References and Notes

1) M. Yamaguchi, T. Yamamoto, and A. Hasegawa, *Chem. Pharm. Bull.*, **27**, 3137 (1979).
2) M. Yamaguchi and T. Yamamoto, *Chem. Pharm. Bull.*, **26**, 2568 (1978).
3) R.J. Robert, C.D. Klaassen, and G.L. Plaa, *Proc. Soc. Expt. Med. Biol.*, **125**, 313 (1967).
4) J.B. Willis, *Nature* (London), **16**, 249 (1960).
5) P.F. Hirsch, E.F. Voelkel, and P.L. Munson, *Science*, **146**, 412 (1963).
6) A.D. Care, L.M. Sherwood, J.T. Potts, Jr., and G.D. Aurbach, *Nature* (London), **209**, 55 (1966).
7) H. Rasmussen, *Amer. J. Med.*, **50**, 567 (1971).
8) P.D. Broulik and V. Pocorsky, *Endocrinol. Exp.*, **7**, 183 (1973).
9) R.V. Talmage, S.H. Doppelt, and C.W. Cooper, *Proc. Soc. Exp. Med. Biol.*, **149**, 855 (1975).

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The Use of Liposomes as Enzyme Carriers. I. Dependence of Enzyme Stability on the Method of Preparation

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The stability of liposomal enzymes and the encapsulation efficiency of reverse-phase evaporation vesicles (REV) were investigated in comparison with those of the original small multilamellar liposomes (SML), by using elastase as a model enzyme. The encapsulation efficiency of REV was greatly superior to that of SML. In addition, REV retarded the inactivation of elastase in the neutral pH region, as did SML. Therefore the REV method should be useful for the preparation of carriers to encapsulate not only elastase but also other macromolecules. However, at lower pH, pH 2.63, where elastase was rapidly inactivated, REV accelerated the inactivation of the enzyme, although SML retarded it, as at neutral pH. Thus, since liposomes prepared by different methods have significantly different physicochemical properties, further and more precise studies on the methods of preparation are required.

Keywords——enzyme; enzyme stability; elastase; liposomes; reverse-phase evaporation vesicles; encapsulation efficiency

The use of liposomes as a drug delivery system has been extensively investigated.[1] In the case of enzyme replacement therapy, deficient enzymes are often administered in the liposomal form so that the delivery of foreign enzymes to cells is facilitated while retaining their activities and avoiding immunological reactions.[2] One of the weak polints of liposomal preparations is low encapsulation efficiency due to the small internal aqueous space. Recently, Szoka and Papahadjopoulos reported a procedure for the preparation of reverse-phase evaporation vesicles (REV) with large internal aqueous space and high encapsulation efficiency.[3]

This report describes the stability of the liposomal enzymes and the encapsulation efficiency of REV in comparison with those of the original liposomal preparation of Bangham *et al.*[4] by using elastase as a model enzyme.

Experimental

Materials——Phosphatidylcholine was prepared from egg yolks according to the method of Rhodes and Lea.[5] Elastase and succinyl-tri-L-alanine-*p*-nitroanilide (STANA) were supplied by Eisai Co., Tokyo,

Japan. Other reagents were purchased from Nakarai Chemicals Co., Kyoto, Japan. All lipids were dissolved in chloroform and stored under nitrogen at −20°.

Assay of Elastase Activity——The activity of elastase was estimated by the modified method of Bieth *et al.*[6] with STANA as a substrate. The calibration curve showed a good proportionality between the optical densities and elastase concentrations, regardless of the presence of Triton X-100.

Assay of Glucose——Glucose was assayed by means of the Glucose-HK Test (Gluco-quant®, Boehringer Mannheim-Yamanouchi Co., Tokyo, Japan).

Preparation of Liposomes——Two kinds of liposomes were prepared in 0.06 M Tris buffer, pH 7.4, containing 0.12 M NaCl and elastase (500 μg/ml) or glucose (200 mM) with the lipid composition described in "Results and Discussion." One was the original liposome preparation, small multilamellar liposomes (SML), of Bangham *et al.*[4] with brief sonication (2.5 min). The other was REV, which was prepared by the method of Szoka and Papahadjopoulos.[3] Both procedures were carried out under nitrogen at 0—5° to prevent oxidation of the lipids and inactivation of the enzyme. After standing overnight in a refrigerator, enzyme-containing liposomes were separated from the non-encapsulated enzyme by gel filtration. When elastase was incorporated into liposomes, SML and REV were passed through Sephadex G-200 and Sepharose 4B columns, respectively. In the experiments where glucose was encapsulated, chromatography was performed on Sephadex G-25.

Calculation of Encapsulation Efficiencies——Liposomal suspensions were appropriately diluted with Tris buffer, pH 7.4. Total elastase activity was determined after solubilization with Triton X-100, and the activity of non-encapsulated free enzyme was determined without Triton X-100. % Encapsulation was calculated by means of the following equation.

$$\% \text{ Encapsulation} = \frac{\text{T.A.} - \text{F.A.}}{\text{T.A.}} \times 100$$

Here, T.A. and F.A. mean total elastase activity and free elastase activity, respectively. This calculation gave the same results as gel filtration. In the case of glucose preparations, encapsulation efficiencies were calculated in the same manner as above.

Latency of Liposome-Encapsulated Elastase——Latency of encapsulated elastase was estimated in 0.06M Tris buffer containing 0.12 M NaCl, pH 7.4, as follows. After the separation of liposome-encapsulated elastase from the free species, the liposome fraction was appropriately concentrated by ultrafiltration with a Toyo ULTRA FILTER UK-50 (molecular weight limit, 50000; Toyo Filter Paper Co., Tokyo, Japan) under positive pressure of nitrogen. The concentrated liposomal fraction was incubated at 37° for a definite period, then total activity and free activity were determined, and the percentage of liposome-encapsulated elastase activity (% latency) was estimated.

pH-Profiles of Elastase Stability——Two ml of concentrated liposomal elastate (SML and REV were concentrated to about 10 μg/ml and 50 μg/ml, respectively) was mixed with 8 ml of a buffer solution and the mixture was incubated at 37°. The compositions of the buffer solutions were KCl–HCl for pH 2.0, citric acid–Na_2HPO_4 for pH 3.8, HEPES–NaOH for pH 6.5, and Tris–HCl for pH 7.4 (all these buffer solutions have the same salt concentration, *i.e.*, 25 mM). The pH's of the reaction mixtures were 2.63, 4.84, 6.52, and 7.40, respectively. After incubation for 24 hr, the pH of the mixture was returned to 7.4 by appropriate addition of NaOH solution and the remaining total activity of elastase was determined. In the case of free elastase solution, 10 μg/ml or 50 μg/ml of the elastase solution was examined in a similar manner. The results for 10 μg/ml solution and 50 μg/ml solution were compared with those for SML and REV, respectively.

Time Course of Elastase Inactivation at pH 2.63——The time course of the elastase inactivation was also examined at pH 2.63.

Results and Discussion

First of all, the ability of REV to encapsulate elatase was examined in comparison with that of SML. Table I summarizes the encapsulation efficiencies of liposome preparations with various lipid concentrations. REV increased the encapsulation efficiciency almost 20-fold. The percentage of glucose encapsulated was approximately identical to that of elastase in both preparations. This suggests that encapsulated elastase exists mostly in the aqueous phase of the liposomes, like glucose. As shown in Fig. 1a, the amount of encapsulated elastase increased in proportion to the amount of lipid at a constant ratio of lipid composition. It can be considered that in the case of SML the number of liposome vesicles increased as the total amount of lipid was increased. Therefore, the encapsulation efficiency was proportional to the amount of lipid. In the case of REV, when the amount of lipid was increased, the amount of encapsulated elastase of each preparation varied enormously (see Fig. 1b). It seems that various factors such as viscosity, *etc.*, made it difficult to achieve good reproducibility

TABLE I. Encapsulation of Elastase and Glucose in Liposome Preparations

	Lipid composition PC : Ch : DCP	Encapsulation Elastase	Glucose
SML	80 : 20 : 5	1.5	1.2
	120 : 30 : 7.5	2.1	—
	160 : 40 : 10	2.9	—
REV	80 : 20 : 5	27.4	24.5

Amounts of lipids are expressed in μmol. Abbreviations: PC, egg phosphatidylcholine; Ch, cholesterol; DCP, dicetylphosphate.

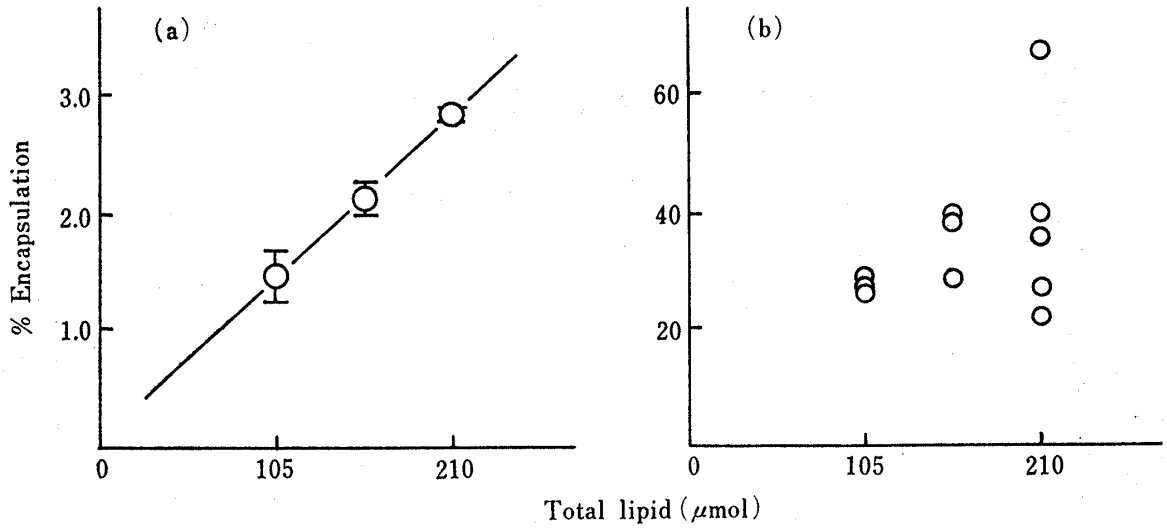

Fig. 1. Effect of Lipid Concentration on the Encapsulation of Elastase in SML (a) or REV (b)

The molar ratio of lipid composition was kept constant (PC: Ch: DCP=80: 20: 5). Values in (a) represent as the means ±S.D. of three experiments.

with REV. On the basis of these findings, subsequent experiments were carried out with SML and REV preparations whose total lipid amounts were 210 μmol and 105 μmol, respectively.

The latency of liposome-encapsulated elastase is shown in Fig. 2. Though the total activity of each preparation was decreased to about 80% of the initial value (data not shown), the latency of SML decreased only slightly and that of REV was not altered at all during incubation for 24 hr at 37°. This result suggests that once encapsulated in liposomes the liposomal elastase is hardly released. Storage in a refrigerator should prevent both the release and the inactivation of the liposome-encapsulated elastase.

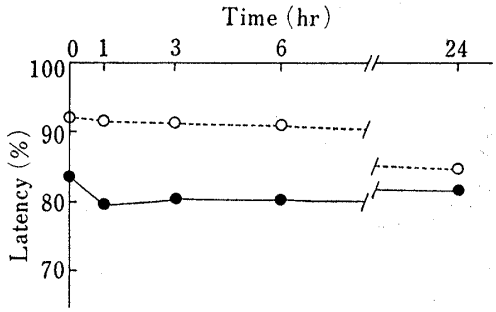

Fig. 2. Latency of Elastase encapsulated in SML or REV at 37°

—○—: SML, —●—: REV.

Next, in order to make clear the differences of physicochemical properties between SML and REV, we tested the effects of modification of the pH 7.4 Tris buffer. The pH-profiles of elastase stability are shown in Fig. 3. In the pH region around neutral, *i.e.*, at pH 7.40, 6.52, and 4.84, elastase in both SML and REV was about 1.5 to 2.5 times more stable than that in free solution. However, at pH 2.63, although the activity of elastase encapsulated

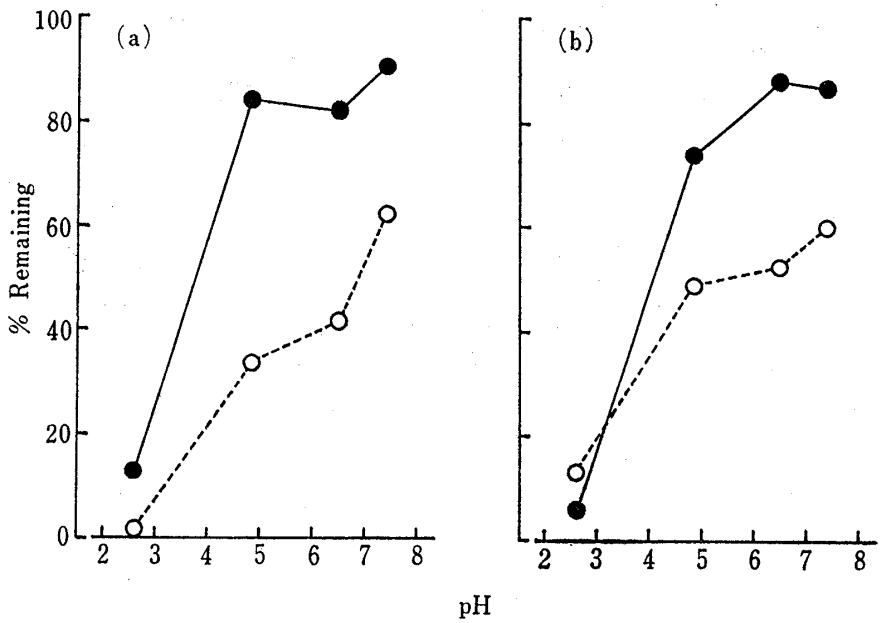

Fig. 3. pH-Profiles of the Stability of Elastase encapsulated in SLM (a) or REV (b)

—●—: encapsulated, ----○----: solution.
The concentrations of elastase in solution were 10 μg/ml in (a) and 50 μg/ml in (b).

in SML was more stable, as in the neutral pH region, that in REV was less stable compared with the free enzyme solution.

In order to clarify the nature of the inactivation processes, the time course of elastase inactivation at pH 2.63 was examined. Fig. 4 shows the results for the free solution (10 μg/ml) and SML, and Fig. 5 shows those for the free solution (50 μg/ml) and REV. In every case, the inactivation of elastase at pH 2.63 seems to be kinetically an apparent biexponential one: a rapid inactivation was observed within 2 min, followed by a slower one. In the case of REV, in contrast to SML, the rapid inactivation was accelerated. About 70% of the total residual activity was present as the encapsulated form after 60 min, which suggests that liposomes retained their integrity under these conditions. Recently, Yotsuyanagi *et al.* reported enhance-

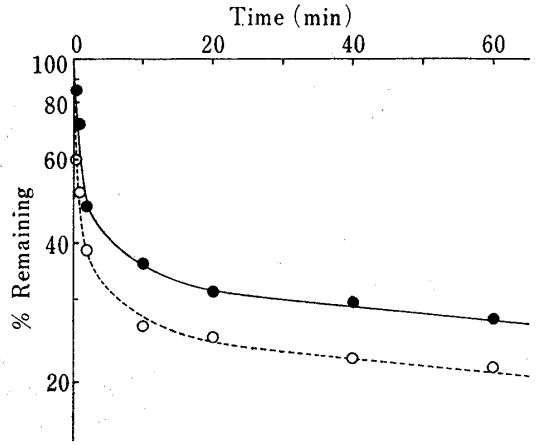

Fig. 4. Time Course of the Inactivation of Elastase encapsulated in SML at pH 2.63 in Comparison with That in Solution

—●—: encapsulated (concentration, about 10 μg/ml) ----○----: solution (concentration, 10 μg/ml)

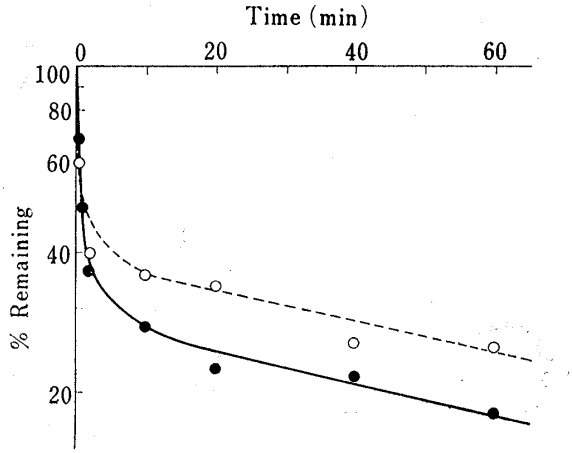

Fig. 5. Time Course of the Inactivation of Elastase encapsulated in REV at pH 2.63 in Comparison with That in Solution

—●—: encapsulated (concentration, about 50 μg/ml) ----○----: soltution (concentration, 50 μg/ml)

ment of the hydrolysis of 2-diethylaminoethyl *p*-nitrobenzoate[7] and *p*-nitrophenyl acetate[8] by liposomes. They explained the former in terms of partition theory and the latter in terms of reaction with lecithin. The mechanism of the enhanced inactivation of elastase by REV remains to be clarified, but it is important to note that the enhancement depends upon the method of preparation of liposomes.

The comparative studies of SML and REV showed that REV provides greatly superior encapsulation and retards the inactivation of elastase in the neutral pH region. Therefore, as Szoka and Papahadjopoulos[3] suggested, the REV method could be very useful for the preparation of carriers to encapsulate not only elastase but also other macromolecules. However, as shown in Fig. 3 or Fig. 5, the REV preparation accelerated the inactivation of the enzyme in the lower pH region, which was not enountered in the case of the SML preparation. The finding that the physicochemical properties of the liposomes depend upon their method of preparation suggests that further and more precise studies on the methods of preparation are required.

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References and Notes

1) B.E. Ryman, R.F. Jewkes, K. Jeyasingh, M.H.N. Tattersall, and D.A. Tyrrell, *Ann. N.Y. Acad. Sci.*, **308**, 281 (1978); G. Gregoriadis, *ibid.*, **308**, 343 (1978); *etc.*
2) C.M. Colley and B.E. Ryman, *Biochim. Biophys. Acta*, **451**, 417 (1976); P.E. Belchetz, I.P. Braidman, J.C.W. Crawley, and G. Gregoriadis, *Lancet*, **2**, 116 (1977); G. Gregoriadis, *Nature* (Lodon), **275**, 695 (1978).
3) F. Szoka, Jr., and D. Papahadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194 (1978).
4) A.D. Bangham, M.M. Standish, and J.C. Watkins, *J. Mol. Biol.*, **13**, 238 (1965).
5) D.N. Rhodes and C.H. Lea, *Biochem. J.*, **65**, 526 (1957).
6) J. Bieth, B. Spiess, and C.G. Wermuth, *Biochem. Med.*, **11**, 350 (1974).
7) T. Yotsuyanagi, T. Hamada, H. Tomida, and K. Ikeda, *Acta Pharm. Suec.*, **16**, 325 (1979).
8) T. Yotsuyanagi and K. Ikeda, *J. Pharm. Sci.*, **69**, 745 (1980).

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The Analgesic Effects of The Decomposition Products of Sulpyrine, N-[2-(5-Hydroxymethyl-2,3,4,5-tetrahydro-2,3,4-trihydroxy)furyl]methyl-N-methylantipyrine and Antipyrinyl-4-peroxide

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The analgesic effects of the decomposition products of sulpyrine, N-[2-(5-hydroxymethyl-2,3,4,5-tetrahydro-2,3,4-trihydroxy)furyl]methyl-N-methylantipyrine (MAAG)and antipyrinyl-4-peroxide (AP), which were reported by us previously, were determined by measurement of the inhibition of writhing caused by the injection of acetic acid into male mice, and compared with the effects of sulpyrine and antipyrine. MAAG and AP showed 62% and 48% of the potency of sulpyrine, respectively, in spite of having hydrophilic substituents. It is suggested that the analgesic effects of these compounds are intrinsic to the compounds themselves.