Interaction of an Amine Oxide Detergent with Lecithin Vesicles as Studied by Nuclear Magnetic Resonance†

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ABSTRACT: The interaction of an amine oxide detergent with single bilayer lecithin vesicles was investigated with proton and phosphorus magnetic resonance. The addition of the detergent micelles to vesicle suspensions leads to rapid detergent incorporation into the vesicle bilayer, resulting in a heterogeneous vesicle population. Initially, some vesicles take up the equivalent of one detergent micelle, whereas others contain no detergent. Subsequently, the detergent is distributed between the vesicles by vesicle-vesicle collisions. This can be followed by the change in the Pr^{3+}-shifted spectral positions of the detergent and lecithin head groups with time. From the intensity of the head-group signals, it can be concluded that after about 20 h the detergent is almost equally distributed between the outer and inner vesicle membrane monolayers. Vesicles obtained by cosonication of the detergent and lecithin take up metal ions. This ion permeability depends on the vesicle concentration and can be attributed to vesicle-vesicle or vesicle-mixed micelle collisions. Egg lecithin vesicles are stable against the detergent up to molar ratios of detergent to lecithin of 0.2–0.3. At larger ratios mixed micelles and multibilayers are formed. Measurements of proton spin-lattice relaxation times confirmed that the internal architecture of the vesicle bilayer is almost unaffected by the incorporated detergent.

Detergents of a wide variety have been used for the disruption of biological membranes in order to isolate membrane-bound proteins (Helenius & Simons, 1975; Tanford & Reynolds, 1976). Less is known about the role of detergents in the reconstitution of detergent-solubilized proteins into artificial phospholipid membranes (Kagawa & Racker, 1971; Dixon & Hokin, 1974; Racker et al., 1975; Krämer & Klingenberg, 1977). Phospholipids in a vesicular state are required for the functional reconstitution of the membrane proteins which are involved in transport processes across natural membranes. Transport phenomena can be studied advantageously in such reconstituted systems, because artificial phospholipid vesicles are of relatively uniform size and contain small internal solution compartments, which are inaccessible to the substrates of the transport proteins.

Phospholipid vesicles obtained by ultrasonic irradiation have been studied extensively by a variety of physical methods. Nuclear magnetic resonance has been widely used for the determination of the mobility of individual structural elements of phospholipid molecules in multilamellar and bilayer dispersions (James, 1975). The use of various paramagnetic shift reagents such as lanthanide ions (Bystrov et al., 1971; Levine et al., 1973) and ferricyanide (Berden et al., 1973) makes it possible to distinguish between signals from the inner and outer vesicle surfaces.

In the present paper the time course of the detergent incorporation into vesicles and the influence of detergent on the vesicle bilayer structure were investigated with NMR. This information may shed some light on the membrane protein reconstitution process.

For membrane protein solubilization, the mild non-ionic Triton X-100 and similar non-ionic detergents have been very useful. Ribeiro & Dennis (1975) have recently investigated the properties of mixed micelles of Triton X-100 and dimyristoylphosphatidylcholine by proton magnetic resonance relaxation studies. The ratios of Triton to phospholipid examined by these authors were, however, very large and the properties of the mixed micelles reflect mainly those of the surfactant.

Amine oxide detergents have been used for the purification of rhodopsin (Applebury et al., 1974; Sardet et al., 1976) and it is very likely that their application in biochemistry will become more widespread. These detergents have some advantages over the frequently applied polyoxyethylene compounds. (1) There is no UV absorption as with the Triton-type polyoxyethylene detergents. (2) The amine oxides are not subject to autoxidation. (3) They do not exhibit cloud points, i.e., their micelle size remains small at all temperatures. 3-Lauramido-*N*,*N*-dimethylpropylamine oxide (see Figure 1) is a non-ionic detergent, whose structure resembles that of lysolecithin. Recently it was shown that this detergent can be used for the isolation of the ATP, ADP carrier protein from the inner mitochondrial membrane as well as for the reconstitution of this carrier system into egg yolk phospholipid vesicles without loss of inhibitor binding capacity (Krämer & Klingenberg, 1977). The experiments described investigate the process of detergent incorporation into the lipid vesicles by NMR.

Materials and Methods

Crude preparations of egg yolk lecithin were obtained from Merck, Darmstadt. Further purification was accomplished as follows: 5 g of the crude material was dissolved in 150 mL of chloroform–methanol (3:2). Fifty grams of aluminum oxide (Woelm, basic, activity grade I) was added and the mixture was stirred at 4 °C for 2 h. The supernatant was evaporated at room temperature to dryness and the residue chromatographed on silica gel (Biosil HA). Thin-layer chromatography (chloroform–methanol–water, 65:25:4) gave a single spot. LAPAO[1] was obtained from Th. Goldschmidt AG, Chemische Fabriken. Praseodymium oxide and europium oxide were ob-

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[1] Abbreviations used: LAPAO, 3-lauramido-*N*,*N*-dimethylpropylamine oxide; CMC, critical micelle concentration.

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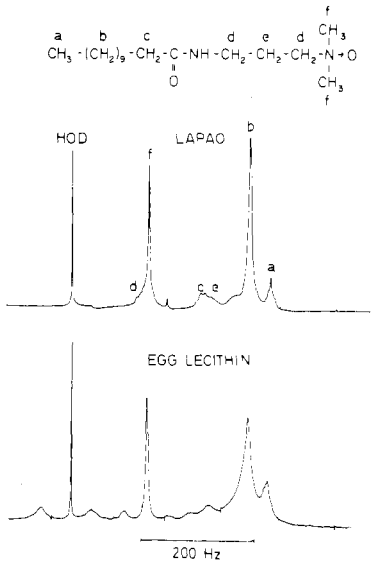

FIGURE 1: ^1H NMR spectra of egg lecithin vesicles and 3-lauramido-*N*,*N*-dimethylpropylamine oxide. Both 5% w/v in 100 mM KCl in D_2O. Temperature: 35 °C.

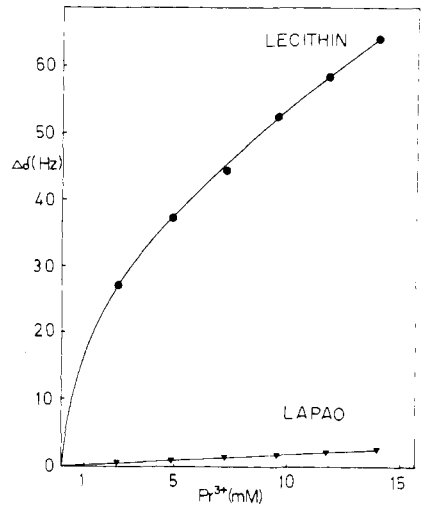

FIGURE 2: Pr^{3+}-induced pseudo-contact shift of the head-group signal of LAPAO and of the "outer" choline methyl signal of lecithin vesicles, both 5% w/v in 100 mM KCl in D_2O. Temperature: 35 °C.

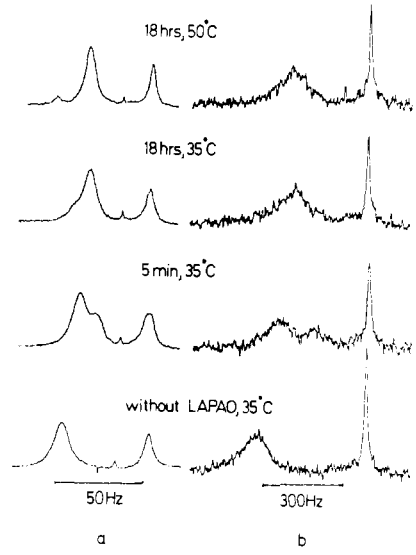

FIGURE 3: Time-dependent change of the head-group signals of lecithin vesicles in the presence of Pr^{3+} after LAPAO addition as indicated. (a) ^1H NMR. Lecithin, 52 mM; LAPAO, 11 mM; Pr^{3+}, 8.9 mM. (b) ^{31}P NMR. Lecithin, 54 mM; LAPAO, 11 mM; Pr^{3+}, 1.5 mM. Incubation at 35 °C.

tained from Sigma and were converted to $Pr(NO_3)_3 \cdot 6D_2O$ and $Eu(NO_3)_3 \cdot 6D_2O$, respectively, with nitric acid and recrystallization from D_2O. Vesicles were prepared by intermittent sonication of lecithin dispersions in 100 mM KCl, 0.5 mM EDTA in D_2O with a Branson type sonicator with microtip in 6-mL portions in an ice bath under a stream of nitrogen. The total sonication time at power level 4 was about 40 min. The lecithin concentration was 5% in most cases.

The CMC of the detergent was determined according to the method of Shinitzky et al. (1971) as described by Yedgar et al. (1974). Phosphorus determinations were carried out by the method of Chen (1956).

^1H and ^{31}P measurements were made at 90 and 36.4 MHz, respectively, with a Bruker SXP4-100 pulsed NMR spectrometer interfaced with a Nicolet 12 K computer. All ^{31}P spectra were fully ^1H broad-band decoupled.

^1H relaxation measurements were made by the inversion recovery method. Data were collected after the 90° pulse of a (180°-τ-90°) pulse sequence into 4K of memory. After 20 transients had been collected, another 20 transients from a 90° pulse were accumulated into the second 4K. After Fourier transformation of both FID's, the spectra were subtracted electronically. The logarithms of the remaining signal heights for τ values of 0.1-1.2 s were plotted against the pulse delay times τ. Variable temperature measurements were made with the standard Bruker variable temperature equipment with an accuracy of ±1 °C.

Results

Detergent Uptake by Vesicle Bilayer Membranes. The ^1H NMR spectrum of LAPAO is shown in Figure 1 in comparison with that of egg yolk lecithin vesicles. The chemical shifts of the most prominent signals—arising from the fatty acid methylenes, the fatty acid methyls, and the head-group methyls—are shown to be nearly identical. On the other hand, there is a marked difference in the line widths of the methylenes and methyls of phospholipid vesicles and LAPAO micelles.

On addition of rare earth salts to vesicle solutions the inward and outward facing choline head groups may be discriminated by the pseudo-contact interaction of the paramagnetic metal ion with the head-group methyl protons (Bystrov et al., 1971). This effect is due to complexation of the metal ion to the negatively charged phosphate group of the choline moiety. On the other hand there is no negatively charged group in the LAPAO molecule. Correspondingly on titration with Pr^{3+}, the LAPAO head group exhibits only very small paramagnetic shifts in contrast to the outward facing methyl group of egg yolk lecithin (Figure 2).

The addition of LAPAO to a vesicle suspension in the presence of Pr^{3+} has a distinct effect on the ^1H spectrum, when recorded a few minutes after mixing (Figure 3a). The downfield shifted signal from outward facing methyls appears to be split into two still overlapping signals which then coalesce after 15 to 20 h. In addition a downfield shoulder develops after 5 h which can be shifted further by raising the temperature. The remaining lecithin head-group signal has a quasi-Lorentzian shape with nearly the same line width as the original egg yolk head-group "outer" signal. Addition of $MnCl_2$ to the sample causes considerable broadening of the temperature-sensitive

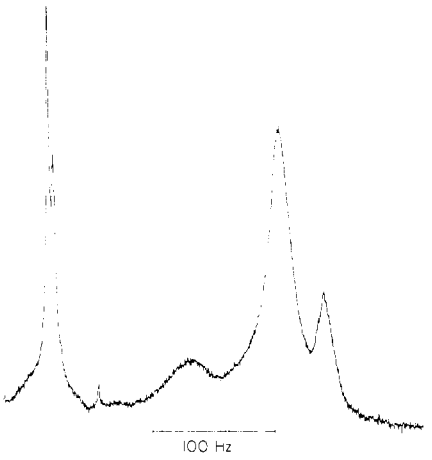

FIGURE 4: ^{1}H NMR spectrum of a mixture of LAPAO and lecithin vesicles, lecithin concentration 28 mM, molar ratio LAPAO to lecithin 2.8, in the presence of 9.5 mM Pr^{3+}. Incubation time: 2 h at 35 °C. Spectrum accumulated at 50 °C.

signal as well as of the "outer" head-group peak (not shown in Figure 3).

The downfield shoulder could be shown to originate from the LAPAO *N*-methyl groups since this signal increases with LAPAO concentration and the temperature induced shift was also found in mixed micelles formed by large ratios of LAPAO to phospholipid. The ^{1}H NMR spectrum of these micelles shows only one head-group signal which is shifted downfield by addition of Pr^{3+}. On raising the temperature this Pr^{3+}-shifted signal splits into two components, the downfield one being that of LAPAO as can be seen from the relative intensities (see Figure 4). Obviously, the incorporation of LAPAO into a phospholipid environment makes the LAPAO head group sensitive to the paramagnetic shift effect of the rare earth ion and this sensitivity increases with temperature.

The behavior of the "outer" head-group signal after addition of LAPAO, i.e., the splitting and coalescence as shown in Figure 3, can be explained as follows. Upon mixing phospholipid vesicles and detergent micelles undergo collisions leading to rapid incorporation of the detergent into the vesicle bilayer. Some other detergents have been shown to be almost fully incorporated into excess phospholipid liposomes, the free concentration remaining below the CMC (Helenius & Simons, 1975; Becker et al., 1975).

For a suspension of particles of the average diameter of the egg yolk lecithin vesicles, 230 Å (Finer et al., 1972), a diffusion constant of 1.9×10^{-7} cm^2 s^{-1} can be calculated from Stokes' law. Assuming a homogeneous distribution of the particles at time zero, the time required for the "first collisions" can be estimated from Fick's law and second-order kinetics (Smoluchowksi, 1917). A half-time of 1.8×10^{-5} s for this collision process can be calculated for a particle concentration of 10^{16} particles per mL—corresponding to a total concentration of 50 mg per mL and an average particle weight of 3×10^6 daltons.

Ordinary mixing cannot be accomplished in such a rapid manner that local excess of the detergent may be avoided. As a result, after mixing the population of vesicles is heterogeneous, consisting of vesicles without detergent and vesicles with a relatively high detergent concentration. Vesicles with a high detergent concentration exhibit a smaller Pr^{3+}-induced pseudo-contact shift of the outward facing choline head-group signals as shown in Figures 3 and 5B. The coalescence with time of the two signals in Figure 3 shows that the detergent is now transferred from highly loaded vesicles to the unloaded

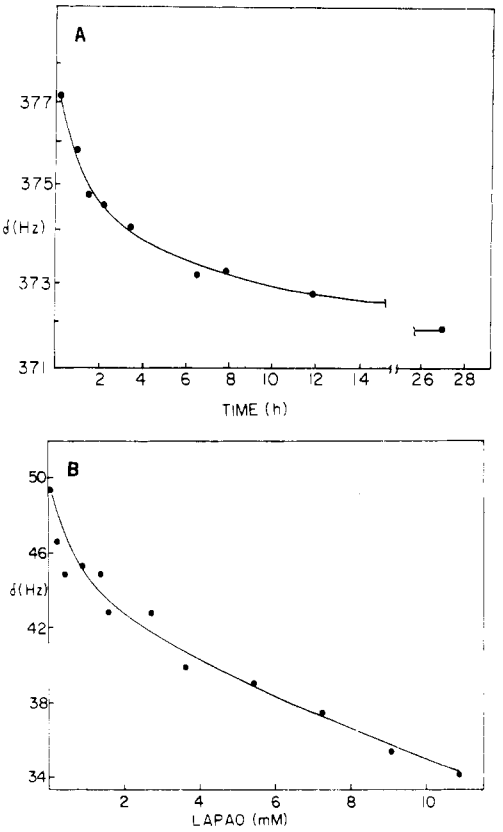

FIGURE 5: (A) Time dependence of the Pr^{3+}-induced shift of the choline head-group signal (downfield peak maximum measured against external tetramethylsilane) after addition of LAPAO. Conditions as in Figure 3. (B) Final Pr^{3+}-induced chemical shift difference between the "inner" and "outer" methyl resonances as a function of LAPAO concentration. Incubation time: 24 h at 30 °C. Lecithin, 54 mM; Pr^{3+}, 9.5 mM.

vesicles until equal distribution of detergent is achieved. The final lanthanide-induced shift is intermediate between that of both types of vesicles. The time dependence of the signal coalescence is shown in Figure 5A. The upfield migration of the low-field maximum was evaluated relative to the signal of tetramethylsilane in an external capillary in dependence on time. This shift can be regarded as a rough measure of the time course of the equilibration of detergent concentrations in the vesicles. The shift rate for various lecithin concentrations (10%, 5%, 2.5%, 1.25% v/v), at fixed LAPAO to lecithin molar ratio of 0.2, was shown to be concentration dependent. This rate approximately increases proportionally to the lecithin and LAPAO concentrations. It can be assumed that the detergent exchange is governed mainly by vesicle–vesicle collisions.

Figure 5B shows the final Pr^{3+}-induced signal shifts which were reached after incubation with the detergent for 24 h. The Pr^{3+}-induced shift decreases with increasing amounts of LAPAO, but the concentration dependence is complex. Below a total concentration of about 0.5 mM LAPAO, the paramagnetic shift changes very rapidly. This may be due to micelle formation at the critical micelle concentration of LAPAO (0.6 mM) which was determined with the perylene–glass beads method of Shinitzky et al. (1971).

Transmembrane Movement of Detergent ("Flip-Flop"). The integral intensity of the "outer" head-group signal increases slightly at room temperature on addition of LAPAO resulting from overlapping head-group signals of LAPAO and of the outward facing lecithin. Therefore, measurement of this intensity with time should show whether the detergent can flip over to the inner part of the membrane which is inaccessible to the rare earth ion. Reproducible results are difficult to obtain

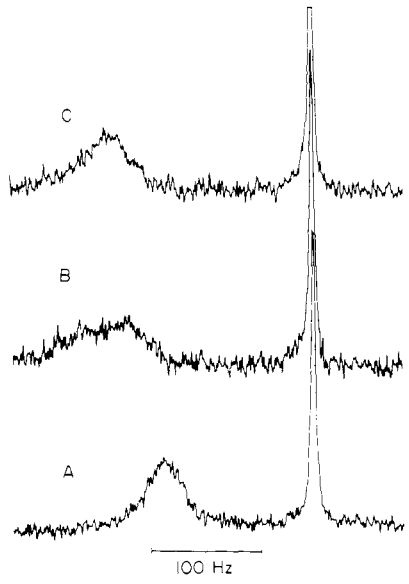

FIGURE 6: (A) ^{31}P NMR of the same mixture as in Figure 3b; total sample volume, 1.3 mL. (B) After addition of 0.6 mL of a fresh vesicle solution containing the same lecithin and Pr^{3+} concentrations as in A; the data accumulation was started 3 min 30 s after mixing; total accumulation time was 1 min. (C) The same sample with data accumulation started 64 min after mixing. All measurements were at 34 °C.

because the change in the ratio of the "outer" to "inner" signal can only be small due to the low contribution of LAPAO. At 50 °C the head-group signal of outside LAPAO is separated and one can calculate from its area (see Figure 3a) the molar ratio of LAPAO in the outer monolayer to about 0.7, by standardizing the total area of the remaining head-group signals with the total lecithin present. One-third of the LAPAO head groups are separated from the outer aqueous phase, presumably because the detergent molecules have diffused into the inner bilayer compartment within the incubation time of 20 h. When Pr^{3+} is trapped in the inner vesicular space by sonication of lecithin in the presence of $Pr(NO_3)_3$ at 50 °C, no LAPAO head-group signal could be separated from the Pr^{3+}-shifted signal of the inner membrane surface. This seems to be in contrast to the Pr^{3+} influence on the outward facing LAPAO molecules. It may be argued that the inner membrane leaf because of its high curvature does not allow the temperature-induced rearrangement of the LAPAO molecule which presumably causes the downfield shift in the outer membrane part.

The influence of external and of internal Pr^{3+} was studied also with vesicles obtained by cosonication of lecithin and LAPAO. The signal shifts of the head-group signals were the same as shown before with vesicles incubated with LAPAO for 24 h within experimental error.

Detergent Transfer from LAPAO Loaded to Unloaded Vesicles. The ^{31}P nucleus of egg lecithin is much more sensitive to the paramagnetic shift effect of Pr^{3+}, because the metal ion is attached to the phosphate group. The lanthanide-induced shift of the ^{31}P signal from outward facing choline is about 12 times that of the *N*-methyl groups in the ^1H spectrum (in Hz). In contrast to the proton head-group resonance, there is a marked broadening of the line of the outer phosphorus nuclei. The rapid detergent incorporation into the vesicle membrane is also reflected in the ^{31}P spectrum. After addition of LAPAO, splitting and subsequent coalescence of the Pr^{3+}-shifted ^{31}P signal takes place (see Figure 3b). Again the upfield part of the "outer" ^{31}P signal can be referred to the vesicles containing high LAPAO concentrations, whereas the downfield part

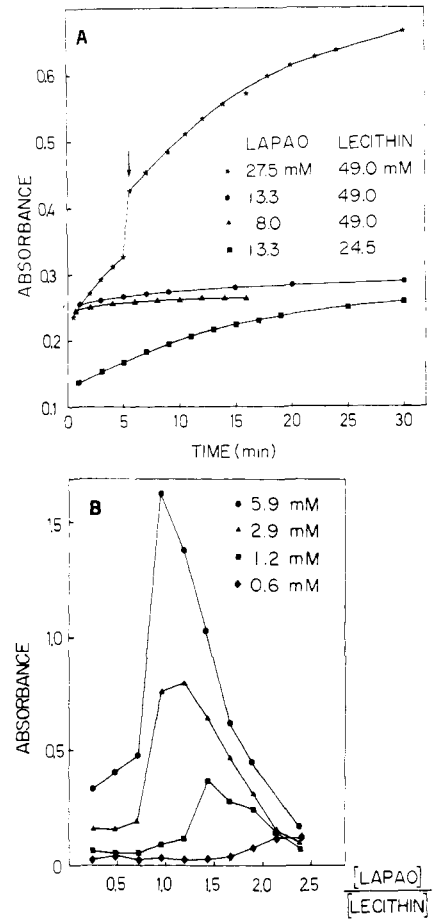

FIGURE 7: (A) Change of absorbance (at 334 nm) of mixtures of LAPAO and lecithin vesicles with time. The arrow indicates shaking of the sample. (B) Turbidity of mixtures of LAPAO and lecithin vesicles after incubation at 35 °C for 2 h as measured by light absorbance. Lecithin concentrations as indicated in the figure.

should arise from those vesicles which contain little or no LAPAO. This was confirmed by the following experiment: When the distribution process was completed (after about 20 h at 30 °C, as observed in the ^1H spectra), a fresh vesicle solution, containing the same Pr^{3+} concentration as the incubation mixture, was added to the LAPAO-loaded vesicles. The ^{31}P NMR spectrum, accumulated immediately after mixing, now shows two overlapping "outer" signals. The downfield signal part can be attributed to the fresh vesicles. The following redistribution process leads to one signal, indicating that all detergent concentrations in the vesicle population are equalized again. The equilibration was reached already after about 1 h at 34 °C (Figure 6). The same results were obtained with ^1H NMR. On the other hand the redistribution effect could be better made visible by ^{31}P NMR because of the larger Pr^{3+}-induced shifts of ^{31}P.

Stability of Vesicles against LAPAO. In order to examine the integrity of the phospholipid vesicles absorbance measurements were carried out after LAPAO addition. Rapid absorbance increase was observed when LAPAO was added to vesicle suspensions in molar ratios of LAPAO to lecithin >0.3 (Figure 7A). It was shown previously (Krämer et al., 1977) that at a ratio of 0.2 the vesicles begin to lose [^{14}C]-sucrose trapped in the inner space. Figure 7B shows the absorbance change with varying LAPAO to lecithin ratios at several lecithin concentrations. The incubation time was 2 h. The absorbance maximum appears near molar ratios of 1:1. However, below an absolute concentration of about 0.5 mM

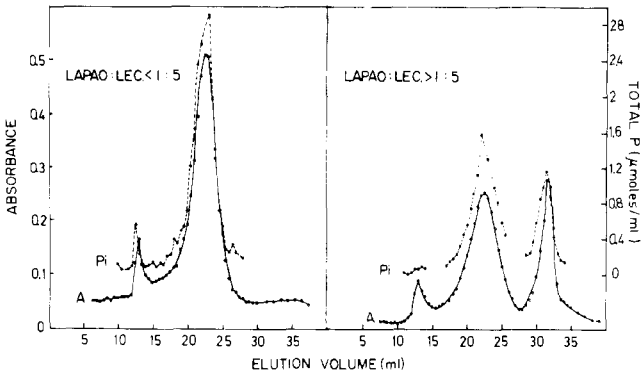

FIGURE 8: Chromatography of mixtures of LAPAO and lecithin vesicles on Sepharose 4B. Incubation time: 20 h at 30 °C. Samples of 0.2 mL of the vesicle suspensions were applied to a column of 1.5 × 37 cm. Molar ratio LAPAO:lecithin was 0.18 and 0.3, respectively. Lecithin concentration was 55 mM.

LAPAO, no maximum is observable. This may be due to the fact that the detergent is not incorporated by the vesicles below its CMC. An analogous behavior was reported earlier for the non-ionic detergent Triton X-100 in the presence of sphingomyelin dispersions (Yedgar et al., 1974). The CMC of LAPAO was determined by the method of Shinitzky et al. (1971) and was indeed found to be 0.5–0.6 mM.

The intensity of the ^1H NMR spectrum at LAPAO to lecithin ratios above 0.3 gradually disappears with increasing LAPAO concentrations. At a ratio of 1:1 all lines are completely broadened.

In Sepharose 4B chromatography (Figure 8) the elution pattern is similar to that of pure egg lecithin vesicle preparations up to a molar ratio of LAPAO to lecithin of 1:5, in agreement with Huang (1969). During the sonication process a small portion (3–5%) of large vesicles is formed (Huang, 1969; Sheetz & Chan, 1972). At higher LAPAO:lecithin ratios another peak of smaller particle size appears, which can be attributed to mixed micelles (Figure 8). The size of these micelles is intermediate between the size of the vesicles and that of the pure LAPAO micelle. The distribution between large and smaller vesicles seems not to be greatly altered by the detergent incorporation.

LAPAO and lecithin form clear solutions when sonicated together at ratios >0.2. In this case a similar elution pattern is obtained as when LAPAO is added after sonication. At LAPAO:lecithin = 0.47, the ratio of phosphorus in the vesicle and in the mixed micelle was almost the same as in the case of vesicles incubated with LAPAO.

The vesicle mixtures, obtained by cosonication of detergent and phospholipid, were found to be slightly permeable to metal ions. Pr^{3+} leads to the expected signal splitting in the ^1H and ^{31}P NMR spectra (Figure 9). Some time after addition of Pr^{3+}, however, the upfield head-group signal becomes broadened and another broad peak appears downfield. This time dependence is shown in Figure 9 for the ^{31}P signal. When Pr^{3+} was added before sonication, the same picture was obtained as after equilibration of Pr^{3+} added after sonication (Figure 9). The assignment of the two overlapping signals in Figure 9 was confirmed with Mn^{2+}. A short time after addition of Mn^{2+} only the narrower upfield shifted signal reflecting the outward facing choline phosphates was broadened almost beyond detectability. These observations indicate that in these vesicles the membrane is not destroyed, although they are somewhat permeable to the metal ions. The downfield shift due to penetrated Pr^{3+} indicates that Pr^{3+} binds stronger to the inward facing phosphates. This is also true for pure lecithin vesicles,

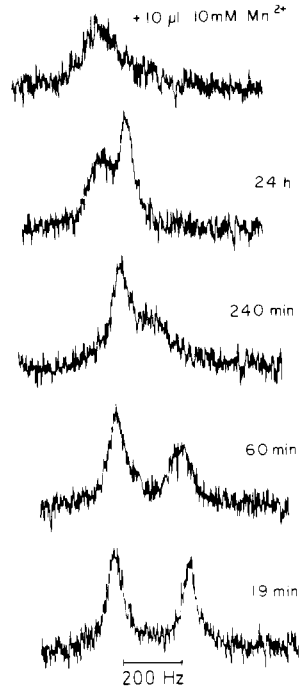

FIGURE 9: ^{31}P NMR spectra of a cosonicated mixture of LAPAO and lecithin after addition of Pr^{3+}. Data accumulation (1 min) started as indicated. Molar ratio of LAPAO:lecithin was 0.45. Lecithin concentration was 50 mM.

TABLE I.

Sample	Head	$(CH_2)_n$	CH_3
Egg lecithin vesicles	0.38	0.38 0.45	0.62
LAPAO	0.24	0.33	0.73
LAPAO:lec. = 0.2 incubation time = 17 h	0.37	0.40	0.75
LAPAO:lec. = 0.2 incubation time = 6 h	0.36	0.40	0.71
LAPAO:lec. = 0.28 cosonicated	0.34	0.35	0.77

[a] All measurements were in 100 mM KCl, 0.5 mM EDTA at 35 °C. T_1 values are in seconds.

as can be shown by ^{31}P NMR of egg lecithin cosonicated with praseodym.

The ion influx rate was found to be dependent on the vesicle concentration. A plot of the rate of the decrease in frequency separation between the "inner" and "outer" ^1H signals vs. the square of the total phospholipid concentration gave a straight line with a slope of 0.12 Hz $min^{-1}/(mg^2\ mL^{-2})$ and an intercept of 0.04 Hz min^{-1} (molar ratio LAPAO to lecithin 0.45; temperature 40 °C).

Spin–Lattice and Spin–Spin Relaxation Measurements. In order to get information about the segmental mobility in LAPAO-loaded vesicles, measurements of spin–lattice relaxation times were carried out. In Table I, T_1 for the methyl and methylene proton signals of pure egg lecithin vesicles, pure LAPAO and LAPAO-loaded vesicles are compared.

Unfortunately the signals of the head groups of the fatty acid methylenes and of the methyl terminals of egg lecithin and LAPAO completely overlap, so that the high resolution T_1 measurements for the mixed vesicles are undefined. But at the ratio of LAPAO:lecithin = 0.2, there is a large excess of methyl

protons and of fatty acid methylene protons from lecithin. As a result, for the first inversion-recovery pulses, almost the exact T_1 values for the lecithin signals are measured. Computer simulation of the $\ln(A_\infty - A_\tau)$ vs. τ curves (using the T_1 values of the pure substances) shows that the deviation from T_1 values of pure lecithin is only 1.6% for the fatty acid methyls and 3.2% for the head-group methyls. This is within the error encountered in T_1 measurements by the inversion recovery method.

The differences between relaxation times of pure lecithin and LAPAO-loaded vesicles are only small. The $\ln(A_\infty - A_\tau)$ vs. τ plots are linear except for the methylene protons of pure egg lecithin vesicles, as already observed by Lee et al. (1972). This nonlinearity disappears after incubation with the detergent in nondestructive concentrations, resulting in an intermediate T_1 value. The relaxation time for the fatty acid methyl protons is somewhat increased in the presence of the detergent, whereas the head-group methyls are almost unaffected.

The line widths of the phospholipid signals are almost unchanged after LAPAO incorporation. From the observed line widths of pure LAPAO micelles (5% solution in 100 mM KCl at 35 °C), an apparent transverse relaxation time T_2^* of about 55 ms can be calculated. The ratio T_1:T_2^* yields about 6.0. On the contrary for lecithin fatty acid protons a value for T_2^* of 18 ms and for T_1:T_2^* of about 22 was found (at 35 °C). The corresponding values for vesicles incubated with LAPAO (molar ratio = 0.2) were 21 ms and 19, respectively.

Finally it was shown that the T_1 values for the proton groups given in Table I increased with increasing temperature. Plots of ln T_1 vs. the inverse of the absolute temperature exhibited slopes which were almost identical with those reported for pure egg lecithin vesicles (Horwitz et al., 1972).

Discussion

The described mechanism of detergent distribution is of importance for the reconstitution of detergent solubilized membrane proteins. This process leads to dilution of the detergent in the neighborhood of the protein and permits protein–phospholipid contact that may be essential for the biological activity of the protein.

The dimethylamino head-group of LAPAO must be exposed to the aqueous medium when incorporated into the vesicle, because its protons are sensitive to the paramagnetic effect of Pr^{3+} or Mn^{2+}. LAPAO has a rather short fatty acid chain as compared with the side chains of egg yolk lecithin. The fatty acid moiety of LAPAO is a 12:0 chain, whereas a typical analysis of egg yolk phospholipids gives: 42.6%, 18:1; 31.8%, 16:0; 8.2%, 18:2; 4.1%, 18:0; 13.3%, 22:5 (Bolton, 1961). Therefore the LAPAO fatty acid chain cannot extend into the center of the hydrophobic region of the lecithin bilayer.

The distribution of the LAPAO molecules in the bilayer membrane may be facilitated by the relative low order of the liquid-crystalline state. The incorporation process must be very fast, because the signal splitting described for the ^1H signal of the outer membrane half is already complete after about 2 min. The collisions between LAPAO micelles and lecithin vesicles are believed to be similar to the mixing of small and large drops such that all "first" collisions should lead to the absorption of the detergent micelle by the vesicle bilayer.

The experiments described show that NMR shift reagents can be used to distinguish between vesicles of high and low detergent content. Moreover, the redistribution process of the detergent can be conveniently studied. Previously the intervesicular transfer of phospholipids was investigated by ^1H and ^{31}P NMR (Barsukov et al., 1975). In this case, however, a phospholipid exchange protein preparation (Wirtz & Zilversmit, 1968) was used in order to enhance the phospholipid transfer. The exchange rate was found to be very low without the lipid exchange protein. On the contrary, the detergent exchange described here seems not to be catalyzed by other molecules. The concentration dependence of the LAPAO induced signal splitting lets us conclude that the intervesicular detergent transfer is mediated by vesicle–vesicle contact. The involvement of free detergent molecules in the transfer is less probable, because the concentration of free detergent in the presence of phospholipid liposomes is generally below the CMC of the detergent (Becker et al., 1975).

When micelles are incorporated into the vesicular bilayer, a local detergent overconcentration may be not only generated among the vesicles but also in the individual vesicle membranes where a short time after the vesicle–micelle collision a concentrated detergent patch may exist in the outer leaf. It may then be distributed by detergent diffusion over the vesicle surface. The lateral (two-dimensional) diffusion constant of spin-labeled phospholipid molecules in phospholipid bilayers was found to be in the order of 10^{-8} cm^2 s^{-1} (Träuble & Sackmann, 1972; Devaux & McConnell, 1972). This rather large diffusion constant should lead to an equilibration in the vesicle surface within 1 s. However, the observed slow spectral changes after LAPAO addition must be caused by intervesicular detergent transfer as shown by the reverse splitting and downfield shift of the "outer" lecithin head-group signal on addition of a fresh vesicle suspension to vesicles preincubated with LAPAO.

It is rather difficult to give an unambiguous explanation for the large paramagnetic effect exerted by Pr^{3+} on dimethylamine oxide head groups of LAPAO incorporated into the lipid bilayer membrane as well as for the temperature dependence of this effect. For an axially symmetric **g** tensor of the paramagnetic metal complex, the induced pseudo-contact shift is proportional to the inverse temperature and the average quantity $\langle(3\cos^2\theta - 1)/r^3\rangle$ where r is the distance between the paramagnetic center and the observed nucleus and θ is the angle between the axis of symmetry of the complex and the radius vector r. As Pr^{3+} produces only negligible shift with LAPAO micelles alone, the phospholipid bilayer LAPAO head groups can be visualized to be close to the Pr^{3+} binding sites which is presumably the phosphate moiety of lecithin. Furthermore the distance r and the angle θ may be altered on raising the temperature.

Sepharose 4B chromatography shows that all the detergent is trapped by the phospholipid membrane up to a LAPAO to lecithin ratio of about 0.2. LAPAO distributes between the inner and outer monolayers of the lecithin vesicles rather rapidly as compared with the transmembrane diffusion of phospholipid molecules in bilayer membranes (Rothman & Davidowicz, 1975). From the temperature sensitive downfield shift of the LAPAO head-group signal in the outer layer, it can be concluded that the detergent concentration in the two leaves of the membrane is nearly equal. Obviously the detergent molecule can flip over to the inner membrane side faster than lecithin (Rothman & Davidowicz, 1975).

The metal ion permeability of vesicles produced by cosonication with a ratio LAPAO/lecithin $\simeq$0.4–0.5 cannot only be caused by structural defects in the bilayer. Recently the ion permeability of dipalmitoyllecithin vesicles obtained by sonication below the gel–liquid crystalline transition temperature was described (Lawaczek et al., 1976) and was attributed to structural defects in the membrane, which could be annealed by heating the sample above the transition point. Such structural defects should lead to an influx which does not depend

on the total vesicle concentration. In our case, the observed strong concentration dependence of the ion influx suggests that vesicle–vesicle and/or vesicle–micelle collisions are the major reason for the increased membrane permeability. Moroever, fusion seems not to occur with these mixed vesicles as judged from the high resolution methylene signal which did not change in intensity and width over a period of more than 100 h at 4 °C.

The rapid increase in turbidity with time when LAPAO was added to vesicle suspensions in a molar ratio of LAPAO to lecithin >0.2 may be due to the fact that, immediately after the micelle incorporation, part of the vesicles is composed of detergent and phospholipid at a ratio near 1:1. This can be concluded from the finding that in a mixture near ratio 1:1 only very large structures are formed, which do not show a high resolution ^{1}H NMR spectrum. Presumably these structures are mainly planar multilamellar bilayers resembling unsonicated phospholipid preparations. The thermodynamic state of vesicles may then be metastable and is changed to the stable multibilayer state by the incorporation of detergent molecules.

The measurements of spin-lattice relaxation and line broadening show that the presence of LAPAO does not alter the mobility of the lecithin molecules in the vesicular bilayer membrane. T_1 and line widths are very close to those of the pure vesicles, even in the case of maximal nondestructive LAPAO concentrations. With temperature, T_1 for the fatty acid methylene and methyl protons as well as for the headgroup methyl protons increases in the mixed vesicles as is known for pure egg lecithin and dipalmitoyllecithin vesicles (Lee et al., 1972).

The ratio T_1/T_2* determined for the mixed vesicles agrees with those for pure vesicles. Earlier measurements of the ratio of T_1 and T_2 (Horwitz et al., 1972) and of the frequency dependence and temperature dependence (Seiter & Chan, 1973) of phospholipid methyl and methylene protons have been interpreted in terms of two different correlation times usually referred to as $\tau_\perp$ for a slow and $\tau_\parallel$ for a fast reorientation process. Likewise theoretical calculations by Seiter & Chan (1973) and Feigenson & Chan (1974), using the two correlation time model of Woessner (1962), yielded a reasonable model for the observed spectra of sonicated and unsonicated lecithin by the introduction of an anisotropic motion of the fatty acid side chains. The observed positive temperature dependence of T_1 cannot be ascribed to a single relaxation process and activation energy taken from a ln T_1 vs. temperature plot has no well-defined physical meaning. Nevertheless, the temperature dependence signals that at least one of the two correlation times must be within the extreme motional narrowing limit, for which the relation $\omega_0^2\tau_c^2 \ll 1$ holds (ω_0 = Larmor frequency; τ_c = motional correlation time). Therefore, the observed ratios T_1/T_2* for the $(CH_2)_n$ signal of the mixed vesicles can be attributed to the anisotropy of chain motions because T_2 and the line width are dependent on slow motion whereas T_1 is not. In the case of rapid isotropic motion T_1 and T_2 should be equal.

All these observations are very similar to earlier results on the lecithin vesicle membrane. The ratio of $\tau_\perp$ and $\tau_\parallel$ is not greatly affected by the presence of LAPAO in the vesicle bilayer. The small increase in the methyl T_1 can be qualitatively attributed to an increased motional freedom of the methyls and a slightly enhanced degree of disorder in the hydrophobic core of the bilayer. The relatively short hydrocarbon chains of the detergent may not reach to the hydrophobic middle part of the membrane but give rise to a looser packing of the phospholipid chain ends.

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References

Applebury, M. L., Zuckerman, D. M., Lamola, A. A., & Jovin, T. M. (1974) *Biochemistry 13,* 3448.

Barsukov, L. I., Shapiro, Yu. E., Viktorov, A. V., Volkova, V. I., Bystrov. V. F., & Bergelson, L. D. (1975) *Chem. Phys. Lipids 14,* 211.

Becker, R., Helenius, A., & Simons, K. (1975) *Biochemistry 14,* 1835.

Berden, J. A., Barker, R. W., & Radda, G. K. (1973) *Biochim. Biophys. Acta 375,* 186.

Bolton, W. (1961) in *Biochemists' Handbook* (Long, C., Ed.) p 762, E. & F. N. Spon Ltd., London.

Bystrov, V. F., Dubrovika, N. I., Barsukov, L. I., & Bergelson, L. D. (1971) *Chem. Phys. Lipids 6,* 343.

Capaldi, R. A., Komai, H., & Hunter, D. R. (1973) *Biochem. Biophys. Res. Commun. 55,* 655.

Chen, P. S. (1956) *Anal. Chem. 28,* 1756.

Devaux, P., & McConnell, H. M. (1972) *J. Am. Chem. Soc. 94,* 4475.

Dixon, J. F., & Hokin, L. E. (1974) *Arch. Biochem. Biophys. 163,* 749.

Feigenson, G. W., & Chan, S. I. (1974) *J. Am. Chem. Soc. 96,* 1312.

Finer, E. C., Flook, A. G., & Hauser, H. (1972) *Biochim. Biophys. Acta 260,* 49.

Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415,* 29.

Horwitz, A. F., Horsley, W. J., & Klein, M. P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 590.

Huang, C. (1969) *Biochemistry 8,* 344.

James, T. L. (1975) *Nuclear Magnetic Resonance in Biochemistry,* p 299, Academic Press, New York, N.Y.

Kagawa, Y., & Racker, W. (1971) *J. Biol. Chem. 246,* 5477.

Krämer, R., & Klingenberg, M. (1977) *Biochemistry 16,* 4954.

Krämer, R., Aquila, H., & Klingenberg, M. (1977) *Biochemistry 16,* 4949.

Lawaczek, R., Kainosho, M., & Chan, S. I. (1976) *Biochim. Biophys. Acta 443,* 313.

Lee, A. G., Birdsall, N. J. M., Levine, Y. K., & Metcalfe, J. C. (1972) *Biochim. Biophys. Acta 255,* 43.

Levine, Y. K., Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C., & Robinson, J. D. (1973) *Biochim. Biophys. Acta 291,* 592.

Racker, E., Chien, T. F., & Kandrach, A. (1975) *FEBS Lett. 57,* 14.

Ribeiro, A. A., & Dennis, E. A. (1975) *Biochemistry 14,* 3746.

Rothman, J. E., & Davidowicz, E. A. (1975) *Biochemistry 14,* 2810.

Rydström, J. (1976) *Biochim. Biophys. Acta 455,* 24.

Sardet, C., Tardieu, A., & Luzzati, V. (1976) *J. Mol. Biol. 105,* 383.

Seiter, C. H. A., & Chan, S. I. (1973) *J. Am. Chem. Soc. 95,* 7541.

Sheetz, M. P., & Chan, S. I. (1972) *Biochemistry 11,* 4573.

Shinitzky, M., Dianoux, A. C., Gitler, C., & Weber, G. (1971) *Biochemistry 10,* 2106.
Smoluchowksi, M. (1917) *Z. Phys. Chem. 92,* 129.
Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457,* 133.
Träuble, H., & Sackmann, E. (1972) *J. Am. Chem. Soc. 94,* 4499.
Wirtz, K. W. A., & Zilversmit, D. B. (1968) *J. Biol. Chem. 243,* 3596.
Woessner, D. E. (1962) *J. Chem. Phys. 36,* 1.
Yedgar, S., Hertz, R., & Gatt, S. (1974) *Chem. Phys. Lipids 13,* 404.

In Vitro Biosynthesis of Phospholipids by Chondrocytes and Matrix Vesicles of Epiphyseal Cartilage†

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ABSTRACT: Matrix vesicles are extracellular structures involved in endochondral calcification. They have a phospholipid composition distinct from that of chondrocytes from which they appear to be derived, but controversy exists concerning their origin. To elucidate the pathways involved in their formation, phospholipid biosynthesis by chondrocytes and matrix vesicles, either in tissue slices or as isolated fractions, was studied utilizing ^{14}C-labeled lipid precursors: acetate, palmitate, eicosatrienoate and L-serine. *Cartilage slices* incorporated L-serine into phospholipids of both chondrocytes and matrix vesicles; however, matrix vesicles were labeled more slowly than the cells. Serine was incorporated first into LPS (lyso form of phosphatidylserine) and then later into PS (phosphatidylserine), indicating a precursor–product relationship. This monoacyl base-exchange pathway may be unique to epiphyseal cartilage. *Isolated chondrocytes* actively incorporated all precursors into phospholipids, while *isolated matrix vesicles* did not. Chondrocytes incorporated significant amounts of L-serine into several nitrogenous phospholipids, whereas matrix vesicles incorporated only small amounts into PS and LPS. Since this was markedly inhibited by EDTA, it appears to have been by the non-energy-requiring base exchange. Chondrocytes incorporated significant amounts of acetate into all lipids, indicating a capacity for de novo fatty acid synthesis. Exogenous fatty acids, both saturated and unsaturated, were much more actively metabolized, but incorporation into specific phospholipids was selective. In particular, PS, diphosphatidylglycerol, and the lysophospholipids had significantly slower fatty acid turnover than the other phospholipids. Since isolated vesicles essentially lacked lipid synthetic capability, our findings indicate that matrix vesicles must be actively produced by chondrocytes. Although matrix vesicles were enriched in SPH, PS, and the lyso forms, none of these showed enhanced biosynthesis by either chondrocytes or matrix vesicles. This indicates that selective degradation of phospholipids and shedding of the modified membrane are involved in matrix vesicle formation.

Vesicles present in the extracellular matrix of epiphyseal cartilage have been shown to be associated with the initiation of endochondral calcification (Anderson, 1967, 1969; Bonucci, 1967, 1970; and others). They have been shown to be rich in alkaline phosphatase (Ali et al., 1970; Majeska & Wuthier, 1975) and phospholipids (Peress et al., 1974; Wuthier, 1975), both of which have been implicated in vesicle-mediated calcification (Fleisch et al., 1966; Eisenberg et al., 1970; Wuthier & Eanes, 1975). The phospholipid composition of matrix vesicles is distinctive, being enriched in PS,[1] SPH, and various lysophospholipids, and depleted in PC compared with chondrocytes (Wuthier, 1975). PS is known to have a strong affinity for Ca^{2+} (Nash & Tobias, 1964; Abramson et al., 1964; Cotmore et al., 1971; and others) and has been shown recently to be involved in phospholipid–calcium–phosphate complexes in matrix vesicles (Wuthier & Gore, 1977). Our recent studies have shown that matrix vesicle phospholipids are rapidly labeled in vivo with [^{32}P]orthophosphate (Wuthier et al., 1977). Curiously, however, most of the lipids specifically enriched in the vesicles (PS, SPH, LPC, and LPE) were not as rapidly labeled, relative to the cells, as PC which, in contrast, was depleted in the vesicles. We wished to further explore this paradoxical finding using a variety of other lipid precursors to elucidate the pathways of matrix vesicle phospholipid formation.

A further goal of this study was to unequivocally establish whether isolated matrix vesicles were capable of lipid biosyn-

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[1] Abbreviations used: ATP, adenosine 5′-triphosphate; CoA, coenzyme A; CTP, cytidine 5′-triphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; DPG, diphosphatidylglycerol; SPH, sphingomyelin; LPC, LPE, and LPS, lyso forms of PC, PE, and PS, respectively; Tes, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]taurine.

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