Biochimica et Biophysica Acta, 729 (1983) 229–236
Elsevier Biomedical Press

BBA 71593

A NEW METHOD OF VESICLE FORMATION BY SALTING-OUT AND ITS APPLICATION TO THE RECONSTITUTION OF SARCOPLASMIC RETICULUM

TAKAHISA TAGUCHI and MICHIKI KASAI

Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560 (Japan)

(Received November 1st, 1982)

Key words: Liposome; Salting-out; Triton X-114; Membrane reconstitution; (Sarcoplasmic reticulum)

This paper describes a new method of forming membrane vesicles. It was found that the addition of salt such as KCl into a solution containing lipid (asolectin) and a non-ionic surfactant, Triton X-114, led to the formation of closed membrane vesicles. The vesicles were separated from Triton X-114 by hydrophobic interaction chromatography. Electron microscopy revealed that the mean diameter of the vesicles was 110 nm ± 69 nm (S.D.). Measurement of osmotic volume change showed that the permeability of the vesicle was very low to salts, sugar (glucose) and amphoteric ion (glycine), but very high to glycerol, ethylene glycol and water. Vesicle formation by this 'salting-out' method is very useful for reconstitution of transport systems in biomembranes because of its advantages: completion within a short time; high yield; and the possibility of utilizing samples in non-ionic surfactant solution. When we applied the method to the reconstitution of sarcoplasmic reticulum, Ca^{2+}-ATPase was incorporated into the reconstituted vesicles and was enzymatically active in the membrane.

Introduction

Reconstitution of transport systems in biomembranes is an elegant approach to investigation of their molecular mechanisms. In the first state of the reconstitution experiments, the proteins bound to the membrane must be solubilized and purified with surfactant. Non-ionic surfactants such as Triton X-100 are more appropriate in this state than ionic ones, because the proteins solubilized in non-ionic surfactant solution can be purified by ion-exchange chromatography. In the last decade, successful reconstitution experiments have been reported: Ca^{2+}-ATPase [1,2]; H^+-ATPase [3,4]; glucose transporter [5]; acetylcholine receptor [6,7]; and so forth. However, we can find only a few cases of the reconstitution with Triton X-100. In the case of Kasahara and Hinkle [5], Triton X-100 in solubilizing solution was removed with Bio-beads SM-2 before the incorporation of purified protein into membrane. This is because the formation of vesicles is made difficult by the large micelle size and the low critical micelle concentration of Triton X-100.

As mentioned by Maclay [8], non-ionic surfactants such as the Triton X series in solution form large aggregations and they make the solution turbid above a well-defined temperature; this temperature is called the 'cloud point'. Recently, Bordier [9] reported that the phase separation in solutions of the non-ionic surfactant, Triton X-114, separated integral membrane proteins from hydrophilic ones. The low cloud point of Triton X-114 at about 20°C made this separation possible. We investigated the possibility of utilizing Triton X-114 for the formation of membrane vesicles.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

In this paper, we described a new method to form membrane vesicles. It was found that an addition of salt to the solutions containing Triton X-114 and lipid forms membrane vesicles and this phenomenon can be utilized to reconstitute a transport system in biomembranes. The proteins solubilized and purified with non-ionic surfactant such as Triton X-100 are directly incorporated into the reconstituted membrane vesicles by the addition of salt.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit dorsal and hind leg muscle as a microsomal fraction according to Weber et al. [10] with slight modification [11]. This was stored in 100 mM KCl and 5 mM Tris-maleate (pH 6.5) at 0°C and washed 5 mM sodium Hepes (pH 7.4) before use.

ATP (sodium salt) and *Naja naja* snake venom were purchased from Sigma Chemical Co. (U.S.A.). Phospholipase A was prepared from the venom according to Fleischer and Fleischer [12]. Triton X-114 was purchased from Nakarai Chemical Co. (Kyoto, Japan), and asolectin from Daigo Chemical Co. (Osaka, Japan). The other chemicals used were of reagent grade.

An ω-amino-*n*-octyl derivative of Sepharose 4B was prepared by the reaction of Sepharose 4B activated with BrCN and 1,8-diaminooctane (1 mmol/ml wet volume of Sepharose 4B) according to the manual of Pharmacia Fine Chemicals, referring also to Cuatrecasas [13].

The osmotic volume change of vesicles was investigated by measuring the scattered-light intensity (450 nm) according to Kometani and Kasai [14]. SDS polyacrylamide gel electrophoresis was performed according to Fairbanks et al. [15]. Ca^{2+} uptake was measured by the Millipore method using $^{45}Ca^{2+}$ and a Millipore filter (0.22 μm) as described by Kasai and Miyamoto [11]. The activity of Ca^{2+}-ATPase was assayed for 5 min at 37°C and the inorganic phosphate was determined colorimetrically [16]. The concentration of protein was determined by the method of Lowry et al. [17], using bovine serum albumin as a standard.

Results

Cloud point of Triton X-114 solution in various conditions

A solution of a non-ionic surfactant became turbid at the cloud point when the solution is heated. A solution of 1% (v/v) Triton X-114/5 mM sodium Hepes (pH 7.4) has a cloud point at about 20°C. But this cloud point disappeared when lipid was added, as shown in Fig. 1.

The addition of 200 mM (final) NaCl made the 1% (v/v) Triton X-114 solution with lipid turbid at 20°C as well as that without lipid, as shown in Fig. 2 (solid circles). But the appearance of the solutions was different, depending on the lipid concentration. The solutions containing lipid at less than 3 mg/ml were light gray and became clear with the decrease in temperature in the ice bath. On the other hand, those containing lipid at more than 5 mg/ml were ivory-white and remained turbid with the decrease in temperature. These results suggest that the structures produced by the addition of salt are different in these two cases.

We next tried to separate the two different materials. As shown in Fig. 2 (open circles), the

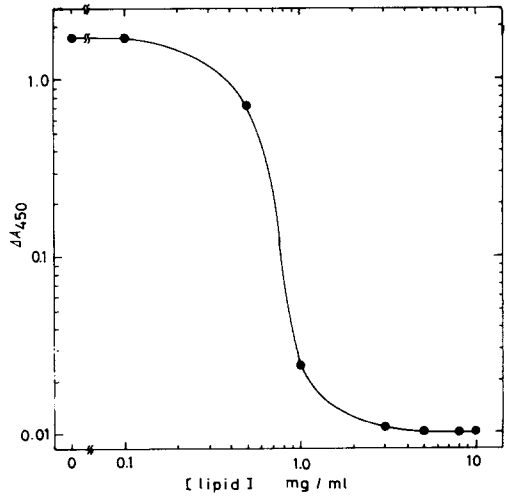

Fig. 1. Effect of lipid on the cloud point of the solution of 1% (v/v) Triton X-114. The increments of absorbance at 450 nm were measured when the temperature in the solution containing 1% (v/v) Triton X-114, different concentrations of asolectin, and 5 mM sodium Hepes (pH 7.4) was changed from 20°C to 30°C. The mixture of asolectin and Triton X-114 was stirred by a vortex mixer.

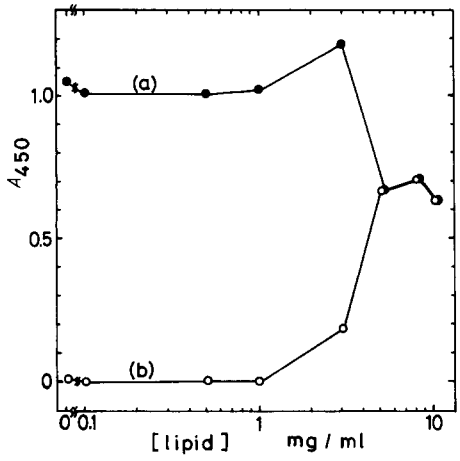

Fig. 2. Effect of lipid of the turbidity change of the solution of Triton X-114 and NaCl. (a) Absorbance measured at 20°C after the addition of 200 mM (final) NaCl into the same solution in Fig. 1. (b) Absorbance of the supernatant solution measured at 20°C after the centrifugation (1300 × *g*, for 10 min). The value of the background, or the absorbance before the addition of NaCl, was subtracted from each datum.

supernatants obtained by low-speed centrifugation of those solutions containing lipid at less than 3 mg/ml were clear, but those of the solutions of more than 5 mg lipid/ml were still turbid. Further experiments were performed with a solution of 1% (v/v) Triton X-114 with 8 mg lipid/ml, and that without lipid, as shown in Table I. In the case of the solution without lipid, Triton was eliminated by the first centrifugation. The supernatant after the centrifugation hardly contained Triton X-114 and no longer had the cloud point. On the contrary, in the case with lipid, Triton X-114 still remained in the supernatant after the second centrifugation, though the supernatant was clear due to the precipitation of material. Accordingly, this supernatant became turbid after heating. This turbidity was due to the large aggregation of Triton X-114, since it could be separated by the third low-speed centrifugation. These results indicate that the material obtained by the addition of salt into the Triton X-114 solution with lipid was due not to the aggregation of Trition X-114, but of lipid.

TABLE I

SEPARATION OF TRITON X-114 AND LIPID

200 mM (final) NaCl was added into the solution containing 1% (v/v) Triton X-114 and 5 mM sodium Hepes (pH 7.4) with or without 8 mg/ml asolectin. The turbidity was measured at 450 nm and the concentration of Triton X-114 (%, v/v) was measured at 280 nm after appropriate dilution with distilled water to avoid interference with turbidity. Sup-*i* in the table means the supernatant after the *i*th centrifugation. First and third centrifugations: 1300 × *g* for 10 min at 30°C. Second and fourth centrifugations: 100000 × *g* for 60 min at 4°C. The pellet from each centrifugation was discarded. Sample temp. is the temperature at which the turbidity measurement was carried out.

Sample	Sample temp. (°C)	− lipid		+ lipid	
		A_{450}	Triton X-114	A_{450}	Triton X-114
Solution	30	1.50	1.00	0.990	1.00
Sup-1	30	0.00	0.054	1.00	0.869
Sup-2	4	0.00	0.054	0.095	0.671
	30	0.00	0.054	1.60	0.671
Sup-3	30	0.00	0.041	0.210	0.336
Sup-4	4	0.00	0.042	0.034	0.220

Elimination of Triton X-114 from aggregation of lipid

The aggregated lipid could be separated, to some extent, from Triton X-114 by high speed centrifugation (100000 × *g*, for 60 min at 4°C), but the co-precipitated Triton X-114 remained with the lipid (Table I). This contaminated Triton X-114 could be lowered nearly to 0.01% (v/v) after washing ten times with 10 vol. 5 mM sodium Hepes (pH 7.4) (data not shown).

For better elimination of Triton X-114, hydrophobic interaction chromatography was used. This procedure could eliminate more than 99% of Triton X-114 by the direct application of the turbid solution of Triton X-114, lipid and salt, and was completed within 15 min. As shown in Fig. 3, the concentration of Triton X-114 could be reduced finally to 0.001% (v/v) by followed several washings.

Formation of closed vesicles by the addition of salt

The structure of the aggregated lipid, which was formed by the addition of 200 mM (final) KCl into mixture of 1% (v/v) Triton X-114, 8 mg/ml asolectin and 5 mM sodium Hepes (pH 7.4), was confirmed to comprise closed membrane vesicles by electron microscopy (Fig. 4). The vesicles were relatively large, their mean diameter being 110

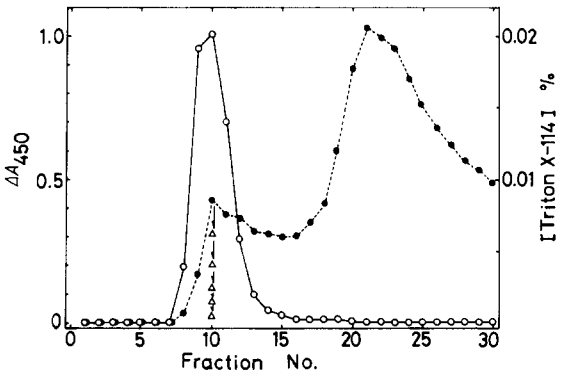

Fig. 3. Hydrophobic interaction chromatography. The solution (10 ml) with 1% (v/v) Triton X-114, 200 mM KCl, 8 mg/ml asolectin and 5 mM sodium Hepes (pH 7.4) was applied to a column (2.2×30 cm) with the ω-amino-*n*-octyl derivative of Sepharose 4B. The flow rate was 4 ml/min. This chromatography was carried out at 20°C. The turbidity (solid line) and the Triton X-114 concentration (dotted line) of each fraction (3 ml) were measured at 450 nm and 280 nm, respectively. The absorbance at 280 nm of the turbid fraction was measured after centrifugation (100000× *g*, for 60 min) at 20°C. Triangles and arrows in the figure show that the concentration of Triton X-114 in the No. 10 fraction decreased with repetitive washing by centrifugation (100000× *g*, for 60 min) at 4°C.

nm ± 69 nm (S.D.) for 1693 vesicles in 12 pictures. Most of vesicles were surrounded by unilamellar membrane, but many vesicles included smaller vesicles within them.

It was found that the membrane of these vesicles was semipermeable. As shown in Fig. 5, the change of the scattered light intensity was observed after an increase in the osmolarity of the solution. According to Kometani and Kasai [14], the increase in the scattered light intensity corresponds to the shrinkage of the vesicles due to the outflow of water caused by the osmotic pressure difference across the membrane, and the following decrease corresponds to their swelling due to the water

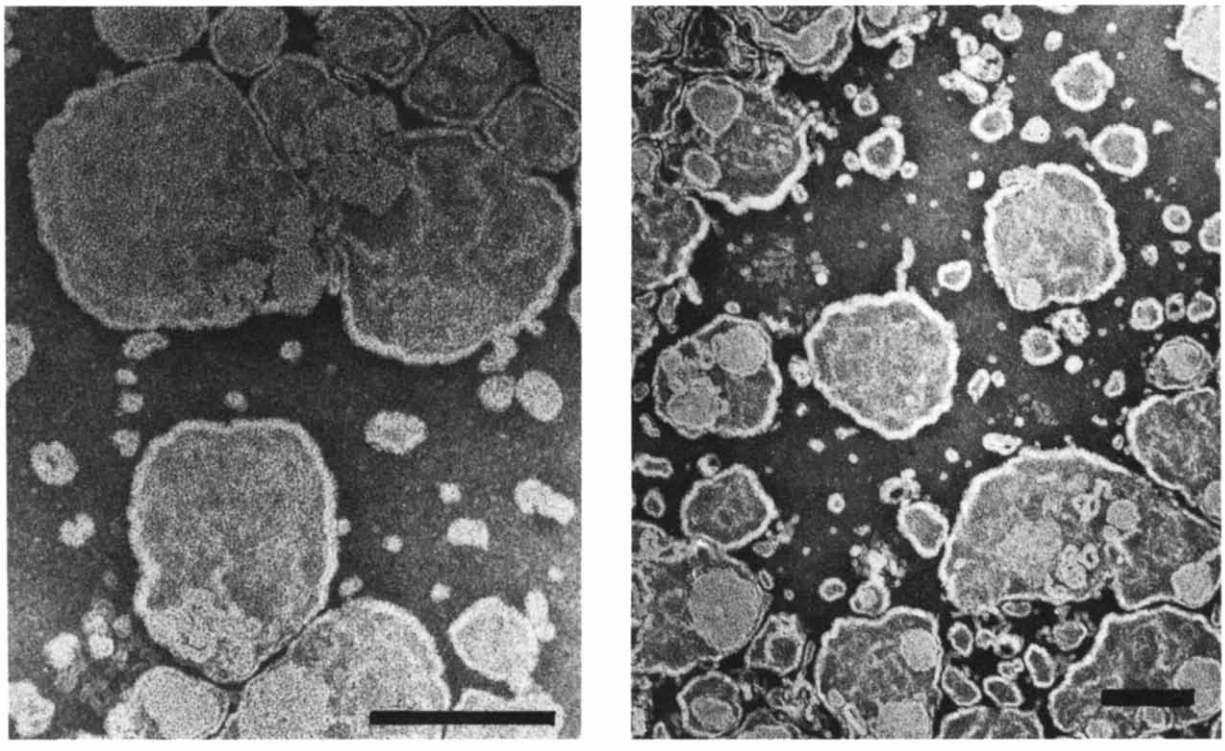

Fig. 4. Electron micrographs of vesicles formed by the salting-out method. The vesicles were formed by the addition of 200 mM (final) KCl into the solution containing 8 mg/ml asolectin, 1% (v/v) Triton X-114 and 5 mM sodium Hepes (pH 7.4). Triton X-114 in vesicle solution was eliminated by hydrophobic interaction chromatography as shown in Fig. 3, and then washed three times with 5 mM sodium Hepes (pH 7.4). Samples were negatively stained with uranyl acetate. Bars represent 200 nm.

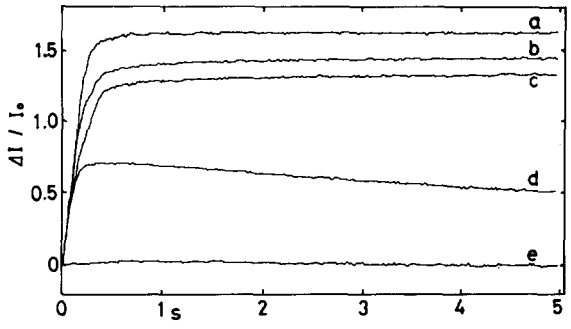

Fig. 5. Osmotic volume change of vesicles. The vesicles were formed as described in the legend of Fig. 4 and suspended in the same buffer overnight at 4°C. These vesicle solutions ($A_{450} = 0.40$) were mixed at 23°C with an equal volume of 5 mM sodium Hepes buffer containing various solutes. Then the changes in the intensity of the scattered light in these mixed solution were measured at 450 nm by a stopped-flow apparatus. The ordinate indicates the ratio of the increment of the intensity of scattered light (ΔI) to the intensity before the shrinkage of the vesicles (I_0); it indicates the degree of shrinkage of the vesicles. (a) 50 mM KCl, (b) 100 mM glycine, (c) 100 mM glucose, (d) 100 mM glycerol, (e) 100 mM ethylene glycol (final concentrations).

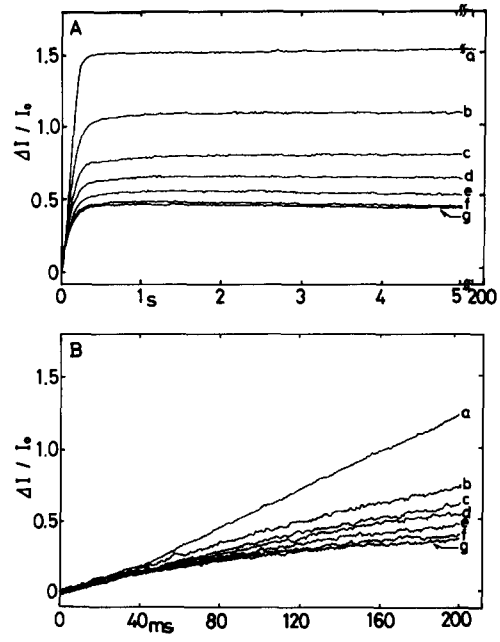

Fig. 6. Osmotic volume change of vesicles treated with phospholipase A. The vesicles were formed and washed as described in the legend to Fig. 5. Phospholipase A (final 5 μg/ml) was added into the vesicle solution ($A_{450} = 0.40$) at 23°C. Osmotic volume changes were measured as described in Fig. 5 after the mixing the vesicle solution with the equal volume of 100 mM (final 50 mM) KCl. The measurements were carried out at (b) 1.5 min, (c) 3.5 min, (d) 7 min, (e) 11 min, (f) 20 min, (g) 30 min, after the addition of phospholipase A. (a) control without phospholipase A. The ordinates are the same as in Fig. 5.

inflow accompanied with the inflow of molecules or ions.

Fig. 5, therefore, indicates that the membrane of the vesicles has no permeability to salt (KCl), amphoteric ion (glycine), and sugar (glucose), but has high permeability to glycerol, ethylene glycol and water. In the experiment of Fig. 5(a), valinomycin exerted no effect on the curve (data not shown). This shows that the permeability to Cl^- is also low. In the case of ethylene glycol, the shrinkage of the vesicles did not take place, as shown in Fig. 5(e), which means that the permeability to ethylene glycol is comparable with that to water. When the elimination of Triton X-114 from the vesicle solution was not sufficient, the permeability to sugar remained high. For example, when the same experiment as shown in Fig. 5 was performed with the vesicles formed by addition of salt and washed twice, the permeability of membrane to KCl was very low, as in Fig. 5(a), but that to glucose was as high as that to glycerol shown in Fig. 5(d) (data not shown).

Further, it was confirmed by the experiment shown in Fig. 6 that the curves in Fig. 5 corresponded to the permeability of membrane vesicles composed of lipid. The degree of shrinkage of the vesicles decreased as the reaction of phospholipase A proceeded. This phenomenon can be explained as follows; the membrane attacked by phospholipase A became as leaky that the vesicles did not respond any longer to the difference in osmotic pressure Fig. 6(B) indicates that the changes of shrinkage levels in Fig. 6(A) were not caused by artificial drifts.

Formation of vesicles by the addition of salt under various conditions

The dependence of vesicle formation on NaCl concentration was studied as illustrated in Fig. 7. More than 100 mM NaCl was required to obtain the maximal increment of absorbance. Similar experiments were carried out by using various kinds of salts (Table II). Neutral molecules, such as

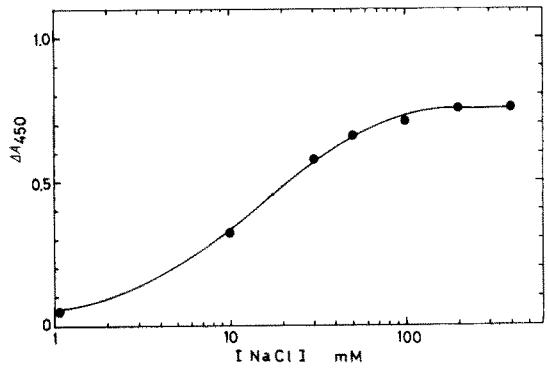

Fig. 7. Vesicles formations at various concentrations of NaCl. The increments of absorbance (450 nm) of the solution of 8 mg/ml asolectin, 1% (v/v) Triton X-114 and 5 mM sodium Hepes (pH 7.4) were measured at 20°C after the addition of concentrated NaCl solution.

sucrose (final 400 mM) or urea (final 400 mM), were not effective on the vesicle formation (data not shown). We, therefore, name this new method of forming vesicles by addition of salt, the 'salting-out method'.

TABLE II

VESICLE FORMATION BY ADDITION OF VARIOUS KINDS OF SALTS

The experiments as described in Fig. 7 were carried out on various kinds of salts. The maximum increment of absorbance at 450 nm and the concentration of salt at the half increment of absorbance are listed for each salt. KMS, potassium methanesulfonate.

Salt	A_{450} maximum	[Salt] (mM)
NaCl	0.750	13.5
KCl	0.790	11.0
K_2SO_4	0.890	5.1
KNO_3	0.690	7.5
KBr	0.710	7.8
KI	0.640	7.2
K_2oxalate	0.940	3.4
KSCN	0.580	5.3
KMS	0.840	9.0
KF	0.920	13.0
Choline-Cl	0.680	12.0
Tris-HCl	0.630	2.8
LiCl	0.830	9.6
CsCl	0.840	11.0
$MgCl_2$	0.680	0.95
$CaCl_2$	0.510	0.9

The yield of vesicles was virtually invariant in the range of biochemically important pH (5–10), but increased with the temperature from 0°C to 30°C (data not shown). Effects on the contamination of other surfactants upon the formation of vesicles by the salting-out method are shown in Fig. 8. The high concentration of the second surfactant prevents vesicle formation. These data are important in the reconstitution experiments for the selection of the suitable surfactant and of its concentration to solubilize and to purify the membrane-bound proteins.

The application of salting-out method to the reconstitution of sarcoplasmic reticulum mambrane

The reconstitution of sarcoplasmic reticulum vesicles by the salting-out method was performed as follows. The solution of sarcoplasmic reticulum vesicles (12 mg protein/ml), 1.0% (v/v) Triton X-100 and 5 mM sodium Hepes (pH 7.4) and the

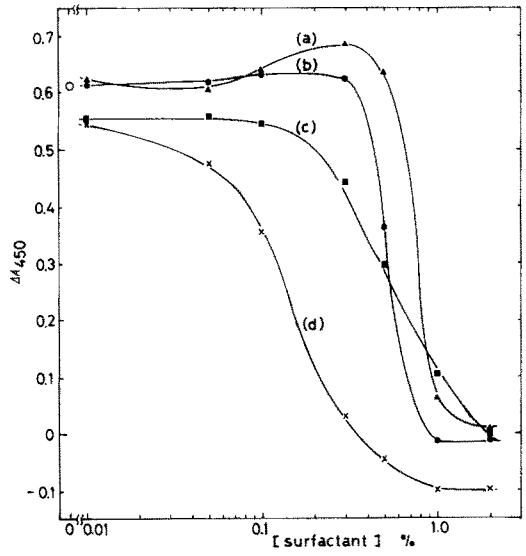

Fig. 8. Effect of the second surfactant on the vesicle formation by the salting-out method. The change of absorbance at 450 nm was measured at 20°C when 200 mM (final) NaCl was added into the solution containing 8 mg/ml asolectin, 1% (v/v) Triton X-114, 5 mM sodium Hepes (pH 7.4) and various concentrations of the second surfactants: (a) Nonidet P-40, (b) Triton X-100, (c) Lubrol PX, (d) sodium cholate. The abscissa indicates the concentration of the second surfactants. The unit of the concentration is % (v/v) for the non-ionic surfactants (Nonidet P-40, Triton X-100 and Lubrol PX) and % (w/v) for ionic surfactant (sodium cholate).

solution of 8 mg/ml asolectin, 1.0% (v/v) Triton X-114 and 5 mM sodium Hepes (pH 7.4) were centrifuged (100000 × g, for 60 min) separately at 4°C to remove unsolubilized materials. The supernatant of the former solution (1.5 ml) and that of the latter solution (13.0 ml) were mixed at 20°C, and then concentrated KCl (or oxalate) was added into it (final concentration 200 mM). This turbid mixture with salt was applied to a column (2.2 × 30 cm) containing ω-amino-*n*-octyl derivative of Sepharose 4B which has been washed with 200 mM KCl (or oxalate) and 5 mM sodium Hepes (pH 7.4), and then eluted with the same solution. The turbid fractions were collected and washed three times with a suitable buffer solution by centrifugation (100000 × g, for 60 min) to remove the residual Triton X-114. The reconstituted vesicles obtained in this way contained 40–60% (w/w) of the total proteins of the sarcoplasmic reticulum vesicles. The proteins incorporated into the membrane of the reconstituted vesicles are represented in Fig. 9. Ca^{2+}-ATPase (integral protein) was incorporated into the membrane, but calsequestrin (peripheral protein) was not. Some proteins of molecular weight more than 100000 were also incorporated into the reconstituted vesicles.

As shown in Fig. 10, the membrane of the reconstituted vesicles was more permeable to KCl than the membrane containing no protein (compare with Fig. 5(a)). The reconstituted sarcoplasmic reticulum vesicles prepared by the addition of oxalate and washed with 300 mM KCl and 5 mM sodium Hepes (pH 7.4) three times could hydrolyze ATP (363 nmol ATP/min per mg protein) and take up Ca^{2+} (+ ATP: 115 nmol Ca^{2+}/min per mg protein; −ATP: 5.3 nmol Ca^{2+}/min per mg protein). These results show that the ATPase was reconstituted functionally with respect to Ca^{2+} transport. But activities are lower than those reported by Zimniak and Racker [1]. This may be due to the unsuitable ratio of protein to lipid in reconstituted vesicles.

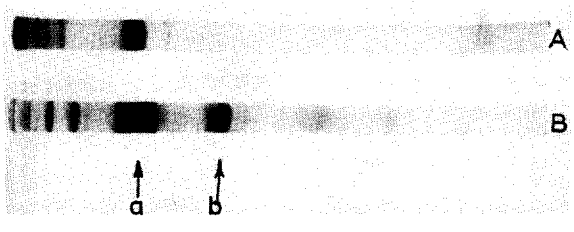

Fig. 9. SDS polyacrylamide gel electrophoresis. B. Native sarcoplasmic reticulum vesicles (40 μg protein). A. Reconstituted sarcoplasmic reticulum vesicles (40 μg protein). 7.5% (w/v) polyacrylamide gels were run and stained with Coomassie blue R. Band a and band b indicate Ca^{2+}-ATPase (molecular weight 100000) and calsequestrin (55000), respectively.

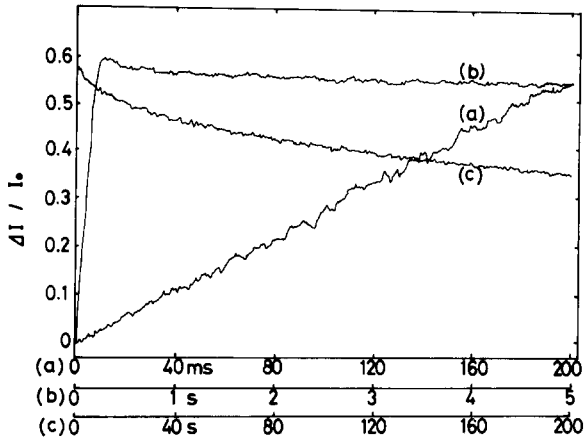

Fig. 10. Osmotic volume change of reconstituted sarcoplasmic reticulum vesicles. The reconstituted vesicles were prepared as described in Results. The vesicles were washed and suspended in 5 mM sodium Hepes (pH 7.4) overnight. The volume change of the vesicles was measured as described in the legend to Fig. 5 after mixing of the vesicle solution ($A_{450} = 0.40$) and 50 mM (final) KCl. The full scales of abscissae of (a), (b) and (c) represent 200 ms, 5 s and 200 s, respectively.

Discussion

In this paper, we present a new method of forming membrane vesicles, which we name the salting-out method. The phenomenon that the addition of salt develops the lipid vesicles in the solution containing lipid and Triton X-114 is interesting not only biochemically but also physicochemically. The cloud point of the solution of a non-ionic surfactant, such as Triton X-100, decreases by the addition of some kinds of salts [8,18]. This phenomenon can be interpreted in the fact that the added salt weakens the attractive interactions between the surfactant and water. The decrease in the hydrophilicity of Triton X-114 might play an important role in vesicle formation by addition of salt.

A similar vesicle formation can be expected

when Triton X-100 is used instead of Triton X-114. The cloud point of 2% (w/v) Triton X-100 solution is 64°C and decreases to 20°C by the addition of approx. 0.55 molality (final concentration) of Na_2SO_4 [8]. We can expect that vesicle formation by the addition of salt occurs in the solution containing an appropriate concentration of Triton X-100 and lipid if the salt (about final 200 mM) is added into this solution at 50°C to 60°C, or if the large amount of salt (about final 600 mM) is added into it at about 20°C.

In all experiments presented in this report, asolectin was utilized as phospholipid. The concentration of asolectin was 8 mg/ml in the experiments concerning the formation of vesicles. As shown in Fig. 2, the concentration of 5–10 mg/ml is appropriate, but that of more than 10 mg/ml is not suitable because the lipid is not sufficiently soluble in 1% (v/v) Triton X-114 solution. Further examination of the relation between the concentration of Triton X-114 and that of asolectin is required as well as the effect of various kinds of lipid on salting-out.

We have applied this method to the reconstitution of sarcoplasmic reticulum vesicles. Triton X-100 was used in order to solubilize sarcoplasmic reticulum vesicles because Triton X-100 extracts more proteins from membrane than does Triton X-114 [19]. Triton X-114 and other surfactants are also available to extract proteins bound to membrane.

The new salting-out method to form vesicles has some advantages for the reconstitution experiments of transport systems in biomembranes as follows. (1) Completion of the reconstitution process in a short time. This is favorable for incorporation of unstable proteins into membrane vesicles. (2) Availability of non-ionic surfactants to solubilize the membrane proteins. (3) High yield and good reproducibility. This method, therefore, will be widely used for reconstitution experiments as well as for dialysis, freeze-thaw, dilution and sonication methods. In our laboratory, reconstitution of other transport systems by this method is in pregress.

Acknowledgements

The authors wish to thank Dr. Kazutomo Imahori for valuable suggestions and Mr. Kazuki Nunogaki for the electron micrograph. This investigation was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

1 Zimniak, P. and Racker, E. (1978) J. Biol. Chem. 253, 4631–4637
2 Chiesi, M., Peterson, S.W. and Acuto, O. (1978) Arch. Biochem. Biophys. 189, 132–136
3 Kagawa, Y. (1972) Biochim. Biophys. Acta 265, 297–338
4 Kagawa, Y. (1978) Biochim. Biophys. Acta 505, 45–93
5 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
6 Lindstrom, J., Anholt, R., Einarson, B., Engel, A., Osame, M. and Montal, M. (1980) J. Biol. Chem. 255, 8340–8350
7 Popot, J.-L., Cartaud, J. and Changeux, J.-P. (1981) Eur. J. Biochem. 118, 203–214
8 Maclay, W.N. (1956) J. Colloid Sci. 11, 272–285
9 Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607
10 Weber, A., Herz, R. and Reiss, I. (1966) Biochem. Z. 345, 329–369
11 Kasai, M. and Miyamoto, H. (1976) J. Biochem. (Tokyo) 79, 1053–1066
12 Fleischer, S. and Fleischer, B. (1967) in Methods in Enzymology Vol. 10 (Estabrook, R.W. and Pullman, M.E. eds.), pp. 406–433, Academic Press, New York
13 Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065
14 Kometani, T. and Kasai, M. (1978) J. Membrane Biol. 41, 295–308
15 Fairbands, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
16 Fiske, C.H. and SubbaRow, Y. (1925) 66, 375–400
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
18 Deguchi, K. and Meguro, K. (1975) J. Colloid Interface Sci. 50, 223–227
19 Egan, R.W., Jones, M.A. and Lehninger, A.L. (1976) J. Biol. Chem. 251, 4442–4447