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Preparation and characterization of long chain amino acid and peptide vesicle membranes

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Four amino acid dicarboxylic amphiphiles which contain cysteine or homocysteine were synthesized. Each forms synthetic bilayer membranes upon hydration. Extensive sonication above the lipid phase transition temperature, 61 to 82°C, produced 1000 Å diameter vesicles. Treatment of the vesicles with water-soluble carbodiimides during and after sonication induced oligopeptide formation at the vesicle surface with retention of vesicle size and shape. Size exclusion chromatography indicates the products are predominantly di- to decapeptides. The permeability characteristics of the amino acid and peptide vesicles to [^{3}H]glucose and 6-carboxyfluorescein are reported. The amino acid vesicles are among the least permeable nonpolymerized bilayer vesicles described in the literature to date. Formation of the peptide vesicles increases the membrane permeability, whereas in other polymerizable lipid vesicles the permeability decreases upon polymerization. The amino acid vesicles can be immobilized on Sephadex beads by reaction with carbodiimide. The impermeability, biodegradability, and ease of immobilization make this class of vesicles attractive materials for the encapsulation of reagents.

Introduction

In 1977, Kunitake and Okahata [1] first demonstrated that the ability to form vesicles or liposomes is not restricted to naturally occurring phospholipids; it is a common property of various amphiphiles. Since then, the synthesis of new amphiphilic compounds and the study of their behavior in membrane models such as vesicles (liposomes), black lipid membranes, and monolayers has become a field of growing interest. An increasing number of bilayer forming compounds, either polymerizable or nonpolymerizable, some of them with structures not found in biological membranes, have been realized. Their properties and potential applications have already been reviewed [2–5].

Although amino acids have been applied in the synthesis of various lipids, most of them have been used as multifunctional backbones for the attachment of both the hydrophobic chains and the head group [6–8]. Less attention has been paid to the synthesis of amphiphilic amino acids where the amino acid itself forms the head group. Marr-Leisy et al. [9] synthesized amphiphilic tyrosine derivatives and investigated chiral discrimination in monolayers at the air/water interface. Based on monolayer experiments from Katchalsky et al.

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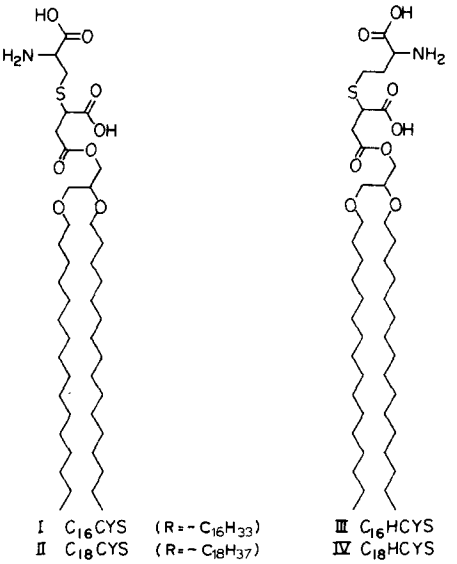
[10] and Fukuda et al. [11], the first polyamino acid formation in liposomes was reported by Folda et al. [12]. They took advantage of the aminolysis of amino acid esters; however, their resulting peptides were too hydrophobic and the peptide liposomes precipitated (Fig. 1). This problem was overcome by Neumann and Ringsdorf [13], who described the synthesis of amino dicarboxilic acids. These materials form vesicles upon hydration and may be condensed with a water-soluble carbodiimide to yield a colloidal suspension of peptide vesicles. The difference in behavior is due to the additional polar group as illustrated in Fig. 1.

We have now characterized the permeability of vesicles of amphiphilic amino acids, as well as peptide vesicles formed by condensation of the monomeric vesicles. The amphiphilic amino acids shown below are isoionic to phosphatidylserine. These aminodicarboxylic acids have either L-cysteine (I, II) or D,L-homocysteine (III, IV) as head groups.



Amphiphilic Amino Acids

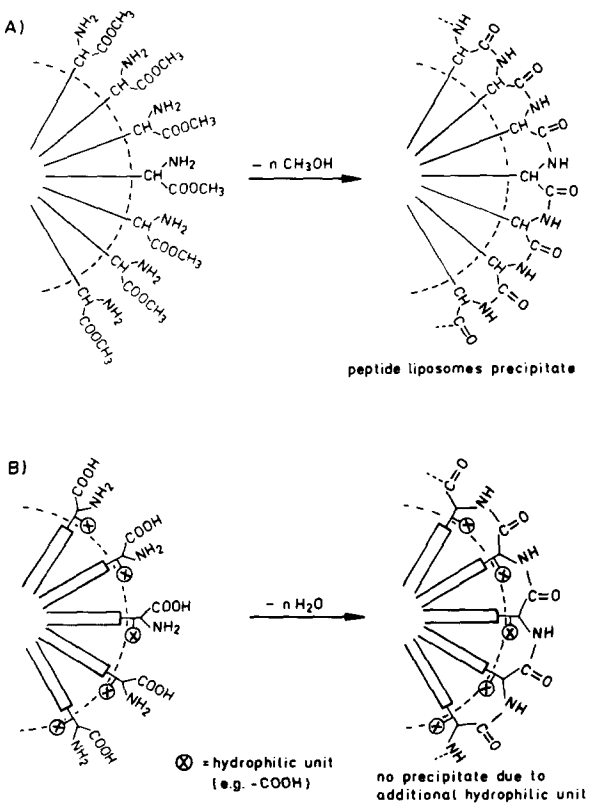


Fig. 1. Routes to peptide vesicle memrbanes: (A) aminolysis of amino acid esters; (B) condensation of amino dicarboxylic acids.

Experimental

Materials

6-Carboxyfluorescein was obtained from Kodak Laboratory Chemicals (Rochester, NY) and recrystallized from acetone. [^{3}H]Glucose from New England Nuclear (Boston, MA) had an activity of 30 Ci/mmol. 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate was obtained from Merck-Schuchardt (F.R.G.).

Amphiphilic amino acids

The 1,2-bisalkylglycerol ethers, which form the hydrophobic portion of the molecules, were prepared according to procedures found in the literature [14–16]. The reaction with maleic anhydride in the presence of triethylamine gave the monoesters. In the final step, the nucleophilic attack of the cysteine or homocysteine thiol group on the activated double bond of the maleic monoester (Michael addition) was used to prepare the amphiphilic amino acids (I–IV), previously described in detail [13].

Vesicle preparation

The lipids, with or without carbodiimide (mole ratio 0.55), were weighed into polycarbonate screw-cap vials and buffer was added. The mixture was sonicated in a Heat Systems cuphorn sonifier at 70°C for 15 min to 2 h. The lipid concentration was 2 mg/ml. The vesicles sonicated in the presence of carbodiimide were allowed to stand at room temperature for 16 to 24 h to ensure complete reaction.

Size-exclusion chromatography (SEC) and Fourier transform infrared (FT-IR) measurements

The vesicles were destroyed by addition of 1 M HCl, and the precipitate pelleted by centrifugation. The residue was washed several times in water by suspension and centrifugation, then dried under vacuum. The peptides were dissolved in tetrahydrofuran and chromatographed on high performance Ultrastyragel (Waters Associates) columns with porosities of 1000, 500, 100 Å. The columns were calibrated with monodisperse polystyrene standards and the results are given in equivalents of polystyrene. A part of each sample was transferred into a KBr pellet and the infrared spectra were recorded using a Nicolet 60 SX Fourier transform infrared spectrophotometer.

Carboxyfluorescein permeability

The vesicles were prepared by sonication of the lipids (2 mg/ml) in 50 mM 6-carboxyfluorescein, 50 mM Tris-HCl buffer (pH 8.3) for 15 min at 70°C. After cooling, the vesicle suspension (2 ml) was separated from the free dye on a Sephadex G-50 M column (prepacked pD 10 columns, Pharmacia). The elution buffer was a 50 mM Tris-HCl buffer of the same pH containing 50 mM Na_3PO_4. Fraction 4 (1 ml fractions), which contained most of the vesicle entrapped dye, was used for the permeability measurements. Usually an aliquot of 0.2 ml was diluted with 2 ml of buffer in a quartz cuvette, and increase of the fluorescence maximum at 517 nm (excitation 493 nm) with time at 24°C was determined with a SPEX fluorimeter. A 6-carboxyfluorescein solution served as a standard for correction of the measured intensities. In the case of the peptide vesicles, a similar procedure was used except that the 6-carboxyfluorescein solution contained 2 mg/ml of carbodiimide. The vesicles were applied to the column for chromatography either directly after cooling or after 16 h reaction. The entrapped dye 100% value was obtained by adding 0.2 ml 5% Triton X-100 surfactant solution to the vesicles in the cuvette to release all the entrapped dye.

The percentage of the released 6-carboxyfluorescein was calculated according to

$$I(\%) = \frac{I(t) - I_0}{I_\infty - I_0}$$

where I_∞ is the 100% fluorescence intensity determined by Triton X-100 surfactant treatment, and I_0 and $I(t)$ are the fluorescence intensities of the sample at $t = 0$ and t, respectively.

[^{3}H]Glucose in vesicles

The procedure used here is similar to that reported by Dorn et al. [17]. A 250 μl aliquot of the [^{3}H]glucose in ethanol/water was taken to dryness with N_2 in a test tube, and 10 mg of the lipid and 1 ml of the Tris-HCl buffer described above was added. The mixture was sonicated for 15 min at 70°C. First, in order to evaluate the peptide vesicles, the lipids and the carbodiimide were sonicated for 1 h and allowed to react overnight. These peptide vesicles were combined with the [^{3}H]glucose and sonicated again for 15 min to speed diffusion of the glucose through the membranes. The lipid concentration was 5 mg/ml and the molar ratio of carbodiimide to lipid was approx. 1.8.

Gel permeation chromatography (GPC)

The vesicle solution (1 ml) was chromatographed on Sephadex G-25 M prepacked columns (1 ml fractions). The vesicle fraction with the entrapped glucose appeared after 3 ml, the free glucose after 7 ml. The activity found in fraction 4 for glucose in the absence of vesicles or in the presence of empty vesicles was < 5–7% of the activity of vesicle samples with entrapped glucose. Gel permeation chromatography of the vesicles with Sephadex G-50 M or Bio-Gel P6 DG columns resulted in some vesicle adherence to the column. The recovery was 60 to 70% of the total activity applied to the column. The residual activity could only be removed with a detergent (Triton X-100 surfactant).

Scintillation counting

Aliquots (25–75 μl) of the sample were pipetted into 20 ml screw-cap vials containing 10 ml scintillation cocktails (Kodak ready-to-use II scintillator solution 132777). After mixing, the vials were counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3380. Column samples were counted for 1 min; dialysis samples were counted for 10, 20, or 50 min to give better statistics. Each sample was counted at least three times and the total activity applied to the column was determined. A 25 μl aliquot was diluted to 25 ml, and aliquots of 25, 50, and 75 μl were counted. The average value was taken for the total activity. The recovery of [^{3}H]glucose was > 90%.

Dialysis

Fraction 4, which contained most of the vesicles and entrapped glucose, was placed in a dialysis bag and dialyzed against 200 ml of the Tris-HCl buffer. The membrane (Union Carbide) had a cut-off of approx. 10000 daltons. At different time intervals, 100-μl aliquots were taken and the activity of the released glucose was determined by scintillation counting. Upon completion, the dialysis bag was opened, or Triton X-100 surfactant was added (final concentration 0.5%) to determine the infinity value. This value agreed well with the original activity placed in the bag.

Glucose permeability

The method of Johnson and Bangham [18,19] was used to analyze the permeability data. It assumes that the bilayer represents a much higher barrier for the entrapped molecule than the dialysis bag. We found the permeability of free glucose out of the dialysis bag to be much faster than the vesicle-entrapped [^{3}H]glucose, and it was not influenced by the presence of empty vesicles. The analysis leads to

$$\ln\left[\frac{NV_0}{V_1+V_0}-N_t\right]=\ln\left[\frac{NV_0}{V_1+V_0}\right]-k_1\frac{V_0+V_1}{V_0}t \qquad (1)$$

where $k_1=(A_1/V_1)P_1$, A_1 = surface area of the vesicles, N = counts in the membrane, V_1 = internal volume of the membrane, V_0 = volume of the dialyzate, P_1 = permeability of the vesicles, and N_t = counts in the dialyzate at $t=t'$. A plot of the release data according to Eqn. 1 allows the estimation of k_1 from the slope and, after evaluation of the vesicle size by light-scattering or electron microscopy, the calculation of the permeability P_1.

Inelastic light scattering

The liposomes were prepared as described above in vesicle preparation. The Tris-HCl buffer (2 ml), previously filtered through a 0.45 μm Nucleopore filter, was placed in a test tube and 50 μl of the vesicle solution was added. The measurements were performed at a scattering angle of 90° with a Brookhaven light-scattering photometer equipped with a helium-neon laser. The data were analyzed in a Brookhaven Instruments digital correlator BI-2030.

Immobilization of vesicles

A sample of C_{16}HCYS (5 mg) and carbodiimide (5 mg) were sonicated for 10 min in a 50 mM 6-carboxyfluorescein solution. After cooling, the vesicles were chromatographed to remove external dye. The vesicles with the entrapped dye were diluted to 6 ml with Tris-HCl buffer to yield the stock solution. Prepacked Sephadex G-25 M columns were equilibrated with buffer, then washed with 10 ml of buffer containing different amounts of carbodiimide (2 to 20 mg/ml), followed by 1 ml of the vesicle stock solution for each column. After 4 h at room temperature, the columns were rinsed with 24 ml of buffer to remove the nonimmobilized vesicles and the free carboxyfluorescein, and the eluent was collected in a 25 ml volumetric flask to which 1 ml 5% Triton X-100 surfactant was added. In order to release dye that was entrapped in the retained vesicles, a 1 ml aliquot of 5% Triton X-100 solution was added to the column and washed as before. The fluorescence intensities of both solutions were determined. The 100% value was obtained by adding 1 ml of the vesicle stock solution and 1 ml of the surfactant solution to 23 ml of buffer. The ratios of the fluorescence intensities of the fractions to the intensity of the 100% value were taken as the percentage of nonimmobilized and immobilized vesicles, respectively. The sum of both was between 92 and 98%. In a second set of experiments the carbodiimide concentration was

constant at 20 mg/ml and the incubation times varied from 20 min to 7 h.

Results and Discussion

Vesicle formation

Hydration of compounds I–IV, followed by sonication at 70°C (above the lipid phase transition), yields aqueous suspensions of variable-size particles. Prolonged sonication (usually 0.5 to 1 h) produced a suspension with an average particle size of 1000 Å. These materials were identified as vesicles by their ability to entrap water-soluble markers (see below) and by electron microscopy [13]. These monomeric vesicles served as the starting material for the polycondensation reaction and as the control vesicles for evalution of the effect of the polycondensation on vesicle permeability.

Polycondensation

The use of water-soluble carbodiimides is the method of choice for condensation of amino acids at the vesicle/water interface. Water-soluble carbodiimides have been extensively used in peptide synthesis [20] for the modification [21–25] and crosslinking of proteins [26,27]; however, the optimum pH for the coupling reaction has not been reported. Timkovich [27] demonstrated that, in the modification of proteins, crosslinking is more dominant at higher pH. We found it necessary to first evaluate the effect of pH on the condensation reaction, as well as the time of vesicle sonication. The reaction products were characterized by infrared spectroscopy, size-exclusion chromatography (SEC), and thin-layer chromatography (TLC).

The sonication time in the presence of carbodiimide has a clear influence on the conversion of starting amphiphilic amino acid to product. If the sonication time is < 10 min, a spot corresponding to the monomer was still found on TLC. The monomer content did not change if the sample remained at room temperature. However, the starting material disappeared if the vesicle sample was sonicated from 15 to 20 min. Thus extensive sonication is necessary to ensure that the carbodiimide reaches all of the head groups. The condensation reaction requires several additional hours incubation to competely convert the monomers to peptides. Sonication for longer than 50 min did not result in further change of the TLC or SEC data.

Fig. 2 shows some typical SEC profiles of the condensation products obtained from C_{18}HCYS (IV) after 1 h sonication at pH 6.5 (Fig. 2a) and pH 8.3 (Fig. 2b). The values of number average (M_n) and weight average (M_w) molecular weights are given. The columns were calibrated with polystyrene standards and, therefore the reported molecular weights are not necessarily the true molecular weights of the peptides. About 40% of the condensation products have an apparent molecular weight > 2000. TLC shows no monomer present in either preparation. The monomeric amphiphilic amino acids are insoluble in tetrahydrofuran; therefore, their SEC profiles could not be recorded. The major peak at log (mol. wt.) ≈ 3.1 in Figs. 2a and b is probably due to the dimer. Additional distinct peaks (which are probably due to tetramers, hexamers, etc.) are clearly seen in

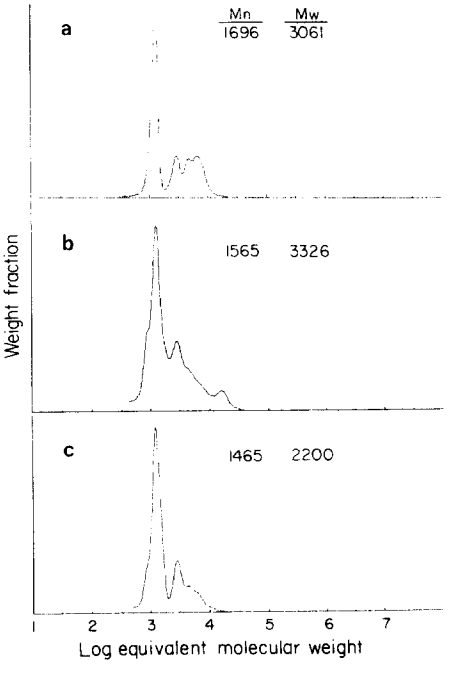


Fig. 2. Size-exclusion chromatography profiles of the peptides obtained after condensation of vesicles from C_{18}HCYS at (a) pH 6.5 and (b) pH 8.3; (c) C_{18}CYS at pH 8.3. The number average (M_n) and weight average (M_w) molecular weights are shown on each profile.

thep rofiles. If the reaction was carried out under more alkaline conditions (Fig. 2b), a broader peak at around log (mol. wt.) 3.1 was found with a shoulder at log (mol. wt.) 2.9. This shoulder is currently assigned to the lactam. Vapor pressure osmometry of the peptide from III was previously reported to give a DP of 4 [13].

The infrared spectra of the same products are depicted in Fig. 3. At pH 6.5 (Fig. 3a), the amide I band is relatively narrow at 1660 cm^{-1}. This band is ascribed to a *trans*-amide bond expected for a peptide. The typical bands for 2,5-piperazinedione that are usually found at 1680 cm^{-1} are not found in these samples. A broadening of the 1660 cm^{-1} band with a shift to 1640 cm^{-1} is found when the reaction pH is 8.3 (Fig. 3b). This broadening in the infrared spectra corresponds to the broadening of the peak at log (mol. wt.) 3.1 in the SEC

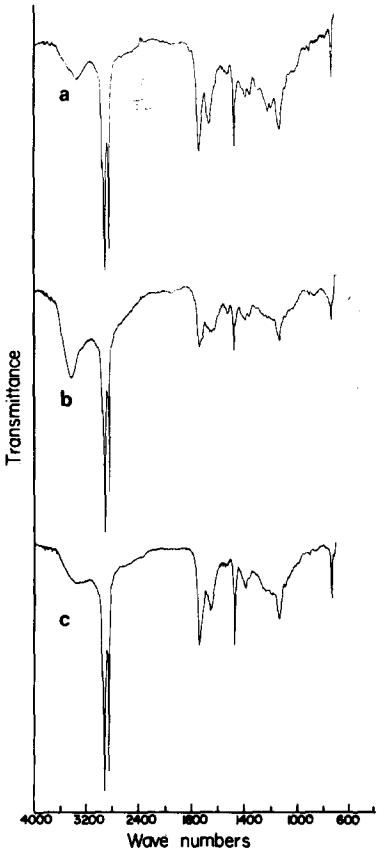


Fig. 3. Infrared spectra of the peptides obtained after condensation of vesicles from C_{18}HCYS at (a) pH 6.5 and (b) pH 8.3; (c) C_{18}CYS at pH 8.3.

profiles, which suggests that more than one type of amide bond is formed during the reaction at higher pH. It should be noted that this effect of pH on product composition is also found in phosphate buffer, as well as the Tris-HCl buffer.

In case of the C_{18}CYS (II), the infrared spectra of the reaction products (Fig. 3c) look similar to those of the C_{18}HCYS (IV), with the exception that the amide II band is weaker. Only 30% of the reaction products of II have a mol. wt. > 2000 according to the SEC profile (Fig. 2c). This indicates greater lactam formation (6-ring lactam) with II than with IV. Neither *N*-acylurea or 2,5-piperazinedione formation could be detected in the infrared spectra under any reaction conditions utilized.

The kinetics of polycondensation reactions differ drastically from those of radical polymerizations. High conversions must be achieved to obtain high molecular weight products. Compounds I–IV were expected to give oligomers rather than high polymers for the following reasons: the compounds do not have the preferred 1 : 1 molar ratio of the reactive groups (e.g., -COOH and $-NH_2$); they may react to form a lactam by intramolecular reaction; and each condensation step on both sides of the membranes has to be initiated by available carbodiimide. Since the interior volume of sonicated vesicles is only 0.1 to 1% of the exterior volume, the number of carbodiimide molecules available within the vesicles for reaction is limited, unless carbodiimide permeability through the bilayer is high. But, as is shown below, the vesicle permeability to glucose and 6-carboxyfluorescein is extremely low; therefore, prolonged sonication was necessary to aid the diffusion of the carbodiimide through the bilayer membrane.

Although the degree of polymerization in these compounds is low, in cases where the lactam formation is less likely (homocysteine-derivatives), a detectable amount of the products with mol. wt. > 10000 was observed. These data clearly show that the structure of the amphiphilic amino acids, and not the nature of the polycondensation reaction itself, determines the molecular weight. It seems likely that higher molecular weight condensates could be formed from amphiphilic amino acids that have only one carboxylic acid (rather

than the two in compounds I to IV), in order to eliminate the lactam side reaction. The second anionic group could be a phosphate or a sulfonic acid.

Carboxyfluorescein permeability

Vesicles can entrap water-soluble compounds in their interior. The permeability of encapsulated molecules is proportional to the partition coefficient of the compound from the aqueous phase into the lipid bilayer, and the rate of diffusion of the compound across the bilayer. Both of these properties are dependent on the size and charge of the compound, and the physical properties of the bilayer membrane. A convenient method to assess the effects of changes in the bilayer membrane, e.g. condensation, is to monitor its permeability with a self-quenched fluorescent dye. The 6-carboxyfluorescein assay first described by Weinstein et al. [28] is a common choice.

The increase of the fluorescence vs. time at 24° C for C_{18}HCYS (IV) vesicles is shown in Fig. 4). The plot also shows the release curves for the peptide vesicles of IV prepared by reaction with carbodiimide for 1 h and 16 h. There is very little, if any, increase in fluorescence with time from the vesicles of IV; even after 30 h the change is less than 0.1%. Vesicles of each of the amphiphilic amino acids show this behavior (see also Fig. 5).

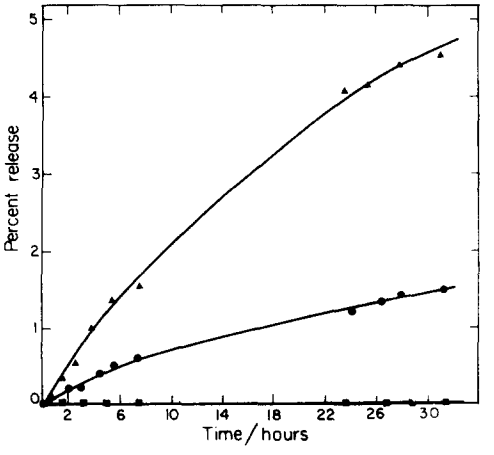


Fig. 4. Percent release of 6-carboxyfluorescein vs. time at 24° C from vesicles of C_{18}HCYS, ■———■; peptide vesicles obtained after a reaction time of 1 h, ▲———▲, and 16 h, ●———●.

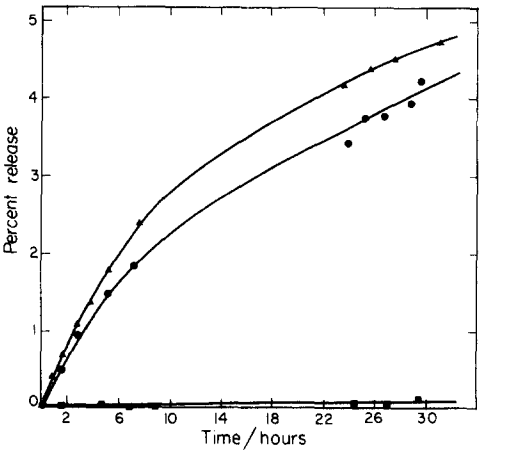


Fig. 5. Percent release of 6-carboxyfluorescein vs. time at 24° C from vesicles of C_{18}CYS, ■———■; peptide vesicles obtained after a reaction time of 1 h, ▲———▲, and 16 h, ●———●.

The permeability of 6-carboxyfluorescein with these vesicles is $< 4.2 \cdot 10^{-14}$ cm · s^{-1}. This value is significantly less than that normally observed with monomeric lipid vesicles, and is comparable to that reported for 6-carboxyfluorescein in polymerized vesicles [4].

The reaction of carbodiimide for 1 h with amino acid vesicles increases the vesicle permeability. Approximately 5% of the 6-carboxyfluorescein is lost from the vesicles of IV (Fig. 4) and of II (Fig. 5) over 30 h. In some cases 20% loss over 30 h was observed. Vesicles from these amino acids contain two negative charges per lipid at pH 8.3, which diminishes the partition of the anionic dye into the bilayer. The carbodiimide-induced condensation reaction decreases the charge of the bilayer membrane and leads to enhanced 6-carboxyfluorescein loss. However, if the condensation reaction is allowed to proceed for 16 h, the membrane permeability to 6-carboxyfluorescein is diminished to an intermediate value between that of the monomeric vesicles and that of the 1 h vesicles (Figs. 4 and 5). The longer reaction time leads to somewhat larger peptides, which may account for this effect. It should also be noted that the formation of peptide bonds places a constraint on the allowed conformations of the molecules, which could alter the lipid chain packing. This is consistent with the observed lower phase transitions (determined by differential scan-

ning calorimetry) of the peptide vesicles, compared to the corresponding amino acid vesicles [13] (e.g., monomeric II, 64°C and peptide II, 51°C; monomeric IV, 82°C and peptide IV, 65°C).

[^{3}H]Glucose permeability

The permeability of the nonionic glucose should be influenced primarily by the chain packing of the bilayer amphiphiles rather than the surface charge. The permeability of glucose through vesicles of phosphatidylcholine (above the phase transition) is approx. 10^{-10} cm · s^{-1}, which is 10^3 faster than monovalent cations [29,30]. This is a reasonably rapid permeation which allows the convenient determination of permeability in a matter of hours.

The permeability data show a fast initial rate followed by a slow approach to equilibrium for both monomeric and condensed bilayers of the amphiphilic amino acids. This behavior is likely due to the presence of multilamellar vesicles in the sample. A typical permeability profile for glucose is shown in Fig. 6 for vesicles of IV and of distearoylphosphatidylcholine (DSPC). The data from Fig. 6 are used in Eqn. 1, according to the analysis of Bangham [18,19], to give the semilogarithmic plot shown in Fig. 7. Also included are the data for DSPC and the dialysis bag. Glucose diffusion from the dialysis bag is fast and does not interfere with the analysis. The slopes of the semilog plot (k_1) and the permeability constant (P) derived from them are given in Table I for the vesicles studied. The permeability constant was estimated from the vesicle sizes as determined by inelastic light scattering (see above). The diameters were about 1000 Å, and that value was used in the calculation of P, since $k_1 = (A_1/V_1)P_1$, where A = the surface area of the membranes,

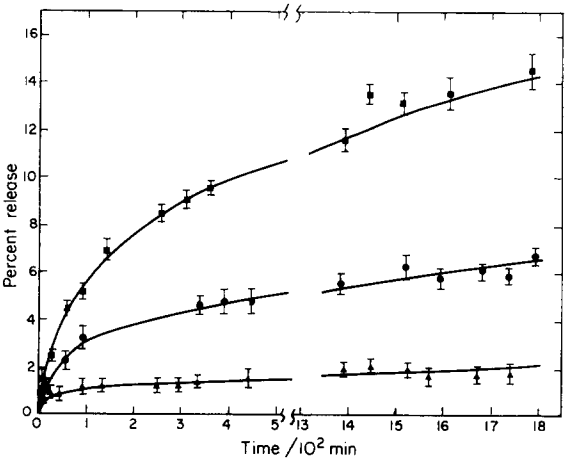


Fig. 6. Percent release of [^{3}H]glucose vs. time at 24°C from vesicles of C_{18}HCYS, ▲———▲; peptide vesicles after 16 h condensation, ●———●; DSPC vesicles, ■———■.

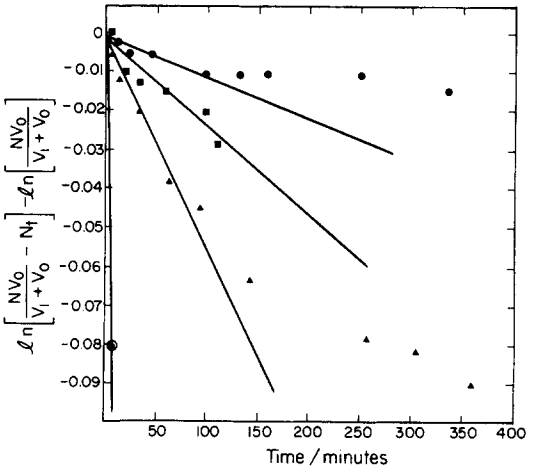


Fig. 7. Semilogarithmic plot of the fraction $\ln[NV_0/(V_0+V_1) - N_t]$ vs. time for the permeation of [^{3}H]glucose through the dialysis bag, ⊙; membrane DSPC vesicles, ▲———▲; C_{18}HCYS vesicles, ●———●; peptide vesicles from C_{18}HCYS, ■———■.

TABLE I

PERMEABILITY OF AMINO ACID AND PEPTIDE VESICLES TO [^{3}H]GLUCOSE AT 24°C

Lipid	k_1 (10^{-6} s)	P [a] (10^{-12} cm·s^{-1})
DSPC	10.6 ±1.3	18
C_{16}CYS		
amino acid	2.37±0.2	3.9
peptide	18.6 ±2.0	31
C_{18}CYS		
amino acid	2.8 ±0.2	4.5
peptide	9.7 ±2.1	15
C_{18}HCYS		
amino acid	1.5 ±0.6	2.5
peptide	3.6 ±1.3	6.0

[a] Calculated on the basis of vesicles of 1000 Å diameter as determined by light scattering.

V = internal volume of the membranes, and P = permeability of the membrane. Analysis of the scattering data showed that a relatively homogeneous poulation of vesicles is present in the samples.

The data in Table I show very similar permeabilities for the C_{16}CYS (I) and the C_{18}CYS (II) vesicles, and each are about twice as permeable as the C_{18}HCYS vesicle. The calculated values of P are about 2% of those previously reported by Dorn et al. [17] for monomeric vesicles of cationic ammonium lipids and 1% of those found for unsaturated phosphatidylcholines [29,30]. The previous data are for lipid vesicles at temperatures above the phase transition, T_c, and the amino acid vesicles were measured at 24°C below the transition. Therefore, it was useful to determine the glucose permeability of a phosphatidylcholine below its phase transition for comparison. DSPC was chosen for comparison since the hydrocarbon chains are 18 carbons long and the T_c is 56°C. The P for vesicles of DSPC is about 5-times that of I and II vesicles, and an order of magnitude less than that of cationic ammonium vesicles [17].

Vesicles from the amphiphilic amino acids described here are the least permeable to glucose of any monomeric vesicles described in the literature to date. This is due, in part, to their high phase-transition temperature, yet even phosphatidylcholine (e.g., DSPC) bilayers in the solid analogous state are more permeable. Since the chain lengths are similar in the two cases, it seems likely that the data reflect a difference in head-group packing between the glycerol ester phospholipids and the glycerol ether compounds reported here. The greater permeability of cationic ammonium lipids described earlier [17] is believed to be due to the lower T_c and looser packing of the head group.

Table I also shows data for the peptide vesicles formed by carbodiimide-induced condensation of the corresponding amino acid vesicles. Permeability of the vesicles from the homocysteine (C_{18}HCYS) is 0.2 to 0.4 that of the cysteine vesicles I and II. The difference in permeability behavior is likely due to the higher lactam content and, hence, lower amounts of oligopeptides in vesicles from I and II than in vesicles from IV. In each case, the major effect of the condensation reaction is to make the vesicles more permeable to glucose, whereas polymerization of cationic ammonium lipid vesicles and zwitterionic dienoyl lipid vesicles results in a decrease in membrane permeability [17].

Immobilization of vesicles

The immobilization of water-soluble enzymes has been intensively investigated during the last few years [31,32]. Various techniques for immobilization have been developed, including water-soluble carbodiimides. Immobilization of vesicles of the type described in this paper can also be achieved by reaction with carbodiimides. In general, immobilized vesicles offer some interesting attributes, such as the immobilization of intrinsic membrane proteins in a lipid bilayer environment, and the immobilization of vesicle-entrapped water-soluble enzymes. Enzyme immobilization in this manner avoids chemical modification of the enzyme or membrane protein. Ideally, the vesicle membrane should be permeable to substrate and product, but impermeable to the enzyme.

Vesicles from the amino acids I–IV are almost impermeable to carboxyfluorescein; therefore, immobilization of these vesicles with entrapped 6-carboxyfluorescein is easy to monitor. The reaction of the carboxylic groups on the vesicle surface with the OH groups on the sugar units of Sephadex G-25 columns leads to ester formation. The amount of nonimmobilized vesicles was determined by eluting them from the column and measuring their fluorescence intensity after lysis with detergent. Some non-entrapped 6-carboxyfluorescein is eluted as well. The fraction of label retained on the column by the carbodiimide reaction was determined by the addition of detergent to the column, which disrupts the immobilized vesicles and releases the 6-carboxyfluorescein.

High levels of carbodiimide result in peptide formation and enhanced 6-carboxyfluorescein leakage; thus, it is necessary to find the minimum carbodiimide concentration for vesicle immobilization. Preliminary experiments at a constant carbodiimide concentration of 20 mg/ml, while varying the incubation times, showed that an incubation time of about 4 h is necessary for an almost complete immobilization. Next, a series of experiments at different carbodiimide concentra-

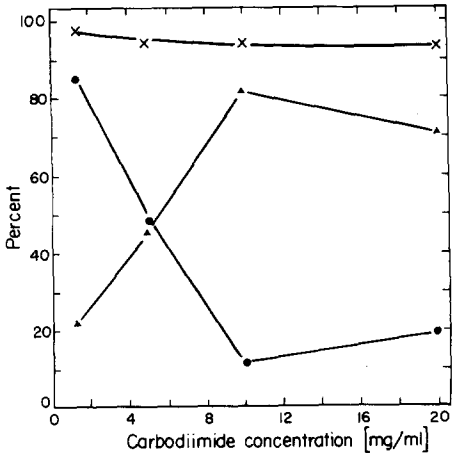


Fig. 8. Percent of immobilized, ▲——▲, and nonimmobilized, ●——●, and sum of both, ×——×, vesicles on Sephadex G-25 column as a function of carbodiimide concentration.

tions indicated that maximum vesicle immobilization occurred at 10 mg/ml with 4 h incubation (Fig. 8). More than 80% of the fluorescence was released from the column by vesicle lysis. The same percentage or greater of the vesicles were immmobilized. Thus, a very efficient immobilization of amino acid vesicles occurs using this simple technique.

Conclusion

Hydration and sonication of the amphiphilic amino acids described in this paper gave vesicles that are exceptionally impermeable to both anionic and neutral molecules. These vesicles were modified by reaction with water-soluble carbodiimide to condense the amphiphiles into oligopeptides. Oligomers were formed because of the nonequivalence of reactive groups and the possibility of lactam formation. Polycondensation should be possible with similar amino acid amphiphiles that contain an equivalent number of amines and carboxylates. The facile synthetic accessibility of the monomers and the limited permeability of the vesicles make these compounds interesting alternatives to phosholipids for the long-term encapsulation of various reagents. Furthermore, the ability to immobilize, condense, and biodegrade these vesicles opens possibilities for their utilization.

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