Polymer Bulletin 15, 397–403 (1986)

Polymer Bulletin

Polymerizable Glycerophosphocholines Containing Terminal 2,4-Hexadienyloxy Groups and Their Polymerized Vesicles[1]

Etsuo Hasegawa[1], Yoh-ichi Matsushita[1], Kiyoshi Eshima[1], Hiroyuki Ohno[2], and Eishun Tsuchida[2]*

[1] Research Institute, Taiho Pharmaceutical Co., Ltd., Tokushima, 771-01, Japan

[2] Department of Polymer Chemistry, Waseda University, Tokyo, 160, Japan

Summary

Polymerizable glycerophosphocholines containing one or two 2,4-hexadienyloxy groups at the terminal of the acyl chains were prepared. Those were 1-[11-(2,4-hexadienyloxy)undecanoyl]-2-O-alkyl-*rac*-glycero-3-phosphocholines 1, 1-acyl-2-[11-(2,4-hexadienyloxy)undecanoyl]-*sn*-glycero-3-phosphocholines 2 and 1,2-bis[11-(2,4-hexadienyloxy)undecanoyl]-*sn*-glycero-3-phosphocholine 3. Those having one hexadienyloxy group formed small unilamellar vesicles. One having two groups formed lipid bilayers, but not unilamellar vesicles. 1 and 2 could form stable microcapsules (polymerized vesicles) with the diameters ranging from 20 to 40 nm.

Introduction

Liposomes (vesicles) which have lipid bilayers and spherical structures are of interest from the standpoint of membrane models and drug carriers (2-4). In addition to natural membrane lipids, synthetic lipids and totally synthetic surfactants can form bilayer membranes (5).

Stabilization of liposomes has been achieved by introducing the new concept which includes polymerizable lipids and polymerization of their bilayer membranes (6-8). Glycerophospholipids having diyne, diene, styrene or methacrylate groups were reported (6-14). The introduction of such groups in acyl chains may disturb or destabilize the bilayer or liposome structures due to their bulkiness or liophilicity. For example, glycerophosphocholines having one styrene group with a spacer form liposomes, but those having two styrene groups can not (12). The liposome formation of glycerolipids containing one styrene group is also remarkably dependent on the polar group introduced in the acyl chain (13).

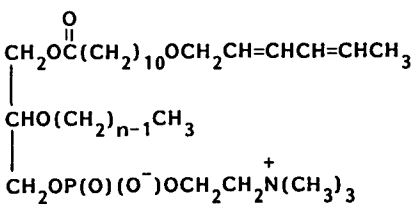
We would like to report here the synthesis of new polymerizable glycerophosphocholines containing diene groups at the terminal of an acyl chain and the effect of *trans*-2,*trans*-4-hexadienyloxy groups on the vesicle formation is discussed.

Experimental

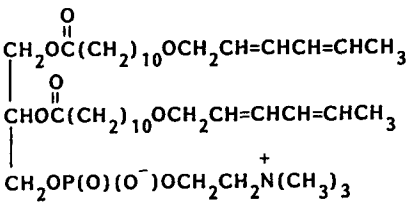
Reagents 4-(N,N-dimethylamino)pyridine (DMAP), dicyclohexylcarbodiimide (DCC) and sorbic alcohol (*trans*-2,*trans*-4-hexadien-1-ol) were purchased and used without further purification. N,N-Dimethylformamide (DMF) and dichloromethane were dried over molecular sieves 4A. 2-Alkyloxy-1,3-propanediol (15) and 2-chloro-2-oxo-1,3,2-dioxaphospholane (16) were prepared according to the literatures.

Differential scanning calorimetry The phase-transition temperatures of lipids in swollen lamellar phases were determined by a Seiko SSC-560U DSC

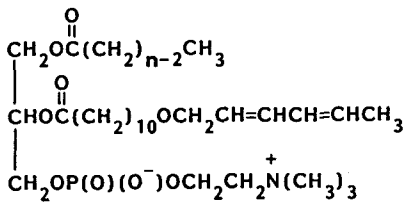
* To whom offprint requests should be sent



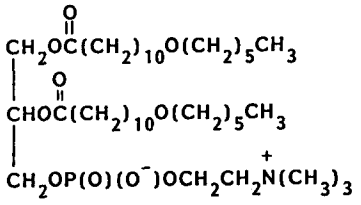
1



3



2



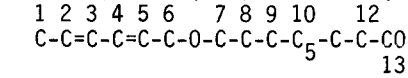
4

meter (5°C/min).
^{1}H and ^{13}C NMR, fast atom bombardment mass (FABMS) and IR spectra and electron microphotos were recorded as previously reported (13).

11-(2,4-Hexadienyloxy)undecanoic acid 5

In a flask sodium hydride (60% in oil, 0.5 mol) was washed with hexane and to the residue was added 700 ml of dry DMF. To the ice-cold and stirred mixture was added dropwise 50 g of sorbic alcohol (0.51 mol) for 1 h. The mixture was stirred for 1 h at room temperature and then for 4 h at 50°C. It was then cooled in an ice-water bath and methyl 11-bromoundecanoate (131.2 g, 0.47 mol) was added dropwise for 1 h and then warmed to 60°C. After 6 h it was cooled again to 0°C and ice-cold water (2 liter) was added. It was extracted with n-hexane twice. The organic layer was washed with brime, dried over sodium sulfate and filtered. The filtrate was concentrated under reduced pressure to give oil. It was purified by column chromatography on silica gel by elution with benzene and benzene/diethyl ether (20/1). The desired fractions having the Rf values (0.29 and 0.38 on TLC, silica gel benzene), which correspond to methyl 11-(2,4-hexadienyloxy)undecanoate and 2',4'-hexadienyl 11-(2,4-hexadienyloxy)undecanoate, respectively, were collected. Removal of the solvents gave 84.5 g of the esters.

To the ester mixture dissolved in methanol (700 ml), 285 ml of 2N NaOH was added. After stirring at room temperature for 1 day, water (1.5 liter) was added and the pH was adjusted to 3 by the addition of conc. HCl. The precipitate formed was collected, washed with water and dried. Recrystallization from petroleum ether gave 48.2 g of the title compound 5: yield 36%; TLC(silica gel,benzene/diethyl ether(2/1)):Rf=0.44; IR(KBr): 1700 (carbonyl), 1100 cm^{-1}(ether); Anal.($C_{17}H_{30}O_3$): C 72.07(72.30), H 10.99(10.71); mp 46-47 °C; MS: 282 (M^+); ^{13}C NMR ($CDCl_3$,TMS) δ in ppm units (carbon number): 18.1 (1), 24.7(11), 26.2(9), 29.1-29.7(7,10), 34.2(12), 70.2(7), 71.2(6), 126.9, 129.7, 130.9, 132.9(2-5) and 179.9(13).



13

1-[11-(2,4-Hexadienyloxy)undecanoyl]-2-O-octadecyl-rac-glycero-3-phosphocholine 1(n=18)

5 (0.8 g, 3 mmol) dissolved in dry dichloromethane (40 ml) was stirred and cooled in a ice-water bath. DCC (0.62 g, 3 mmol) was added to this and the mixture was stirred for 3 h at room temperature and then filetred. The filtrate was added to the solution of 2-octadecyloxy-1,3-propanediol (2.1g, 6 mmol) dissolved in dry dichloromethane (100 ml). DMAP (73 mg, 0.6 mmol) was added to this and it was stirred for 20 h in the dark. The precipitate was filtered off and the filtrate was concentrated under reduced pressure. The residue was chromatographed on silica gel by elution with benzene/diethyl ether(10/1). The desired fraction was collected and solvents were removed under reduced pressure. 0.95 g of 1-[11-(2,4-hexadienyloxy)undecanoyl]-2-O-octadecyl-*rac*-glycerol was obtained: yield 52%; mp: 32-33°C; TLC(silica gel, benzene/diethyl ether(9/1)): Rf=0.3; MS: 608(M^+): IR(KBr): 3420(OH), 1725 ester carbonyl), 1635 cm^{-1}(diene); Anal.($C_{38}H_{72}O_5$): C 74.68(74.95), H 12.19 (11.92); ^{13}C NMR($CDCl_3$, TMS) δ(ppm): 62.0,62.7,77.6(glycerol backbone), 127.0,129.6,130.8,132.8(diene), 173.6(ester carbonyl).

This compound (1.5 g, 2.5 mmol) dissolved in dry benzene (30 ml) containing dry triethylamine (0.27 g, 2.7 mmol) was cooled with stirring. 2-Chloro-2-oxo-1,3,2-dioxaphospholane (0.38 g, 2.7 mmol) dissolved in dry benzene (5 ml) was added dropwise to this. Then 10 mg of DMAP was added and the mixture was stirred for 1 day in the dark. The precipitate formed was filtered off and the filtrate was evaporated to dryness and the residue was dried *in vacuo*. It was allowed to react with dry trimethylamine (20 ml) in dry benzene /acetonitrile(8ml/12 ml) in a pressure bottle at 60°C for 5 h. Solvents were removed by evaporation under reduced pressure and the residue was purified by column chromatography on silica gel by elution with chloroform/methanol/water (65/25/4). The desired fraction having the same Rf values as purified egg yolk phosphatidylcholine (EYPC, Sigma) was collected. Solvents were removed by evaporation and the residue was freeze-dried from dry benzene to give 1.0g of 1(n=18): yield 54%; FAB MS: 747(M+1); IR(KBr): 1735(ester carbonyl), 1640 cm^{-1}(diene); UV(methanol): λ_{max} 227 nm, ε_{max} 2.8×10^4 liter/mol.cm; Anal. ($C_{43}H_{84}N_1O_8P_1$): N 1.66(1.81); ^{13}C NMR spectral data summarized in Table 1 are in complete agreement with the indicated structure.

1(n=14,16) were also prepared by the same procedure described above using the corresponding 2-alkyloxy-1,3-propanediols. FAB MS (M+1) and analytical (N) data were 718, 2.08(1.95) and 746, 2.20(1.88) for 1(n=14) and 1(n=16), respectively.

1-Hexadecanoyl-2-[11-(2,4-hexadienyloxy)undecanoyl]-sn-glycero-3-phosphocholine 2(n=16)

In a flask 5 (2.28 g, 8 mmol) dissolved in dry dichloromethane (70 ml) was cooled in an ice-water bath. DCC (1.65 g, 8 mmol) was added and it was stirred at room temperature for 1.5 h. To this 1-hexadecanoyl-*sn*-glycero-3-phosphocholine (Sigma) (1.5 g, 3.2 mmol) and DMAP (0.49 g, 4 mmol) were added. The mixture was stirred for 2 days in the dark. The precipitate formed was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in 100 ml of methanol/chloroform/water(5/4/1), filtered and passed through a column containing 300 ml of the mixed bed resin (Bio-Rad, AG 501-X8(D)). Solvents were removed and the residue was chromatographed on silica gel by elution with chloroform/methanol/water(65/25/4) and the fraction having the same Rf value with that of EYPC was collected. Solvents were removed and the residue was freeze-dried from dry benzene to give 1.13 g of 2(n=16): yield 46%; FAB MS: 760(M+1); IR(KBr): 1735(ester carbonyl), 1645 cm^{-1}(diene); Anal. ($C_{41}H_{78}N_1O_8P_1$): N 1.86(1.82); ^{13}C NMR spectral data summarized in Table 2 are in complete agreement with the indicated structure.

2(n=14) was also prepared by the same procedure as described above with 1-tetradecanoyl-*sn*-glycero-3-phosphocholine (Sigma). Yield 26%; Anal. ($C_{38}H_{74}N_1O_8P_1$): N 1.59(1.91); FAB MS: 732(M+1). 2(n=14) gave almost the same NMR spectra as that of 2(n=16).

1,2-Bis[11-(2,4-hexadienyloxy)undecanoyl]-sn-glycero-3-phosphocholine 3

L-α-glycerophosphocholine.$CdCl_2$ (Sigma, 1.0 g, 2.3 mmol) was allowed to react with 5 (1.6 g, 5.7 mmol), DCC (1.28 g, 6.25 mmol) and DMAP (0.37 g, 3 mmol) in dry dichloromethane (50 ml). The mixture was vigorously stirred with glass beads (25 ml) for 2 days in the dark at room temperature. Precipitate was removed by filtration and the filtrate was evaporated to dryness. The residue was treated with the mixed bed resin and was chromatographed as described for 2. After freeze-drying from benzene 0.83 g of 3 was obtained: yield 19%; FAB MS: 786(M+1); Anal. ($C_{42}H_{76}N_1O_{10}P_1$): N 1.65(1.78); ^{13}C NMR ($CDCl_3$, TMS) δ (ppm): 63.0,70.7(glycerol backbone), 54.3(choline methyl), 59.3,66.2(choline ethyl), 127.0,129.6,130.0,132.8(diene), 173.1,173.4 (ester carbonyl).

11-(n-hexyloxy)undecanoic acid 6

This compound was synthesized by the same procedure described for 5 using n-hexylalcohol in place of sorbic alcohol. mp: 37-38°C; IR(KBr): 1700 cm^{-1}(carbonyl); Anal.($C_{17}H_{34}O_3$): C 71.39(71.29), H 12.46(11.96); ^1H NMR ($CDCl_3$, TMS) δ(ppm): 0.88(t,3H), 1.28(br s,18H), 1.56(m,6H), 2.35(m,2H), 3.39 (t,4H), 9.3(br s,1H).

1,2-Bis[11-(n-hexyloxy)undecanoyl]-sn-glycero-3-phosphocholine 4

This compound was synthesized by the same procedure as described for 3 with 6 instead of 5. yield 66%; FAB MS: 794(M+1); Anal.($C_{42}H_{84}N_1O_{10}P_1$): N 1.78(1.76); ^{13}C NMR ($CDCl_3$, TMS) δ(ppm): 54.3(choline methyl), 63.1,70.4 (glycerol backbone), 59.3,66.2(choline ethyl), 71.0(-C-O-C-), 173.3,173.5 (ester carbonyl).

Table 1. Proton decoupled ^{13}C NMR spectral data of 1(n=18) in $CDCl_3$ (internal reference: tetramethylsilane(TMS))

Carbon Number	δ(ppm)
1'	14.2
1	18.1
2'	22.7
11	25.0
9,5'	26.2
8,10,4'	29.8-29.4
3'	31.9
12	34.3
f	54.4
d	59.5
a,c	64.5
e	66.4
6,7,6'	70.6
b	76.8
2-5	132.9,130.9, 129.7,127.1
13	173.8

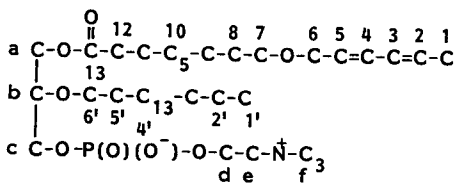
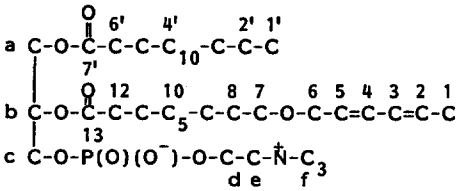


Table 2. Proton decoupled ^{13}C NMR spectral data of 2(n=16) in $CDCl_3$ (internal reference: tetramethylsilane (TMS))

Carbon Number	δ(ppm)
1'	14.2
1	18.1
2'	22.7
5',11	25.0
9	26.3
8,10,4'	29.8-29.3
3'	31.9
12,6'	34.3,34.2
f	54.4
d	59.4
a,c	63.1,63.4
e	66.3
b	70.7
6,7	70.3,71.2
2-5	132.9,130.9, 129.7,127.1
13,7'	173.6,173.3



Results and Discussion

Non-polymerized Vesicle Formation

The ability of the polymerizable lipids, 1(n), 2(n) and 3 and the non-polymerizable lipid, 4, to form lipid bilayers or unilamellar vesicles was studied by a differential scanning calorimeter, a ^1H NMR spectrometer (shift reagent: $Eu(NO_3)_3$), an electron microscope and a laser particle analyzer.

DSC measurements revealed the gel-liquid crystal transition temperatures for all the lipid dispersions as summarized in Table 3, indicating the formation of ordered structures (lipid bilayers).

Unilamellar vesicle formation was examined by the use of the lipid dispersions prepared by the thin film method followed by ultrasonication (13,14). All lipids gave slightly turbid and non-viscous aqueous dispersions. The formation of unilamellar and single-walled vesicles is elucidated by ^1H NMR spectral measurements with a shift reagent, which clarifies the presence or absence of the outer and the inner choline methyl groups (13,14,17,18). Thus, the singlet peak due to the choline methyl groups in the absence of the shift reagent was split by the addition of Eu^{3+} for the dispersions of 1(n=18) and 2(n=16), but no splitting was observed in the case of 1(n=14,16), 2(n=14) and 3. The model compound 4 where the diene groups of 3 were hydrogenated to the saturated hexyl ones gave the spectral change from the singlet to doublet by the addition of Eu^{3+}.

Electron microphotos showed the formation of spherical structures for the dispersions of 1(n=18) and 2(n=16) with the diameters ranging from 20 to 40 nm, of which results agreed well with the average diameters estimated with a laser particle analyzer(Coulter Electronics, Coulter N4D) (29 nm and 30 nm for 1(n=18) and 2(n=16), respectively). The average diameter of the particles formed by 3 was 90-200 nm.

These results concluded: (a) 1(n=18) and 2(n=16) can form small unilamellar vesicles; (b) 1(n=14,16) and 2(n=14) can form ordered structures (lipid bilayer), but not closed small unilamellar vesicles; (c) 3 can form multilamellar liposomes, but not small unilamellar liposomes, while the model 4 can form small unilamellar vesicles.

Thus, it has been clarified that the formation of liposomes is strongly dependent on the 2,4-hexadienyloxy group in an acyl chain and on the chain length of another saturated acyl or alkyl chain of a lipid containing one hexadienyloxy residue. The result that the model 4 can form small unilamellar vesicles strongly suggested that the 2,4-hexadienoyloxy group destabilized the vesicullar structures.

Polymerized Small Unilamellar Vesicles

The polymerizable lipids 1(n=18) and 2(n=16), which can form small unilamellar and closed vesicles, were allowed to polymerize by UV irradiation at 50°C (13,14). The polymerization was followed by measuring UV absorption spectra of the lipids. The absorption band of the non-polymerized vesicles at 227 nm due to the dienyl group disappeared after irradiation. The liophilized powder of the polymerized 1(n=18) and 2(n=16) gave no signals due to the diene group in ^{13}C NMR spectra.

An electron microphoto of the polymerized 1(n=18) confirmed the presence of closed and spherical vesicles having the diameters ranging from 20 to 40 nm (Figure 1). This result agreed with the average diameters determined by the laser particle analyzer.

The ^{1}H NMR spectral measurements in the absence and presence of the shift reagent (Eu^{3+}) supported the formation of the small unilamellar vesicles even after irradiation (Figure 2).

No phase transitions was found for the irradiated particles of 1(n=18) and 2(n=16), probably because of the formation of polymers in bilayers.

Thus, it was found that 1(n=18) and 2(n=16) formed stable (polymerized) small vesicles.

Table 3. Phase transition temperatures of lipids in swollen lamellar phases in H_2O.

Lipids	n	Tc(°C)
1	14	25.9
	16	31.7
	18	54.3
2	14	18.0
	16	19.5
3		28.5
4		12.5

Figure 1. Transmission electron microphotograph of the polymerized vesicles of 1(n=18) stained with uranyl acetate.

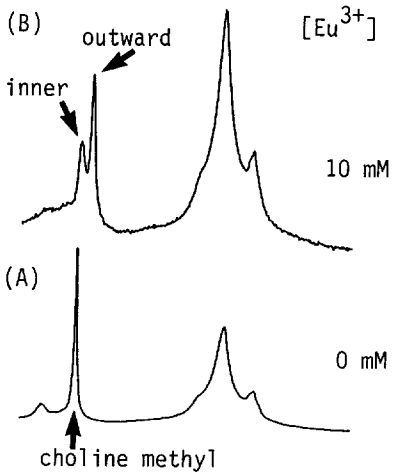


Figure 2. ^{1}H NMR spectra of the polymerized 1(n=18) in the absence (A) and in the presence (B) of $Eu(NO_3)_3$ in D_2O at 50°C. [lipid]= 4 wt/vol%.

References

1. This forms part 4 of a series entitled "Synthesis and Properties of Polymerizable Phospholipids".
2. G. Gregoriadis, "Drug Carriers in Biology and Medicine", Academic Press, London (1979).
3. J. H. Fendler, "Membrane Mimetic Chemistry", John Wiley and Sons, New York (1984).
4. D. A. Tirrell, L. G. Donaruma and A. B. Turek, eds., "Macromolecules as Drugs and as Carriers for Biologically Active Materials", The New York Acad. Sci., New York (1985).
5. T. Kunitake and Y. Okahata, J. Amer. Chem. Soc. 99, 3860 (1977).
6. D. Day, H. H. Hub and H. Ringsdorf, Isr. J. Chem. 18, 325 (1979).
7. S. L. Regen, A. Singh, G. Oehme and M. Singh, Biochem. Biophys. Res. Commun. 101, 131 (1981).
8. D. S.Johnston, S. Sanghera, A. Manjon-Rubio and D. Chapman, Biochim. Biophys. Acta 602, 213 (1980).
9. L. Gros, H. Ringsdorf and H. Schupp, Angew. Chem. Int. Ed. Engl. 20 305 (1982).
10. E. Lopez, D. F. O'Brien and T. H. Whitesides, J. Amer. Chem. Soc. 104, 305 (1981).
11. S. L. Regen, A. Singh, G. Oehme and M. Singh, ibid, 104, 791 (1982).
12. E. Hasegawa, Y. Matsushita, K. Eshima, H. Nishide and E. Tsuchida, Makromol. Chem. Rapid Commun. 5, 779 (1984).
13. E. Hasegawa, K. Eshima, Y. Matsushita, H. Nishide and E. Tsuchida, Polymer Bull. 14, 31 (1985).
14. E. Hasegawa, K. Eshima, Y. Matsushita, H. Nishide and E. Tsuchida, Synthesis in contribution (1986).
15. D. Arnold, H. U. Weltzien and O. Westphal, Justus Liebigs, Ann. Chem. 709, 234 (1967).
16. R. S. Edmundson, Chem. Ind. (London) 1828 (1962).
17. V. F. Bystrov, N. I. Dubrovina, L. I. Bardukov and L. D. Bergelson, Chem. Phys. Lipids 6, 343 (1971).
18. S. B. Andrews, J. W. Faller, J. M. Gillian and R. J. Barrnett, Proc. Nat. Acad. Sci. USA 70, 1814 (1973).

Accepted May 12, 1986

Thus, it has been clarified that the formation of liposomes is strongly dependent on the 2,4-hexadienyloxy group in an acyl chain and on the chain length of another saturated acyl or alkyl chain of a lipid containing one hexadienyloxy residue. The result that the model 4 can form small unilamellar vesicles strongly suggested that the 2,4-hexadienoyloxy group destabilized the vesicullar structures.

Polymerized Small Unilamellar Vesicles

The polymerizable lipids 1(n=18) and 2(n=16), which can form small unilamellar and closed vesicles, were allowed to polymerize by UV irradiation at 50°C (13,14). The polymerization was followed by measuring UV absorption spectra of the lipids. The absorption band of the non-polymerized vesicles at 227 nm due to the dienyl group disappeared after irradiation. The liophilized powder of the polymerized 1(n=18) and 2(n=16) gave no signals due to the diene group in ^{13}C NMR spectra.

An electron microphoto of the polymerized 1(n=18) confirmed the presence of closed and spherical vesicles having the diameters ranging from 20 to 40 nm (Figure 1). This result agreed with the average diameters determined by the laser particle analyzer.

The ^{1}H NMR spectral measurements in the absence and presence of the shift reagent (Eu^{3+}) supported the formation of the small unilamellar vesicles even after irradiation (Figure 2).

No phase transitions was found for the irradiated particles of 1(n=18) and 2(n=16), probably because of the formation of polymers in bilayers.

Thus, it was found that 1(n=18) and 2(n=16) formed stable (polymerized) small vesicles.

Table 3. Phase transition temperatures of lipids in swollen lamellar phases in H_2O.

Lipids	n	Tc(°C)
1	14	25.9
	16	31.7
	18	54.3
2	14	18.0
	16	19.5
3		28.5
4		12.5

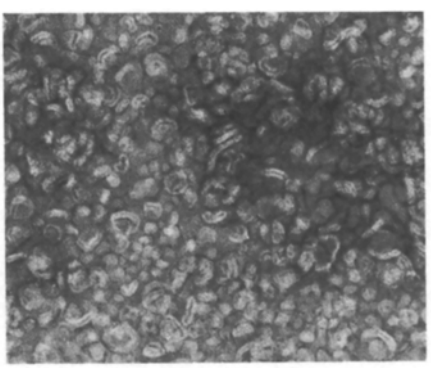


Figure 1. Transmission electron microphotograph of the polymerized vesicles of 1(n=18) stained with uranyl acetate.