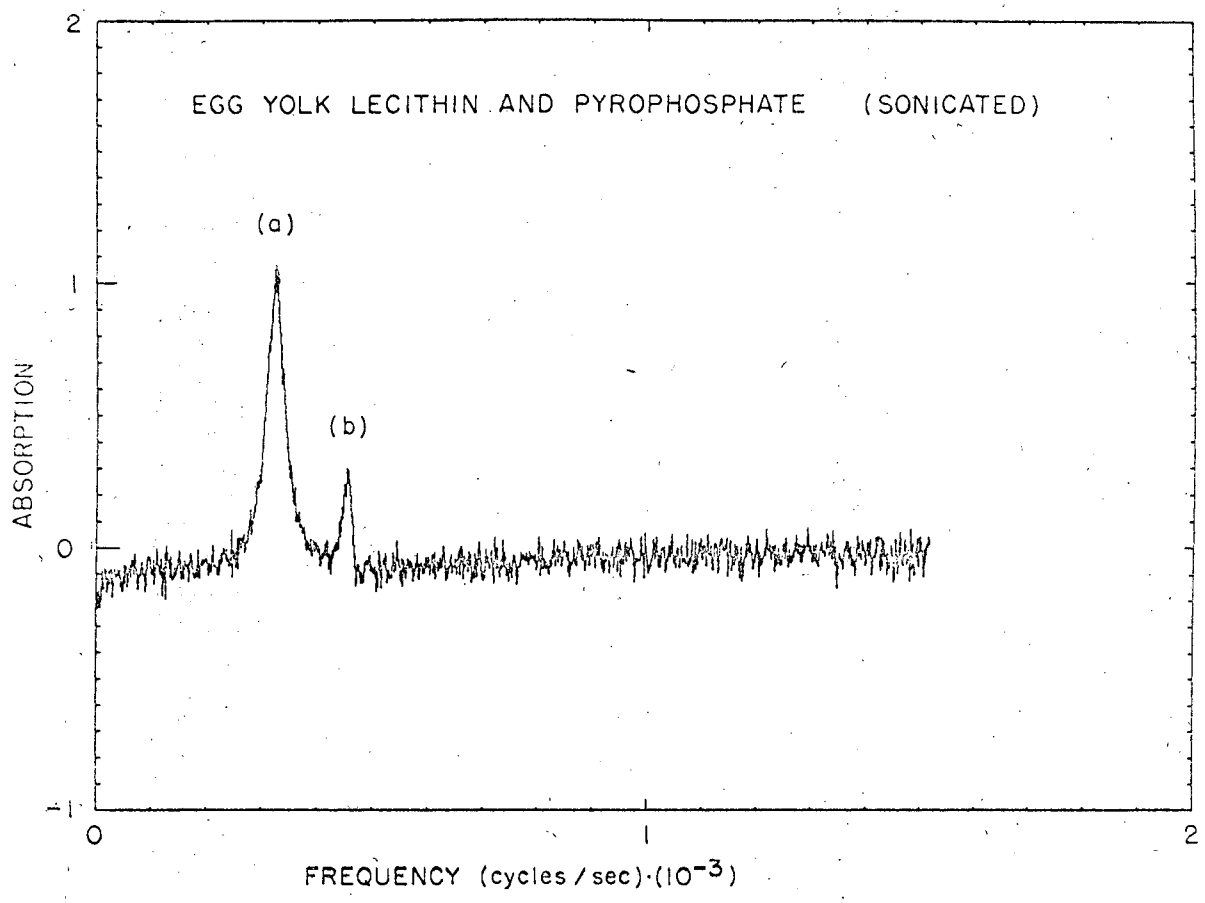

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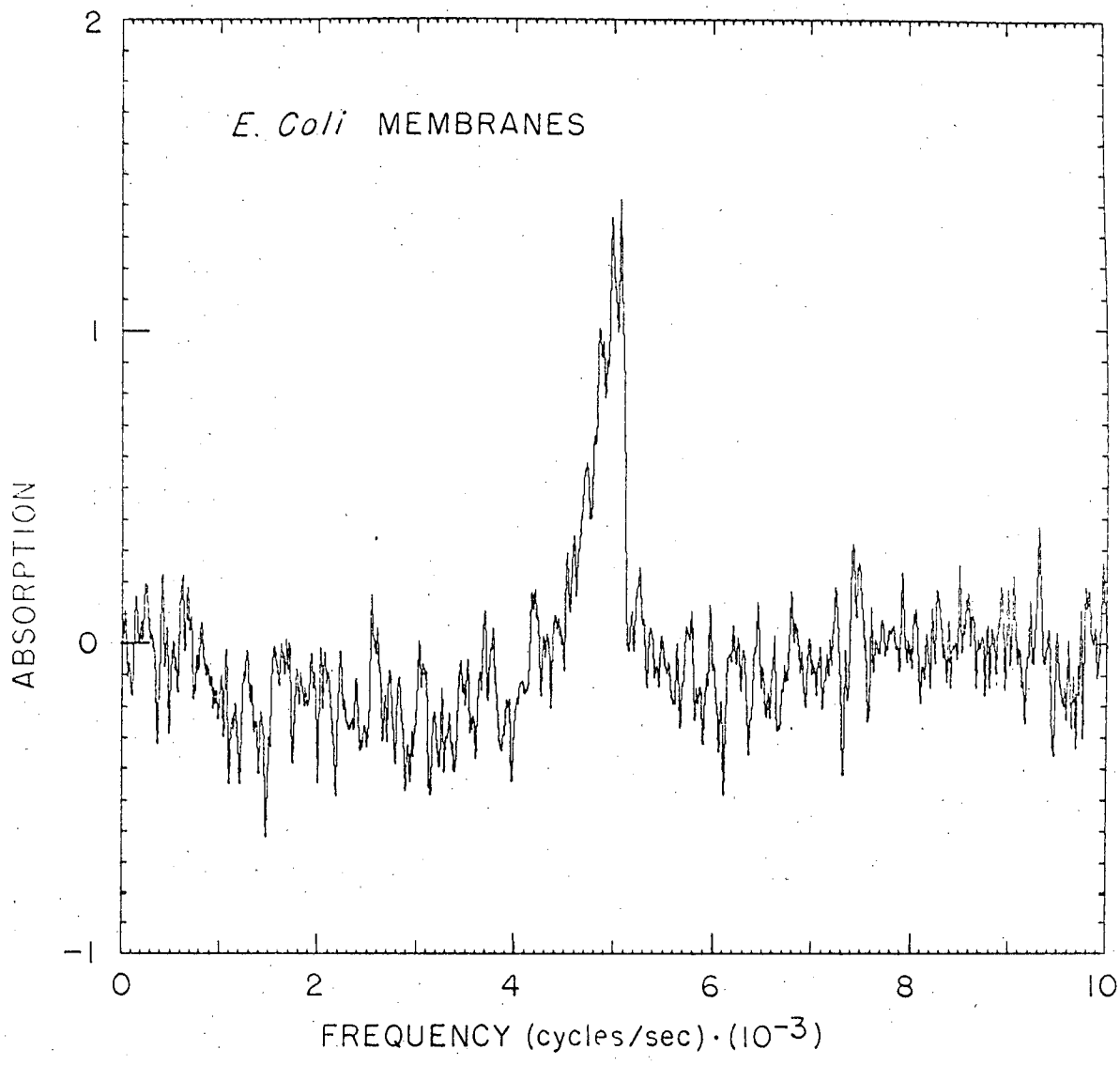

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