Encapsulation of Drugs by Lyophilized Empty Dipalmitoylphosphatidylcholine Liposomes: Effect of Calcium Ion

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The effects of divalent cation (Ca^{2+}) on the characteristics of dipalmitoylphosphatidylcholine (DPPC) liposomes regenerated from lyophilized empty liposomes by rehydration and warming were investigated. The results showed that the volume (ml) of internal aqueous compartment per g lipid (captured volume; V_{cap}) has a maximum at a certain concentration range of calcium chloride and the maximal value is more than ten times the minimal value. This phenomenon can be explained by considering that binding of Ca^{2+} to phosphate groups in DPPC molecules induces an increase in the distance (r) between adjacent bilayer membranes in multilamellar liposomes through electrostatic force and causes an increase in V_{cap}. The dynamic properties of lyophilized liposomes in the rehydration process were examined using a multilamellar vesicle model. The results of simulation suggested that a repulsive force induced between the adjacent bilayer membranes causes rearragement of the constituent lipid molecules in a liposome followed by an increase in the distance r, a decrease in the internal lamellar number, a smaller increase in liposome size and finally a significant increase in V_{cap}.

Keywords liposome; captured volume; entrapment; dipalmitoylphosphatidylcholine; calcium ion; liposomal model

A new method to prepare drug-entrapped liposomes by warming (over the phase-transition temperature, T_c) a mixture of freeze-dried empty dipalmitoylphosphatidylcholine (DPPC) liposomes and aqueous drug solution was proposed in the preceding peper.[1] For example, addition (13 μl/mg lipid) of aqueous insulin to lyophilized, empty multilamellar vesicles (MLV) composed of L-dipalmitoylphosphatidyl choline and dipalmitoyl phosphatidylglycerol (10 : 3) and heating of this lipid suspension at 50 °C for 1 min produced insulin-entrapped liposomes with 71% entrapment. This was explained by the fact that freeze-drying of liposomes produces structural defects in the bilayer membranes and heating ($>T_c$) after rehydration reseals these defects.[1]

Recently, studies on the binding of calcium ion (Ca^{2+}) to phospholipid vesicles have been reported by many authors. Most attention has been focused on the fusion of phosphatidylserine (PS)-based vesicles induced by Ca^{2+}.[2-4] On the other hand, Inoko *et al.*[5] and Ohshima *et al.*[6] elucidated by X-ray diffraction studies that a lamellar phase was produced in an aqueous dispersion of DPPC, and the repeat distance of the lamellar structure changed depending on the concentration of calcium chloride ($CaCl_2$).

These findings suggested the possibility that the presence of Ca^{2+} during the rehydration process of lyophilized liposomes might cause an increase in the distance between adjacent bilayer membranes and an expansion of the aqueous compartment of the regenerated liposomes, but such an expansion requires rearrangement of lipid molecules in MLV. Hence, whether Ca^{2+} in the rehydration medium increases the captured volume (V_{cap}) of regenerated liposomes is an interesting question. In this study, the effects of Ca^{2+} in the rehydration medium on the liposomal structure regenerated by rehydration of lyophilized empty DPPC liposomes were examined with the aim of achieving higher efficiency of drug entrapment and a larger V_{cap} of liposomes. The results are discussed in connection with the V_{cap} and the internal structure of liposomes using a MLV model.

Experimental

Materials DPPC was purchased from Avanti Phospholipids, Inc. Dipalmitoylphosphatidic acid (PA) and dicetylphosphate (DCP) were obtained from Sigma Chemical Co. Stearylamine (SA) was purchased from Tokyo Kasei Co. 5-Fluorouracil (5-FU) was obtained from Daikin Industrial Co. $CaCl_2$ of analytical grade was purchased from Merck Co. These lipids and chemicals were used without further purification.

Lyophilized Empty Liposomes The lipid mixture in chloroform solution was dried to a thin film on the wall of a round-bottomed flask by removal of the organic solvent by rotary evaporation. The lipid film was hydrated with water at 50 °C, and the resultant suspension was then frozen in a dry ice–acetone bath and dried by a freeze dryer (Tokyo Rikakikai Co., Ltd., Type FD-80). The lyophilized liposomes were stored at −20 °C until use.

Regeneration of Liposomes by Rehydration Lyophilized liposomes were rehydrated with aqueous solution (40 ml/g lipid) containing 5-FU (5 mg/ml) and various concentrations of $CaCl_2$, then warmed at 50 °C for 5 min. These rehydrated liposomes were diluted with precooled isotonic aqueous sodium chloride solution and separated from free 5-FU by ultracentrifugation (Hitachi SCP70H; twice at 85000 g for 30 min). Size distribution of liposomes was measured with a Coulter Counter T_A and the dispersion state was observed under an optical microscope.

V_{cap} Liposomes encapsulating 5-FU were disrupted by addition of Triton X-100 and the total amount of the released 5-FU was determined by high-performance liquid chromatography (HPLC) under the following conditions; Nucleosil $10C_{18}$, 0.01 M KH_2PO_4, 265 nm. The encapsulation efficiency and the V_{cap} were calculated by means of the following equations:

$$\text{encapsulation efficiency (\%)} = \frac{\text{total amount of 5-FU entrapped in liposomes}}{\text{total amount of 5-FU applied}} \times 100 \quad (1)$$

$$V_{cap} = \frac{\text{encapsulation efficiency} \times \text{volume of 5-FU solution applied}}{\text{total amount of lipid applied} \times 100} \quad (2)$$

where the V_{cap} implies the volume of the aqueous compartment in liposomes if 5-FU is not adsorbed or partitioned in the lipid bilayer. The effects of Ca^{2+} on the characteristics of liposomes regenerated from lyophilized liposomes were evaluated in terms of the bed volume of liposomes precipitated by ultracentrifugation and the V_{cap}.

Results

In Fig. 1, the V_{cap} (ml/g lipid) of the regenerated liposomes is plotted against the concentration of $CaCl_2$ added with 5-FU to the lyophilized empty DPPC liposomes. The V_{cap}–$CaCl_2$ concentration curve has a maximum in the range from 1 to 50 mM $CaCl_2$, indicating that the internal

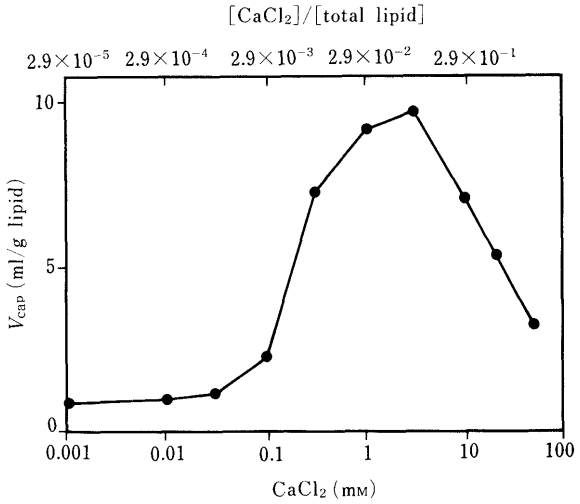

Fig. 1. The V_{cap} of DPPC Liposomes Estimated by Measurement of the Encapsulation Efficiency of 5-FU were Plotted against the Total $CaCl_2$ Concentration in the Rehydration Medium and the Molar Ratio of $CaCl_2$ to Total Lipid

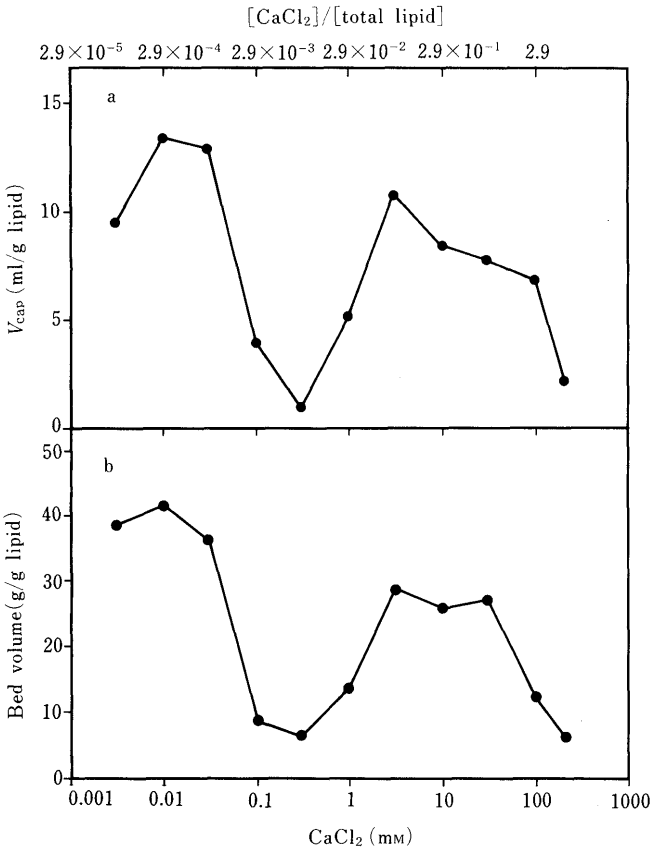

Fig. 2a. The Captured Volumes of Liposomes Composed of DPPC and PA (molar ratio 100 : 1) Estimated by Measurement of the Encapsulation Efficiency of 5-FU were Plotted as a Function of Total $CaCl_2$ Concentration and Molar Ratio of $CaCl_2$ to Total Lipid

2b. The Bed Volumes of Liposomes Composed of DPPC and PA (molar ratio 100 : 1) were Plotted against Total $CaCl_2$ Concentration and Molar Ratio of $CaCl_2$ to Total Lipid

The bed volume was represented as the weight (g) of the liposomal layer separated by sedimentation per g lipid.

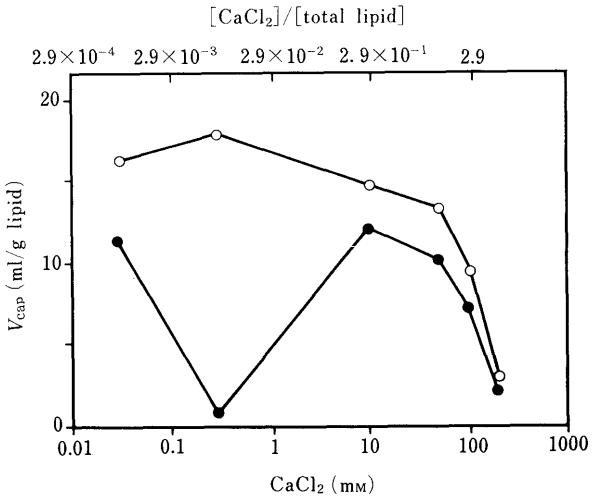

Fig. 3. The Captured Volumes for Negatively Charged Liposomes (●: DCP/DPPC = 1/100) and Positively Charged Liposomes (○: SA/DPPC = 1/100) were Plotted against Total $CaCl_2$ Concentration or Molar Ratio of $CaCl_2$ to Total Lipid.

The captured volumes were estimated by measurement of the encapsulation efficiency of 5-FU.

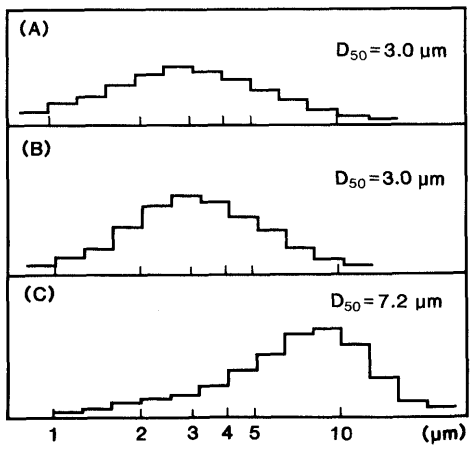

Fig. 4. Size Distribution of PA/DPPC (1 : 100) Liposomes before Freeze-Drying (A) and the Liposomes Regenerated by Rehydration of the Lyophilized Ones at 0.1 (B) and 10 mM (C) $CaCl_2$

structure of the regenerated liposomes was dependent on the medium used for rehydration. Although the equilibrium concentration of free Ca^{2+} was not measured, its value can be estimated from the binding constant of Ca^{2+} to the DPPC membrane described by many authors (for example, a binding constant 21 M^{-1} has been presented by Ohshima and coworkers[6]).

In Fig. 2a, the V_{cap} of the liposomes regenerated from lyophilized negatively charged liposomes (PA/DPPC = 1/100) is plotted against concentration of $CaCl_2$ in the rehydration medium. In contrast with the case of DPPC liposomes shown in Fig. 1, the plotted curve has two maximum regions. This result implies that even a trace amount of charged lipids in liposomes exerts a considerable influence on the structure of the regenerated liposomes. In fact, the liposomes prepared from some commercial DPPC exhibited a similar pattern to that shown in Fig. 2a, suggesting the presence of a negatively charged impurity in DPPC. Figure 2a shows the V_{cap} calculated from the liposomal encapsulation efficiency of 5-FU while Fig. 2b shows the bed volume of liposomes precipitated by centri-

fugation. A similarity of plotted curves between Figs. 2a and 2b indicates that the V_{cap} calculated from the encapsulation efficiency does actually reflect the volume of the aqueous compartment in liposomes. Figure 3 shows V_{cap}–$CaCl_2$ concentration curves obtained for negatively charged liposomes of another composition (DCP/DPPC = 1/100) and positively charged liposomes (SA/DPPC = 1/100). These results show that different charges affect the V_{cap}–$CaCl_2$ concentration curve in different ways.

Figure 4 shows size distributions of PA/DPPC (1 : 100) liposomes used for freeze-drying (A) and the liposomes regenerated by rehydration of the lyophilized ones at 0.1 mM (B) and 10 mM (C) $CaCl_2$. These samples, the size distributions of which were measured with a Coulter counter, were observed to be almost monodispersed under an optical microscope. The values of the mean diameter (D_{50}) were 3.0, 3.0 and 7.2 μm, respectively.

Discussion

The following explanations for the dramatic effects of Ca^{2+} on the characteristics of liposomes reintegrated by rehydration of lyophilized liposomes are put forward on the basis of a comparison of Figs. 1, 2 and 3. In the low $CaCl_2$ concentration range (<0.1 mM), as shown in Fig. 2, mutual electrostatic repulsion of the negative charge involved in the liposomal bilayer membranes can increase the interstitial distance between adjacent bilayer membranes in MLV and can expand the aqueous compartment of the liposomes. With an increase in $CaCl_2$ concentration, binding of Ca^{2+} to negatively charged liposomes (Fig. 2) brings about a decrease in the V_{cap}, resulting in the minimum around 0.3 mM $CaCl_2$, then this is followed by an increase in the V_{cap} in the concentration range from 0.3 to 3 mM, which might reflect an increase in the cationic charge by binding of Ca^{2+} to electrically neutral DPPC. An increase in the V_{cap} in the same $CaCl_2$ concentration range was also observed in the case of DPPC liposomes (Fig. 1), but not in the case of positively charged liposomes (Fig 3). A subsequent decrease in the V_{cap} in the high $CaCl_2$ concentration range (>30 mM) can be explained by a decrease in electrostatic force with increase in the ionic strength.

The remarkable expansion of the internal aqueous compartments of the liposomes by electrostatic repulsion as mentioned above appears to require rearrangement of the internal structure of the DPPC-based liposomes during the rehydration process of the lyophilized liposomes. The plausibility of this rearrangement was further examined by using the following model. If the internal structure of MLV is postulated to be concentric, as shown in Fig. 5, the total volume of the lipid phase (V_1), the total volume of aqueous compartment (V_a) and the V_{cap} of a liposome can be calculated by using Eqs. 3, 4, and 5, respectively,

$$V_1=(2\pi d/3)[a^2n(n+1)(2n+1)-3a\cdot d\cdot n(n+1)+2d^2n] \quad (3)$$

$$V_a=(2\pi/3)[a^2(a-d)n(n+1)(2n+1)-3a(a^2-d^2)n(n+1)+2n(a^3-d^3)] \quad (4)$$

$$V_{cap}=\frac{V_a\cdot\Delta V_m\cdot N_A}{V_1} \quad (5)$$

where a is the repeat distance of the multilamellar structure, d is the thickness of the bilayer membrane, n is the number of membranes and N_A is Avogadro's number. The membrane thickness (d) and phospholipid specific volume (ΔV_m) are assumed to be 37 Å and 1253 Å^3/molecule, respectively,

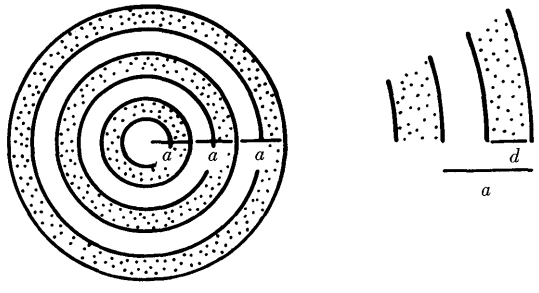

Fig. 5. Model of MLV

TABLE I. V_{cap} Calculated Using a Liposomal Model (Fig. 5) and Eq. 5

Diameter (nm)	Liposome	a (Å)	n	V_1 (Å^3)	V_a (Å^3)	V_{cap} (μl/μmol)
20	SUV	100	1	3.14×10^6	1.05×10^6	0.25
40	SUV	200	1	1.54×10^7	1.81×10^7	0.89
80	SUV	400	1	6.77×10^7	2.00×10^8	2.23
200	LUV	1000	1	4.48×10^8	3.74×10^9	6.30
500	LUV	2500	1	2.86×10^9	6.26×10^{10}	16.49
1000	LUV	5000	1	1.15×10^{10}	5.12×10^{11}	33.49
2000	LUV	10000	1	4.63×10^{10}	4.14×10^{12}	67.48
3000	LUV	15000	1	1.04×10^{11}	1.40×10^{13}	101.47
300	MLV	150	10	3.89×10^9	1.02×10^{10}	1.99
600	MLV	150	20	2.95×10^{10}	8.36×10^{10}	2.14
1200	MLV	150	40	2.30×10^{11}	6.75×10^{11}	2.22
1800	MLV	150	60	7.67×10^{11}	2.29×10^{12}	2.25
2400	MLV	150	80	1.81×10^{12}	5.43×10^{12}	2.26
3000	MLV	150	100	3.53×10^{12}	1.06×10^{13}	2.27
6000	MLV	150	200	2.81×10^{13}	8.50×10^{13}	2.29
9000	MLV	150	300	9.45×10^{13}	2.87×10^{14}	2.29
1500	MLV	1500	5	5.72×10^{10}	1.71×10^{12}	22.58
3000	MLV	1500	10	4.01×10^{11}	1.37×10^{13}	25.82
6000	MLV	1500	20	3.00×10^{12}	1.10×10^{14}	27.72
9000	MLV	1500	30	9.88×10^{12}	3.72×10^{14}	28.40

TABLE II. The Relationship between the Internal Structure and V_{cap} Calculated Using a Liposomal Model for Liposomes Having the Size Shown in Fig. 4

Diameter (nm)	Liposome	a (Å)	n	V_1 (Å^3)	V_a (Å^3)	V_{cap} (μl/μmol)
3000	MLV	45	333	1.16×10^{13}	2.50×10^{12}	0.16
		50	300	1.05×10^{13}	3.66×10^{12}	0.26
		60	250	8.74×10^{12}	5.40×10^{12}	0.47
		70	214	7.50×10^{12}	6.64×10^{12}	0.67
		75	200	7.00×10^{12}	7.14×10^{12}	0.77
		80	188	6.57×10^{12}	7.57×10^{12}	0.87
		90	167	5.85×10^{12}	8.30×10^{12}	1.07
		100	150	5.26×10^{12}	8.87×10^{12}	1.27
		125	120	4.22×10^{12}	9.92×10^{12}	1.77
		150	100	3.53×10^{12}	1.06×10^{13}	2.27
7200	MLV	400	90	1.84×10^{13}	1.77×10^{14}	7.28
		450	80	1.63×10^{13}	1.79×10^{14}	8.27
		500	72	1.47×10^{13}	1.81×10^{14}	9.25
		550	66	1.35×10^{13}	1.82×10^{14}	10.23
		600	60	1.23×10^{13}	1.83×10^{14}	11.20
		650	55	1.14×10^{13}	1.84×10^{14}	12.17
		720	50	1.03×10^{13}	1.85×10^{14}	13.52
		800	45	9.33×10^{12}	1.86×10^{14}	15.05
		1000	36	7.52×10^{12}	1.88×10^{14}	18.85
		1200	30	6.32×10^{12}	1.89×10^{14}	22.58
		1500	24	5.12×10^{12}	1.90×10^{14}	28.06

according to Enoch and Strittmatter.[7] V_{cap} (μl/μmol) calculated for several hypothetical values of a, d and n are shown in Table I. From this calculation, it is obvious that V_{cap} of unilamellar vesicle (SUV and LUV) increases dramatically with particle size, while V_{cap} for MLV is nearly constant against particle size but depends markedly on the repeat distance (a).

Table II shows the relationship between the internal structure and V_{cap} for the liposomes having the size shown in Fig. 4. The calculated values close to the experimental measurements ($V_{cap}=9.7$ and $0.92\,\mu$l/mg) for large ($D_{50}=72000$ Å) and small ($D_{50}=30000$ Å) liposomes are $a=400$ Å, $n=90$, $V_1=1.84\times10^{13}$ Å^3, $V_{cap}=7.28\,\mu$l/μmol ($=9.90\,\mu$l/mg) and $a=70$ Å, $n=214$, $V_1=7.50\times10^{12}$ Å^3, $V_{cap}=0.67\,\mu$l/μmol ($=0.91\,\mu$l/mg), respectively. However, in this case, the V_1 of large liposomes is about 2.4 times more than that of small ones, which suggests that fusion between liposomes might occur during the freeze-drying and rehydration process. On the other hand, it is likely that the 5-FU-containing liposomes (especially the large liposomes) lose some of their contents in the processes of separation of liposomes from free 5-FU and washing. If V_1 is constant, $V_1=7.50\times10^{12}$ Å^3, $a=70$ Å, $n=214$, $V_{cap}=0.67\,\mu$l/μmol ($=0.91\,\mu$l/mg) and $V_1=7.52\times10^{12}$ Å^3, $a=1000$ Å, $n=36$, $V_{cap}=18.8\,\mu$l/μmol ($=25.6\,\mu$l/mg) can be considered as the calculated values corresponding to the experimental measurements. In this case, the gap between experimental and calculated V_{cap} might be assigned to underestimation of the experimental V_{cap} (in particular for the large liposomes). The actual structures of large and small rehydrated liposomes can be considered as intermediate between the two cases mentioned above. These calculations suggest that the dramatic difference in V_{cap} shown in Figs. 1—3 can be mainly attributed to a decrease in the number of the internal lipid bilayers and to an increase in the space between the adjacent layers during the rehydration process.

In summary, the experimental results indicate that Ca^{2+} in the rehydration medium produces a remarkable increase in the V_{cap} of the liposomes regenerated by rehydration of lyophilized DPPC liposomes at a certain concentration range of $CaCl_2$. On the other hand, the results from the simulation using a liposomal model show that the V_{cap} of MLV is dependent only slightly on the liposomal size but mainly on the interstitial distance. It follows from these findings that Ca^{2+} binding at an adequate ratio to phospholipid molecules of DPPC liposomes during regeneration from lyophilized liposomes causes an increase in the interstitial distance accompanied with an enlargement of the aqueous compartments, which implies that a dynamic and drastic change occurs in the internal structure depending on $CaCl_2$ concentration. Proper addition of Ca^{2+} to the rehydration medium is an effective means to get high entrapment efficiency in the liposome preparation method which we have presented.[1] Other additives than Ca^{2+} are also likely to influence the internal structure of liposomes prepared in the same manner as described above. Studies on such additives are in progress.

References

1) H. Jizōmoto, E. Kanaoka and K. Hirano, *Chem. Pharm. Bull.*, **37**, 1895 (1989).
2) D. Papahadjopoulos, W. J. Vail, K. Jacobson and G. Poste, *Biochim. Biophys. Acta*, **394**, 483 (1975).
3) D. Papahadjopoulos, U. S. Patent Appl. 701190 (1976) [*Chem. Abstr.*, **86**, 78698 (1977)].
4) D. Papahadjopoulos, W. J. Vail, C. Newton, S. Nir, K. Jacobson, G. Poste and R. Lazo, *Biochim. Biophys. Acta*, **465**, 579 (1977).
5) Y. Inoko, T. Yamaguchi, K. Furuya and T. Mitsui, *Biochim. Biophys. Acta*, **413**, 24 (1975).
6) H. Ohshima, Y. Inoko and T. Mitsui, *J. Colloid Interface Sci.*, **86**, 57 (1982).
7) H. G. Enoch and P. Strittmatter, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 145 (1979).