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PHYSICAL PROPERTIES OF ARABINOFURANOSYLCYTOSINE DIPHOSPHATE DIACYLGLYCEROL, AN ANTITUMOR LIPONUCLEOTIDE

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Dispersed from a dry film into buffer (5 mM phosphate, 0.15 M NaCl, pH 7.4), the liponucleotide 1-β-D-arabinofuranosylcytosine 5′-diphosphate L-1,2-diacylglycerol (ara-CDPdiacylglycerol) spontaneously forms vesicles which are several microns in diameter and probably unilamellar. Their average size immediately begins to decrease, and after 2 h none can be seen in the light microscope. During 1–2 days in unstirred solutions at 25°C, the vesicles are transformed to spherical or nearly spherical micelles having an apparent partial specific volume of 0.835 ml·g^{-1}, a maximum possible aggregation number of about 150, and an anhydrous radius of about 37 Å. The critical micelle concentration (CMC) is about 10 μM in buffer and 20 μM in distilled water, but micelle-monomer equilibration requires at least 1 week at a total concentration of 66 μM. This exceedingly slow equilibration is unique among reported detergents. The standard enthalpy and entropy of micellization are −13 kJ·mol^{-1} and 87 J·mol^{-1}·K^{-1}, respectively. These values are within the range reported for other detergents. Sonication accelerates the vesicle-micelle transformation to 30 min.

Introduction

The chemical synthesis and potential anticancer activity of a cytotoxic liponucleotide, ara-CDPdipalmitoylglycerol, were first reported by Turcotte and co-workers in 1977 [1,2]. Since that time, several reports of both synthesis and antitumor evaluation of molecules of this structural class have appeared [3–8]. Ara-CDPdiacylglycerol (1-β-arabinofuranosylcytosine 5′-diphosphate L-1,2-diacylglycerol), a multispecies liponucleotide containing the phosphatidyl group derived from egg lecithin, is more active than the clinically used parent drug, ara-Cyt(1-β-arabinofuranosylcytosine), against leukemia L5178Y and P388 ascites cells in mice. It is not a substrate for cytidine deaminase [6], which inactivates ara-Cyt, and is enzymatically converted to cytosine arabinoside 5′-phosphate in rat and human liver and in human brain [2].

A detailed study of the physical properties of ara-CDPdiacylglycerol is warranted, both in order to better understand its mode of biological action and to arrive at rational approaches to formulation for animal testing. As an amphiphile, in aqueous media the compound might be expected to produce supramolecular structures which could have major effects upon biochemical and biological properties. Effects of physical form upon function are well known to occur when liposomes are used to encapsulate pharmaceutical agents [9] or when detergent-like molecules such as dioctanoylphosphatidylcholine [10] and fatty acyl-CoA's [11] are used as enzyme substrates.

Ara-CDPdiacylglycerol is a 3′-epimer of CDPdiacylglycerol, a phosphatidyl donor in normal acidic glycerophospholipid biosynthesis, which has been used as a substrate for studies of

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membrane-bound phosphatidyl transferase in cell-free systems. The physical properties of CDP-diacylglycerol have not been well characterized [12], but recent studies showing that the physical form of this liponucleotide can determine the class of phospholipids synthesized [13] emphasize an interdependence of function and physical form. In view of the near-identity of the two liponucleotides, one might also expect their physical properties to be quite similar. Preliminary studies carried out in this laboratory have in fact demonstrated their similarity. An additional rationale for the work reported here is then the insight it offers into the behavior of the conventional liponucleotide substrate.

Materials and Methods

Ara-CDPdiacylglycerol diammonium salt was synthesized from egg lecithin by published methods [5] and purified by high-pressure liquid chromatography. Thin-layer chromatography with heavy loading on silica gel using chloroform/methanol/water/glacial acetic acid (65:25:4:2, v/v) showed a single spot by charring [14]. Based upon the detection limit determined with known quantities of standards, the purity was >99%. A typical composition of the liponucleotide fatty acid methyl esters, prepared with BF_3-methanol and analyzed by gas chromatography, was 16:0 30.2, 16:1 3.5, 18:0 10.7, 18:1 34.8, 18:2 18.3, 20:4 2.5 percent.

Unless otherwise stated, the buffer solution used throughout this work was 5 mM phosphate (pH 7.4), 0.15 M NaCl, and 0.02% NaN_3. Liponucleotide suspensions were prepared according to the procedure of Papahadjopoulos and Watkins [15]. Sonicated samples were prepared with a Branson Sonifier Cell Disruptor (Model W-185) at 32 W output (calorimetric calibration); titanium particles from the probe were removed by centrifugation at $12000 \times g$ for 10 min. Light microscopy was carried out with a Leitz MPV2 microscope equipped with polarized phase contrast optics; the sample cavity was 1 mm deep, with the microscope focussed at half-depth to avoid effects at the glass interface.

Sepharose and Sephadex were packed in jacketed glass Pharmacia columns thermostatically controlled by circulating water. Samples were injected through three-way valves and eluted with a peristaltic pump. Protein standards were purchased from Pharmacia. Apparent critical micelle concentrations were measured by the method of tail analysis, in which the entire column is uniformly loaded with liponucleotide then eluted with buffer [16,17,18]. Elution patterns were monitored continuously with a flow cell at 254 nm, but concentrations were measured fraction-by-fraction at 271 nm using $\epsilon = 9100\ M^{-1} \cdot cm^{-1}$ [19]. Production of water-soluble hydrolysis products was determined by adjusting the pH to 2.0 in ice with HCl, then extracting with chloroform until the absorbance at 271 nm in the aqueous phase was constant (three or more extractions). The final absorbance in the aqueous phase ($\epsilon = 13400\ M^{-1} \cdot cm^{-1}$ at 280 nm)was taken as the measure of hydrolysis. Controls show no measurable hydrolysis during extraction.

Results

When first dispersed in buffer, ara-CDPdiacylglycerol produces quite turbid suspensions which gradually become less turbid as time passes. The first stage of the structural changes were therefore monitored as the change of absorbance at 350 nm (Fig. 1). During the first few minutes at room temperature, the suspensions becomes opalescent. At this stage vesicles of various sizes, some as large as 20–30 μm in diameter, can be seen in the light microscope (Fig. 2). When a drop of sucrose is introduced, they shrink immediately to dense structures whose shape is below the resolving power of the microscope. The spherical shape, osmotic response, and low contrast of the vesicles suggest that they may be unilamellar.

The vesicles are unstable, and in an unstirred suspension their number and average size continuously decrease, until after about 2–3 h at room temperature they are no longer visible in the light microscope. However, even after this point particles large enough to scatter light at 350 nm remain, and the absorbance continues to change very slowly. Thus, the absorbance changes seen in Fig. 1 monitor the population of the largest vesicles. At 37°C, the visible vesicles vanish slightly more rapidly than at room temperature (data not shown),

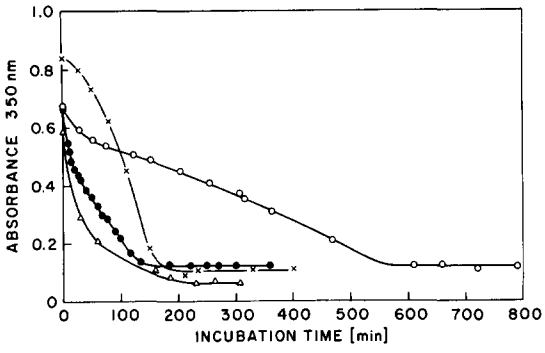

Fig. 1. Turbidity change at 350 nm of ara-CDPdiacylglycerol suspensions (2 mg/ml) dispersed in 5 mM Tris-HCl (pH = 7.4), 0.15 M NaCl at 4°C (○——○) and 22°C (●——●). ×——×, the sample was dispersed in 5 mM phosphate, 0.15 M NaCl buffered at pH 7.4. △——△, a sample was dispersed under the same conditions as (×——×) except at a higher concentration (15 mg/ml). The cuvette used was 1 cm in length except for (△——△) in which a 1-mm cuvette was used.

but the process is retarded considerably in the cold. The time required is little affected by concentration or by the buffer used (Tris or phosphate).

Gel permeation chromatography on Sepharose CL-4B reveals that structural changes continue long after the obvious turbidity changes cease. Fig. 3 shows the results of a series of Sepharose runs in which ara-CDPdiacylglycerol was incubated in phosphate-buffered saline (pH 7.4) and run through the column at the indicated times. Although major turbidity changes had ceased by 3 h (Fig. 1), after 5 h most of the particles are still too large to enter the gel and are eluted at the void volume (exclusion limit $20 \cdot 10^6$ daltons). A small tail, possibly consisting of a continuum of particle sizes, follows the void peak. The peak at fraction 53 on this and subsequent runs emerges coincident

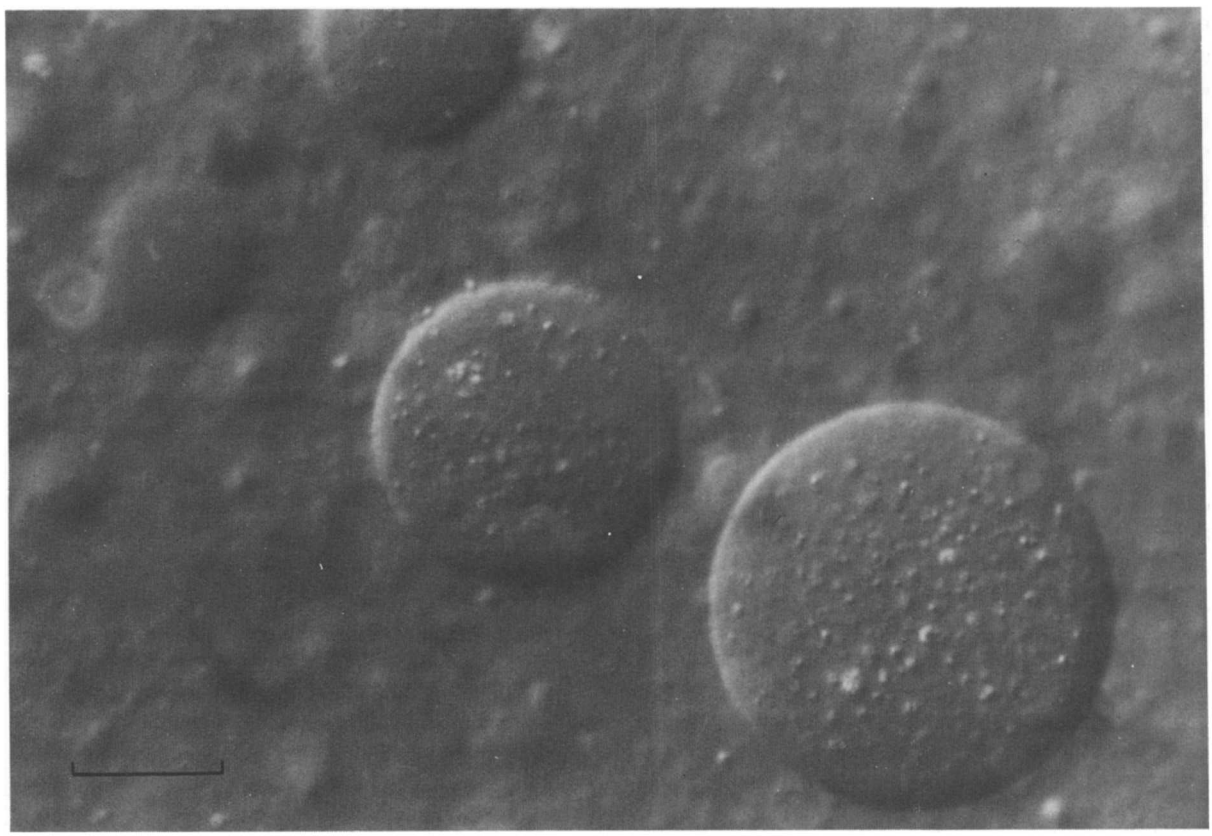

Fig. 2. Light micrograph of ara-CDPdiacylglycerol vesicles 20 min after dispersal of the dry liponucleotide in buffer. The bar indicates 10 μm. Background particles also appear as round vesicles if properly focussed.

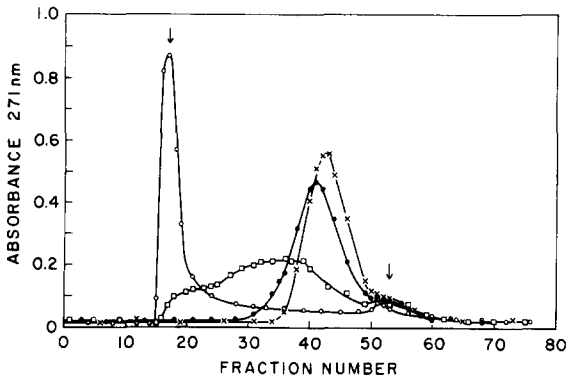

Fig. 3. Sepharose CL-4B elution profiles of ara-CDPdiacylglycerol suspensions incubated at 22°C for 5 h (○——○); 11 h (□——□); 24 h (●——●) and 48 h (×——×). The arrows indicate V_0 and $V_0 + V_i$. Column size 1.6×28 cm, flow rate 26 ml/h, sample size 1 ml of 0.1% liponucleotide suspensions. The small peak at the column volume for this and the following two figures are free liponucleotide molecules and ultraviolet absorbing contaminants.

with sodium dichromate and consists of free liponucleotide molecules and ultraviolet absorbing contaminants. After 11 h, most of the material at the void volume has vanished and is replaced by a broad distribution of particle sizes. Two major regions of the elution profile can be discerned. The smaller one at the void volume apparently consists of particles still large enough to be excluded. The larger one, between fractions 25 and 45, emerges at approximately the same position as sonicated unilamellar vesicles of egg lecithin, which have a diameter of about 250 Å. After 24 h no material remains at the void volume and the average size has further decreased but changes are still taking place. At 48 h a stable symmetrical peak occurs at fraction 42, which is about 10 fractions later than the elution volume of small sonicated unilamellar egg lecithin vesicles. No further change is observed for longer incubation time. It is important to point out that these slow transformations take place without stirring or other mechanical agitation.

Since sonication has been shown to clear liponucleotide suspensions rapidly [4], it would be expected to accelerate the changes seen by chromatography on Sepharose. The results of various regimens of sonication followed by incubation at room temperature are shown in Fig. 4. The elution pattern which requires more than 24 h to reach in an unstirred system is rapidly obtained by sonication for 3 min and incubation for 7.5 h (Fig. 4A); the same pattern is reached by a shorter incubation time of 3.5 h if sonication was carried out for 6 min (Fig. 4B). Prolonged sonication for 30 min eliminates all of the large particles, and the final stable pattern is obtained without further incubation.

The stable form attained in Sepharose CL-4B chromatography, (i.e., more than a day of incubation in an unstirred system or 3 min of sonication

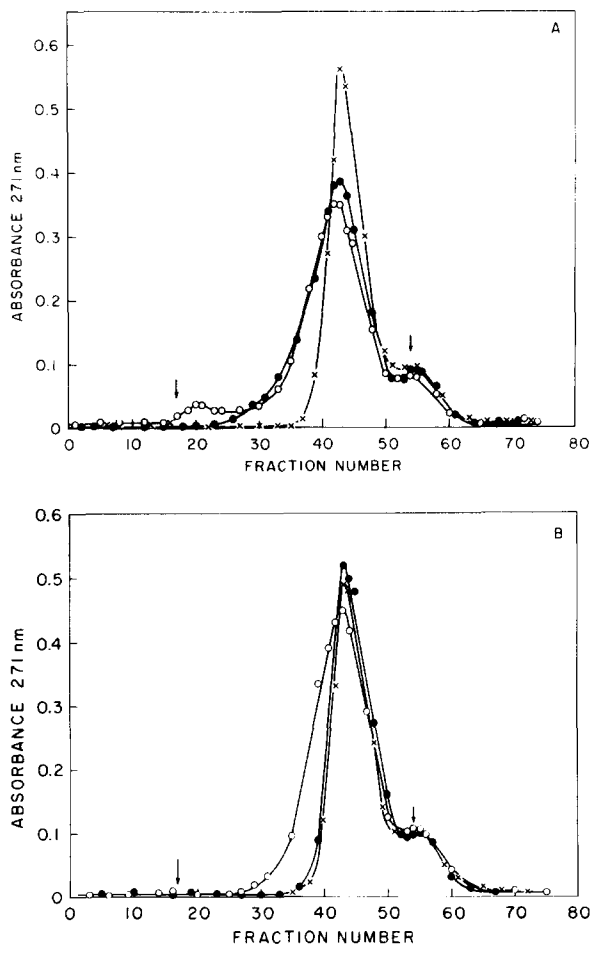

Fig. 4. Sepharose CL-4B elution profiles of sonicated ara-CDPdiacylglycerol suspensions incubated at 22°C. (A) Sample was sonicated for 3 min and incubated for 0 h (○——○); 3.5 h (●——●) and 7.5 h (×——×). (B) Sample was sonicated for 6 min and incubated for 0 h (○——○); 3.5 h (●——●) and 7.5 h (×——×). The arrows indicate V_0 and $V_0 + V_i$. Column size 1.6×28 cm, flow rate 26 ml/h, sample size 1 ml of 0.1% sonicated liponucleotide suspensions.

followed by 7.5 h of incubation) elutes very close to the total volume of the column. Sepharose CL-4B is apparently not adequate to resolve any further structural changes. Sephadex G-150, which has a smaller pore size (exclusion limit $3 \cdot 10^5$ daltons), was therefore selected to continue the studies. Sonication for 3 min and incubation for 7.5 h provides a stable structure in Sepharose CL-4B (Fig. 4A), but most particles are still eluted from Sephadex G-150 in the void volume (Fig. 5). After 23.5 h incubation, the void volume peak has nearly vanished, and a new and apparently stable peak centered at fraction 36 appears. A shoulder of larger particles remains near fraction 29. After 2 days the transformation is completed, and no further changes are observed. The final peak thus appears to consist of the ultimate, thermodynamically stable particles.

If the final stable particles are assumed to be spherical, their anhydrous mass and radius can be estimated by chromatography on Sephadex providing the degree of hydration and partial specific volume are known. Since ara-CDPdiacylglycerol is a hybrid mölecule of lipid and nucleotide, it seems likely that its hydration lies between that of a phospholipid and a polynucleotide; it was taken to be 0.35. The partial specific volume was measured by differential scanning dilatometry [20] of 90.24 mg of the material at a concentration of 11.47 $mg \cdot ml^{-1}$ in distilled water, after incubation for 48 h at room temperature. From 0 to 35°C, an excellent fit is given by $\bar{v} = 0.817 + 4.81 \cdot 10^{-4}\ T + 1.01 \cdot 10^{-5}\ T^2$ $(ml \cdot g^{-1})$; at 25°C, $\bar{v} = 0.835$ $ml \cdot g^{-1}$. Chromatography on Sephadex G-200 (standards: chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase, catalase, ferritin) gives a Stokes radius of 41 Å. If the particle is assumed to be spherical its anhydrous radius would be 37 Å and its anhydrous mass 150000 daltons. Its aggregation number would be about 150. This apparent radius and mass are much smaller than the radius and mass of small unilamellar liposomes, which have a minimum diameter of about 250 Å [21] and are eluted in the void volume from Sephadex G-150. The ultimate particles are apparently micelles rather than vesicles. Since the hypothetical anhydrous radius is reasonably near

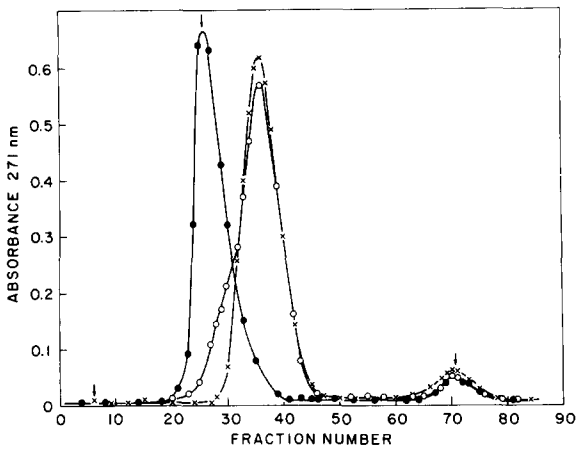

Fig. 5. Sephadex G-150 elution profiles of ara-CDPdiacylglycerol suspensions sonicated for 3 min and incubated at 22°C for 7.5 h (●———●); 23.5 h (○———○) and 2 days (×———×). The arrows indicate the V_0 and $V_0 + V_i$. Column size 1.6×36 cm, flow rate 17 ml/h, sample size 1 ml of 0.1% liponucleotide suspensions.

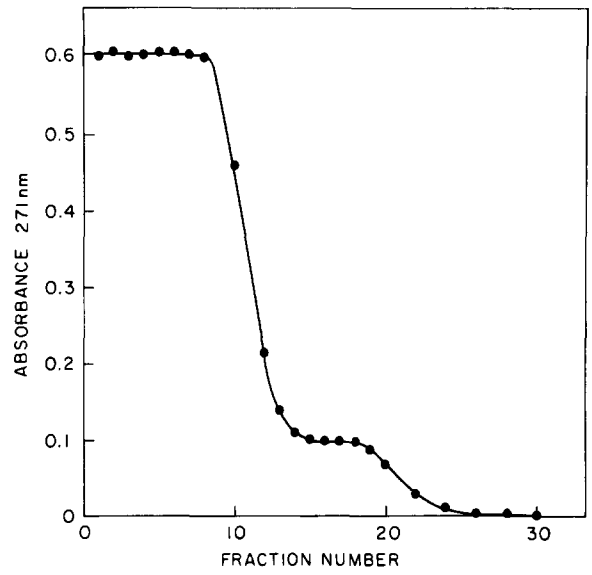

Fig. 6. Elusion profile of a typical tail analysis. About 5 ml of 0.1% ara-CDPdiacylglycerol suspension was sonicated for 3 min and incubated at 22°C for 3 days to ensure the formation of stable micelles. The suspension was then chromatographed on the Sephadex G-200 column to remove free molecules and water soluble ultraviolet light absorbing impurities. The micelle fractions were collected, pooled, and diluted to a total volume of 55 ml. The sample solution was then incubated at 22°C for several days to reach the micelle-monomer equilibrium. A small Sephadex G-150 column (1.0×30 cm) was equilibrated with the incubated sample solution and eluted with buffer. Flow rate 15 ml/h.

that expected for an extended liponucleotide molecule, the real micelle may in fact be spherical. If it is not, the estimated molecular mass of 150000 daltons is an upper limit. The size estimated from gel permeation is in rough agreement with the available sedimentation data. For a sphere of 41 Å hydrated radius and 150000 daltons anhydrous mass, the sedimentation coefficient calculated using the viscosity and density of water at 25°C is 6.0 svedbergs (S); the measured value for ara-CDPdipalmitoylglycerol sonicated for 1 h is reported to be about 4 S in water at 25°C. Although comparison of the calculated and measured values suggests asymmetry, the available data does not justify a detailed analysis.

Critical micelle concentration and kinetics of micellization

Clearly, the production of the final micelles from the initial dispersion procedes very slowly. Such behavior is unusual, since in most other detergents, such as sodium dodecyl sulfate, the process seems to occur nearly as rapidly as the dry material can dissolve. The time required to establish the equilibrium between micelles and molecules in solution at the critical micelle concentration is a separate consideration. For other detergents this process is also nearly instantaneous [22], but, as the data to be presented will show, for ara-CDPdiacylglycerol it can require days. Apparent critical micelle concentrations were measured as a function of time by tail analyses [16] on Sephadex G-150. First, a dry sample is dispersed in buffer, sonicated for several minutes, and incubated for several days at room temperature to form the ultimate micelles. The sample is then passed through a Sephadex G-200 column to remove the impurities and free molecules seen in the peak at fraction 70 of Fig. 5. The monomer-free micelles are collected in a volume of buffer sufficient to give the desired final concentration. After incubation for the requisite time, the sample is loaded uniformly on the entire tail-analysis column then eluted with buffer. A typical elution pattern is shown in Fig. 6, where the higher plateau (fractions 0–10) is the total concentration of liponucleotide and the lower plateau (fractions 15–18) is the apparent critical micelle concentration.

A sequence of tail analyses were carried out at

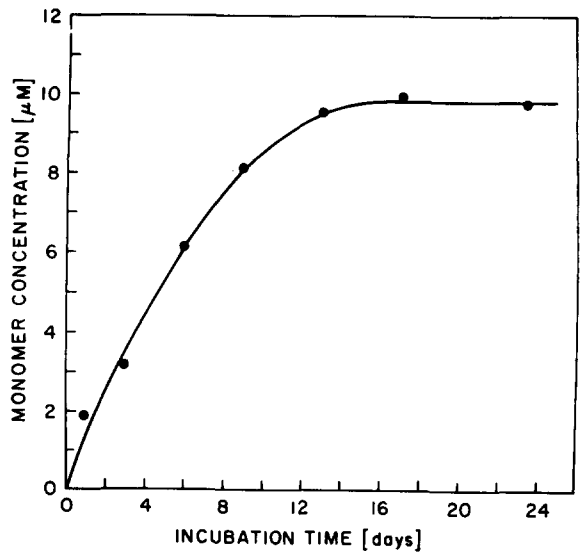

Fig. 7. Kinetics of the micelle-monomer equilibration in buffer at 25°C. Monomer concentrations were determined by tail analysis at the indicated incubation times. The critical micelle concentration is the concentration of the plateau. No hydrolysis was detectable after 17 days incubation, and only minor hydrolysis was found after 3 weeks incubation.

25°C at various incubation times; the concentration of liponucleotide in the lower plateau is plotted as a function of incubation time in Fig. 7. It slowly increases and becomes constant after about 14 days. This constant concentration, about 10 μM, is the equilibrium critical micelle concentration at 25°C. The curve shown in Fig. 7 was obtained at a total concentration of 66 μM, about seven times the critical micelle concentration. A detailed investigation of the effect of concentration upon the velocity of the micelle-molecule equilibration has not been made, but preliminary results suggest that the time required to reach the equilibrium critical micelle concentration is roughly inversely proportional to total concentration. About 1 day might be required for a 1 mM unstirred solution.

Very slow free molecule-micelle equilibration is unprecendented among detergents, and a chemical process rather than a physical change might be responsible. Chemical analysis shows that this is not so. A pooled sample of free molecules from the lower plateau of several tail analyses was acidified with HCl and extracted with chloroform in ice; the chloroform phase was chromatographed by TLC. Detection by charring with $Na_2Cr_2O_7$/

H_2SO_4 showed only a single intense spot of liponucleotide. Hydrolysis was also monitored by measuring the absorbance of the extracted aqueous phase as described in Materials and Methods; none was detected after 17 days of incubation at room temperature, and very minor amounts after 3 weeks. Other evidence for slow free molecule-micelle equilibration comes from the trailing plateau regions in gel chromatography, such as fractions 50–60 in Fig. 5. For rapidly-equilibrating detergents such as SDS, the plateau concentration is independent of flow rate, and is a measure of the critical micelle concentration [23]. For ara-CDPdiacylglycerol, the plateau concentration is a function of both flow rate and total concentration, becoming greater at lower flow rate and higher concentrations. Furthermore, if the columns are eluted with buffer containing free molecules at concentrations below the critical micelle concentration, the apparent critical micelle concentrations in the plateau region are additive. This effect mandates the use of tail analysis for determinations of the critical micelle concentration.

Rigorously speaking, the method of tail analysis does not measure the critical micelle concentration. It measures the concentration of free molecules in the presence of micelles, but providing the free molecule concentration remains nearly constant with changing total concentration at equilibrium above the critical micelle concentration, it can be taken as the critical micelle concentration [18]. This constancy is a well-known property of detergents [22], and the data in Table I indicates that it also holds for ara-CDPdiacylglycerol. Tail analysis performed at total concentrations in the neighborhood of the critical micelle concentration shows that both the equilibrium free molecule concentration and the micelle size become more strongly concentration dependent, and begin to decrease at total concentrations less than about 20 μM. This transition zone extends down to about 8 μM, where micelles can no longer be disconcerned.

At very high concentration ($\sim 1\%$) a rather abrupt change in viscosity occurs which can be easily detected by visual inspection of the rate of bubble rise in shaken preparations. The solution becomes extremely difficult to filter under pressure through an 0.22 μm Millipore. Second associations are common for other detergents at concentrations well above the critical micelle concentration, and have been observed with dipalmitoyllysophosphatidylcholine [24].

Salt and temperature effects

The critical micelle concentration of ionic detergents generally decreases with increasing ionic strength of the solvent [22]. To estimate the effect of ionic strength upon the liponucleotide, the critical micelle concentration was measured by tail

TABLE I

CRITICAL MICELLE CONCENTRATION (CMC) OR ARA-CDP-DIACYLGLYCEROL

CMC was determined by tail analysis at various total concentrations at 25°C.

Total concentration (μM)	CMC (μM)
35.2	9.9
39.5	11.0
44.0	10.7
60.1	10.0
64.8	10.7
74.7	10.0
87.0	10.2
91.7	9.7

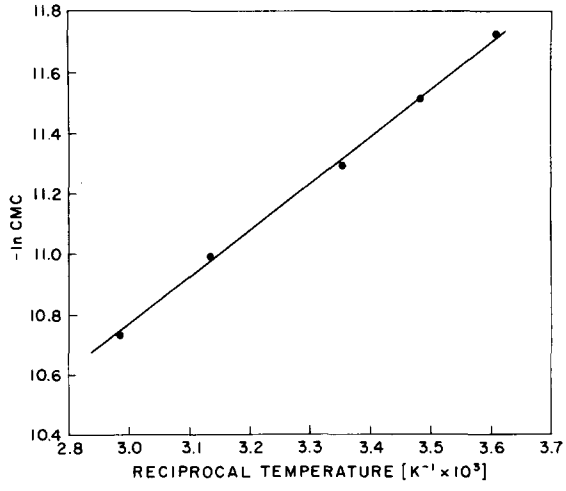

Fig. 8. Temperature effect on the critical micelle concentration (CMC). The critical micelle concentration was determined by tail analysis at temperatures of 4–62°C.

analysis of a solution in deionized distilled water adjusted to pH 7.4 with NaOH and maintained under nitrogen to eliminate absorption of atmospheric carbon dioxide. At 25°C and a total concentration of 70 μM, the critical micelle concentration is near 20 μM. This higher critical micelle concentration reflects the effect of repulsive forces between the polar head groups in the near-absence of salt.

If deviations from ideality and counterion binding are ignored [25,26], the apparent enthalpy of micellization is given by

$$\Delta H_m = -RT^2\left(\frac{\partial \ln \mathrm{CMC}}{\partial T}\right)_P$$

A plot of ln CMC vs. $1/T$, where the critical micelle concentration was measured by tail analysis at various temperatures, is presented in Fig. 8. No hydrolysis was observed at any temperature. Although the critical micelle concentration of most detergents in aqueous media undergoes a minimum in the neighborhood of 25°C [27], within the limits of experimental error the liponucleotide produces a linear plot. The standard enthalpy of micellization, obtained from the slope of the plot, is -13 kJ $\cdot$ mol^{-1} liponucleotide molecules. If the critical micelle concentration is taken as 10 μM, the standard entropy and free energy of micellization are 84 J $\cdot$ mol^{-1} $\cdot$ K^{-1} and -38 kJ $\cdot$ mol^{-1}. These thermodynamic parameters fall within the range measured for other detergents [22,28].

Although measurements of the critical micelle concentration by tail analysis are not invalidated by adsorption providing the detergent remains on the column long enough to reach equilibrium, several lines of evidence indicate that no significant adsorption of the liponucleotides to the gel matrix occurs during chromatography on either Sephadex or Sepharose. First, in runs such as those shown in Figs. 3 and 4 on Sepharose and Fig. 5 on Sephadex, and in runs on Sephadex to prepare pure micelles for tail analysis, free liponucleotide molecules emerge where dichromate emerges. The baseline drops to zero thereafter. Second, where the Stokes radius is determined on Sephadex the micelle peak emerges at precisely the same position whether the column is eluted with buffer or pre-loaded and eluted with buffer containing liponucleotide at concentrations above the critical micelle concentration [23,29]. Third, the same apparent critical micelle concentrations are obtained from tail analysis whether the system is equilibrated on the column or loaded onto the column after exterior equilibration. For the slowly-equilibrating liponucleotide, adsorption of monomers would lower the measured apparent critical micelle concentration. The method of tail analysis was also verified by determining the critical micelle concentration of sodium dodecyl sulfate by both tail analysis and conductivity. Both methods gave 9.0 mM, in agreement with the reported value of 8.1 mM [27].

Discussion

The turbidity measurements, phase contrast microscopy, and gel permeation chromatography clearly indicate that ara-CDPdiacylglycerol undergoes a slow time-dependent structural transformation from vesicles to stable micelles. The liponucleotide thus appears to exhibit some properties of both phospholipids and detergents. The large, possibly unilamellar, osmotically-responsive vesicles initially produced in buffer resemble those produced by the more familiar phosphoacylglycerols such as phosphatidyl choline. The final thermodynamically stable micelles are spherical or nearly spherical and have a maximum possible aggregation number of about 150. They emerge as a narrow, symmetrical peak from Sephadex, and hence are either homogeneous in size or are distributed over a narrow size range. Except for unusually slow micelle-free molecule equilibration, the micelles resemble those produced by common detergents such as sodium dodecyl sulfate. Ordinarily both vesicles and micelles are not formed by a single species, but in this case the unusually large polar moiety may account for both the instability of the initial bilayers and the preference for micelles. The means of transformation from the initial to the final state of dispersion remains unknown, and any suggested mechanism must be quite speculative. Certainly an event which begins early is an attrition in size of the vesicles. Smaller vesicles might be produced from larger by reverse fusion but such a process would be thermodynamically unfeasible unless local patches were

formed having lowered surface tension lasting long enough to allow budding. The small particles seen associated with the large vesicles in Fig. 2 might indicate such a process, although they may also simply be particles which are adsorbed from elsewhere in the suspension. This attrition may be the beginning of a gradual process which terminates at the ultimate micelles. However, vesicles need not necessarily be direct structural antecedents to micelles. Production of micelles may be a parallel process, where slowly dissolving vesicles release free molecules which assemble into micelles once the critical micelle concentration is reached. Sonication greatly accelerates the production of micelles, but the mechanisms by which sonication does so is even more obscure than in the case of unstirred suspensions.

The critical micelle concentration of ara-CDP-diacylglycerol, about 10 μM, is considerably higher than that of phosphatidylcholine [22]. The difference can be rationalized in terms of the structure of the two compounds. Phosphatidylcholine is zwitterionic with no net charge, and has comparatively small polar end. The liponucleotide, with its net negative charge and bulky hydrophilic end, is more soluble in water. One should bear in mind that, expressed as a single value, the critical micelle concentration of any detergent is in a sense a fiction. Measured by most methods, including tail analysis, it characterizes the approximate midpoint of a concentration range extending from the first appearance of small micelles to the point where further addition of detergent no longer appreciably changes the concentration of free molecules in solution [22]. For ara-CDPdiacylglycerol this range is about 8–12 μM.

The most striking property of the compound is the long time required for forming the final thermodynamically stable micelles from the initial suspension, and for establishing the micelle-molecule equilibrium. At a concentration of 100 μM, after removal of free molecules from micelles at least a week is required to re-attain the equilibrium. The reason for the slow kinetics is unknown. Steric factors may play a role, but the presence of a nucleotide residue apparently is not an adequate explanation since palmitoyl-CoA, a liponucleotide with a single hydrocarbon chain and a critical micelle concentration close to that of ara-CDP-diacylglycerol [30] is not reported to exhibit slow equilibration. Nor is the presence of two hydrocarbon chains sufficient; several double-chain detergents have been examined [22,31], and apparently behave in the usual way. However, palmitoyllysolecithin, which has a critical micelle concentration in the neighborhood of 7 μM and forms micelles with a Stokes radius of 34 Å [22,24], is reported to require several hours to achieve free molecule-micelle equilibration [32]. The determining factor may therefore be the glycerol phosphate group, a structural feature which ara-CDPdiacylglycerol shares with other phospholipids. Slow equilibration of phospholipid bilayers with molecules in solution is in fact an essential requirement for liposomes and biomembranes, in order to prevent both structural breakdown and rapid equilibration of lipid classes between the individual membranes of living cells. From this point of view, for phospholipids slow kinetics is a more fundamental requirement than low critical micelle concentration if membrane integrity is to be maintained. Existing measurements of the CMC of phospholipids do not usually consider a kinetic effect.

As a detergent ara-CDPdiacylglycerol offers an exceptional system for studying the kinetics of micellization. For common detergents exchange is examined by NMR and dissolution of micelles by temperature jump or stop flow [33]. The experiments are difficult to interpret, and the time scale severely restricts the information available. For this detergent, the same phenomena can be monitored directly and more precisely by radio-tracers, chromatography, and other techniques heretofore reserves for equilibrium studies.

Studies from this laboratory show that CDP-diacylglycerol, a metabolic intermediate in phospholipid biosynthesis, exhibits essentially the same structural transformations and slow kinetics shown by the arabinose-containing analog. Hence the studies reported here have relevance for kinetic studies of the membrane-bound phosphatidyl transfereases in cell-free systems, where misleading results might be otained if the slow kinetics and polymorphic forms encountered in the liponucleotides are not taken into account. Perhaps complex kinetic effects due to the substrate may be abated or entirely eliminated by the common practise of

presenting the liponucleotide to the enzyme as mixed micelles with nonionic detergents [12], but caution is particularly warranted when foreign detergent is not included in the system.

Preliminary evaluations of the in vivo activity of ara-CDPdiacylglycerol and ara-CDPdipalmitoylglycerol [1,3,4,6,8] against experimental tumors in mice show potential. The drug biodistribution, tumor cell uptake, tumoricidal activity, mode of action at the cellular level, and biotransformation may be closely related to the physical properties of the molecules in solution. The activity of the drug may also depend strongly upon the physical form in which it is administered, whether as liposomes of pure drug, or as micelles, or as liposomes mixed with phospholipids such as phosphatidylcholine. The information obtained in this study therefore becomes an important prerequisite for further understanding and development of this new type of cytotoxic agent, and also defines a protocol which affords uniform and reproducible preparation for animal testing.

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