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THE SCREENING OF ENDOVESICULAR REACTIONS BY CHOLESTEROL

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Summary. Hydroxide ion cleavage of p-nitrophenyl diphenyl phosphate occurs at exovesicular and endovesicular sites of dihexadecyldimethylammonium ion vesicles. The endovesicular reaction can be eliminated upon alteration of the vesicle's permeability to hydroxide ions by the incorporation of cholesterol.

The hydroxide ion cleavage of p-nitrophenyl diphenyl phosphate (1, PNPDPP) distributed in vesicles of dihexadecyldimethylammonium bromide (2, 16_2) is kinetically biphasic.[2] The rapid,

$(C_6H_5O)_2\overset{O}{\overset{\|}{P}}OC_6H_4$-p-$NO_2$ $(n\text{-}C_{16}H_{33})_2\overset{+}{N}(CH_3)_2,Br^-$ $n\text{-}C_{16}H_{33}\overset{+}{N}(CH_3)_3,Br^-$

(1, PNPDPP) (2, 16_2) (3, CTABr)

catalyzed cleavage (k_ψ~0.4-0.6 sec^{-1} at $[OH^-]$ = 0.1M) was attributed to an exovesicular reaction occurring on the exterior surface of vesicular 16_2. The slower process (k_ψ~0.02-0.03 sec^{-1}, 5-10% of reaction) was suggested to be endovesicular, occurring within the vesicular bilayers, on interior vesicular surfaces, or in entrapped aqueous cavities.[2] The principal evidence offered for these assignments was the dependence of the observed kinetic phenomena on the order of reagent addition. Biphasic kinetics were observed when PNPDPP had been bound to and entrapped by the vesicles before the addition of OH^- (i.e., prepositioned in exovesicular and endovesicular sites). In contrast, when PNPDPP was added to vesicular 16_2 already in 0.1 M NaOH solution, only the fast, exovesicular reaction was observed. In this case, the rapidly bound substrate did not survive transit of the outer vesicular surface to endovesicular sites, but was cleaved by exovesicularly approximated OH^-.[2]

Now we present an independent and novel differentiation of the exovesicular and endovesicular hydrolysis reactions which makes use of the known ability of vesicle-incorporated cholesterol to alter vesicular permeability.[3] Additionally, we report significant chemical differences between large 16_2 vesicles formed by the injection method[4] and the smaller 16_2 vesicles resulting from extensive sonication.

Cleavage of PNPDPP, prepositioned in 16_2 vesicles via coinjection with surfactant, by subsequently added 0.10 M NaOH is kinetically biphasic[2] (Table I, case 1). The fast reaction (k_ψ^f ~0.5 sec^{-1}) kinetically resembles the analogous CTABr micellar cleavage (k_ψ ~0.3 sec^{-1}, case 9) and is ~20 times more rapid than uncatalyzed 0.10 M OH^- aqueous cleavage of PNPDPP (k_ψ^{aq} = 0.026 sec^{-1}). The fast process is assigned to exovesicular hydrolysis of bound PNPDPP.

Table I. Cleavage of PNPDPP in Vesicular 16_2[a]

Case	Method[b]	Chol.[c]	Delay[d]	k_ψ^f, s^{-1}[e]	k_ψ^m, s^{-1} (%)[f]	k_ψ^s, s^{-1} (%)[g]
1	coinj.	0	>10 min	0.46	none	0.022 (6)
2	inj.	0	40 msec	0.34	none	0.022 (4)
3	inj.	20	40 msec	0.15	none	none
4	son.	0	>10 min	0.54	0.026 (18)	0.007 (20)
5	coson.	0	>10 min	0.59	0.026 (13)	0.006 (15)
6	son.	20	40 msec	0.55	0.024 (45)	none
7	son.	20	10 sec	0.62	0.025 (44)	none
8	coson.[h]	20	>10 min	0.48	0.020 (19)	none
9	micelle[i]	0	>10 min	0.34	none	none
10	micelle[i]	20	>10 min	0.29	0.018 (23)	none

[a]Conditions (after stopped-flow mixing): [surfactant] = 5.0×10^{-4} M; [PNPDPP] = 1.0×10^{-5} M; [OH^-] = 0.10 M, 25°C. [b]Inj., vesicles created by injection, cf., ref. 4; coinj., surfactant and PNPDPP injected together; son., 16_2 sonicated at 60°C with a Braun-sonic model 1510 probe-type sonicator at 200 W for 30 min, then PNPDPP added; coson., 16_2 and PNPDPP sonicated together. All reactions were initiated by addition of base to solutions initially at pH 7. [c]Wt.-% added cholesterol. The cholesterol was introduced by coinjection or co-sonication with surfactant. [d]Time between the addition of PNPDPP to the 16_2 vesicles and the subsequent addition of OH^-. Short delay times were obtained on a Durrum, model D-132 multi-mixing system. [e]Rate constant of fast, exovesicular reaction; see text. Duplicate runs were reproducible to ±0.02. [f]Rate constant of medium speed, aqueous reaction; see text. Duplicate runs were reproducible to ±0.002. [g]Rate constant of slow, endovesicular reaction; see text. Duplicate runs were reproducible to ±0.003 (cases 1, 2) or ±0.001 (cases 4, 5). [h]16_2, PNPDPP, and cholesterol were cosonicated. [i]Micellar 3 (CTABr); [CTABr] = 8.0×10^{-4} M, other conditions as in a.

Table II. Diameters of 16_2 Vesicles by Dynamic Light Scattering[a]

	Injected 16_2[b]		Sonicated 16_2[c]	
Incorporated cholesterol (%)	Diameter (Å)	Variant[d]	Diameter (Å)	Variant[d]
0	1580	1.09	443	0.71
5	1240	1.38	339	0.84
10	1030	1.49	300	1.08
20	789	0.914	496	0.88

[a]See ref. 10 for light scattering apparatus. [b]See ref. 4 for a description of the injection method. [c]Sonication conditions appear in Table I, note a. [d]Polydispersity index; variant >1.0 indicates marked polydispersity.

The accompanying slow reaction (k_{ψ}^{s} ~0.02 sec^{-1}) occurs at about the same rate as bulk aqueous cleavage, but must occupy a different locus. Thus, vesicles doped with 20 wt-% of coinjected cholesterol only support exovesicular PNPDPP cleavage (case 3). No slow process is observable. Were the k_{ψ}^{s} reactions of cases 1 and 2 due to aqueous cleavages, they would not be inhibited by altering the vesicles. The k_{ψ}^{s} reaction is therefore assigned to an endovesicular process and is thus sensibly subject to inhibition or screening.[5] In the absence of cholesterol, PNPDPP added to a solution of empty 16_2 vesicles appears to distribute between exovesicular and endovesicular sites within ~40 msec (compare cases 2 and 3), so that permeation of PNPDPP across the undoped exterior vesicular membrane is relatively rapid.

Reactions with sonicated 16_2 vesicles are kinetically triphasic. In addition to the rapid (k_{ψ}^{f}) exovesicular process, medium speed (k_{ψ}^{m} ~0.02 sec^{-1}) and slow (k_{ψ}^{s} ~0.007 sec^{-1}) sequential reactions are also apparent (cases 4 and 5). The last process must be endovesicular because it can be screened by intravesicular cholesterol (cf., cases 6-8). The medium speed reaction, k_{ψ}^{m}, is most reasonably attributed to bulk aqueous OH^- cleavage of PNPDPP. Thus, it has an appropriate rate constant, is not screened by intravesicular cholesterol, and, in contrast to k_{ψ}^{s} processes (cases 1, 2, 4, 5), k_{ψ}^{m} does not disappear in inverse addition experiments.[6] Interestingly, doping CTABr micelles with 20% of cholesterol appears to sufficiently inhibit their ability to solubilize PNPDPP so that some of the hydrolysis now occurs in solution, and k_{ψ}^{m} appears (case 10).

Injected and sonicated 16_2 support exovesicular and endovesicular OH^- cleavages of PNPDPP and, in both cases, incorporation of 20 wt-% of cholesterol inhibits endovesicular reactions. A priori, endovesicular screening could be due to reduced permeability of the cholesterol-modified vesicular membrane to either PNPDPP, or OH^- ions, or both. However, the endovesicular reaction was also absent in case 8, where PNPDPP was prepositioned inside the vesicles by ternary cosonication of 16_2, cholesterol, and PNPDPP. This result implicates slow appearance of OH^- in the altered vesicles, in contrast to the results with undoped vesicles where OH^- permeation is not rate-limiting.[2,3c] Indeed, indigo (carmine) disulfonate, entrapped[7] in vesicular 16_2 prepared by cosonication with 20% cholesterol, is deprotonated upon subsequent addition of 0.1 M OH^- with k_{ψ} = 0.0039 sec^{-1}, a value lower than k_{ψ}^{s} for endovesicular PNPDPP cleavage in such vesicles (cases 4 and 5). This supports the suggestion that inhibition of OH^- permeation by intravesicular cholesterol is a key factor in the screening of endovesicular hydrolysis reactions.

Although both injected and sonicated 16_2 vesicles exhibit similar effects of cholesterol doping on endovesicular hydrolytic reactions, they also reveal significant chemical differences. Most apparent is a reduced capacity for binding PNPDPP,[8] manifested by the sonicated vesicles, which results in diversion of ~15% (no cholesterol) to ~40% (20% cholesterol) of the PNPDPP cleavage to the bulk aqueous phase (k_{ψ}^{m}, cases 4-8).[9] The reduced loading capacity of the sonicated vesicles may well be related to their smaller size as revealed by light scattering studies.

Table II displays vesicular diameters for injected and sonicated 16_2 vesicles determined by dynamic light scattering.[10] Although the de novo reproducibilities of specific entries were not particularly good (±20%), the observed trends were reproducible over several series of experiments. The injected vesicles are larger and more polydisperse than their sonicated analogues. Electron microscopy also reveals the unmodified injected vesicles to be multilamellar, whereas the native sonicated vesicles appear to contain fewer lamellae and a large central aqueous pool.[11]

Both types of vesicles contract upon cholesterol incorporation, but the sonicated species expand again when doped with more than 10% of cholesterol. This may reflect surface layering of excess cholesterol after the exhaustion of other binding sites. Although it is premature to further analyze the morphological differences between injected and sonicated vesicles, we believe that they are mechanistically related to the observed chemical differences, particularly the reduced loading capacity of the sonicated species. We are continuing our studies of the chemistry and permeability of doped vesicles.

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References and Notes

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(5) This agrees with the k_{ψ}^{s} assignment based on the order of reagent addition; cf., above and ref. 2.

(6) For example, in an experiment otherwise parallel to case 4, PNPDPP was added to sonicated vesicular 16_2 in 0.1 M NaOH solution. We observed $k_{\psi}^{f} = 0.47\ sec^{-1}$ and $k_{\psi}^{m} = 0.032\ sec^{-1}$ (28%); no k_{ψ}^{s} process was seen.

(7) See ref. 2 for a detailed description of this kind of experiment.

(8) The molar ratio, PNPDPP/16_2, is 1:50. Because the aggregation number of the vesicles is in the thousands,[3c] there are many substrate molecules per vesicle.

(9) Additionally, endovesicular PNPDPP hydrolysis is characterized by a lower rate constant in the sonicated vesicles.

(10) Dynamic light scattering measurements employed on Ar laser (488 nm) and a Nicomp model TC-100 computing autocorrelator. Data were collected at 25°C and a 90° scattering angle, and were evaluated on a Hazeltine microcomputer using the cumulant program. Solutions used for light scattering were passed through a 0.2μ Bio-Rad polycarbonate filter.

(11) Vesicles for electron microscopy were stained with 1% aqueous uranium acetate.

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