Biochimica et Biophysica Acta 937 (1988) 73–80
Elsevier

BBA 73830

Rapid determination of internal volumes of membrane vesicles with electron spin resonance-stopped flow technique

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(Received 11 August 1987)

Key words: Phospholipid vesicle; Sarcoplasmic reticulum; Intravesicular volume; ESR; Stopped flow; (Rabbit muscle)

We have developed an electron spin resonance (ESR)-stopped flow technique and employed it for the simple and rapid determination of internal volumes of biomembrane vesicles and liposomes. A vesicle suspension containing a neutral and membrane-permeable spin label, 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (TEMPONE), was mixed in the stopped-flow apparatus with an isotonic solution of relatively impermeable line broadening agents, potassium tris(oxalato)chromate(III) or potassium ferricyanide, and an ESR spectrum was recorded. From the relative intensity of the sharp triplet signal due to TEMPONE in the aqueous space within vesicles, the determination of the internal aqueous volume was straightforward. Using this technique, it is possible to measure intravesicular volumes in 0.1 s. The internal volume of sonicated phospholipid vesicles was approximately 0.3 μl/mg lipid. The light fraction of sarcoplasmic reticulum membrane vesicles isolated from rabbit skeletal muscle was estimated to have an internal volume of 2.2–2.6 μl/mg protein in its resting state. Activation of Ca^{2+} pumps in the membrane upon addition of ATP and Ca^{2+} ions decreased the internal volume by about 10%. This finding supports the hypothesis that the Ca^{2+} pump is electrogenic and that the efflux of potassium ions compensates for the influx of positive charges. The present technique is widely applicable to the simple and rapid determination of the internal volumes of membrane vesicles.

Introduction

The internal aqueous volume of membrane vesicles is an importnat parameter in the study of their properties. For example, a knowledge of the internal volume of membrane vesicles is a prerequisite for the determination of intravesicular ion concentrations and of their membrane potential by means of probe partitioning techniques. Usually the vesicle internal volume can be determined as a carbohydrate-exclusion volume using ^{14}C-labeled carbohydrate and tritiated water [1]. This method involves procedures of incubation and ultracentrifugation and hence takes time, typically an hour. However, a more rapid technique is desirable since the internal volume can change rapidly depending on the ionic or metabolic conditions. Use of a combination of a membrane-permeable spin label and impermeable paramagnetic ions as spin label broadening agents seems to be

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TEMPONE, 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; (oxalato)chromate, potassium tris(oxalato)chromate(III) trihydrate; ESR, electron spin resonance.

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an appropriate technique to serve this purpose. Keith and co-workers were the first to develop this technique: they used the combination of 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (TEMPONE) and Ni^{2+} [2] or 4-amino-2,2,6,6-tetramethyl-piperidine-1-oxyl and potassium ferricyanide [3] for measuring internal volumes and viscosities in cells. Berg and Nesbitt [4] introduced a new spin label broadening agent, potassium tris(oxalato) chromate(III) ((oxalato)chromate), for use with thylakoids. Vistnes and Puskin [5] used a combination of TEMPONE and (oxalato)chromate to measure internal volumes of phospholipid vesicles.

However, in an attempt to apply these techniques, we noticed that the penetration of these so-called impermeable paramagnetic complex ions through some biomembranes cannot be negligible. Accordingly, we have constructed a stopped flow apparatus coupled with electron spin resonance (ESR) to measure the rate of permeation of these compounds. We then employed it for the rapid determination of the internal volumes of liposomes and sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle. The present method has already been successfully employed to determine the intravesicular volume of cell envelope vesicles of *Halobacterium halobium* by Mukohata and Yoshida [6].

Materials and Methods

Chemicals. Spin label reagent, 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (TEMPONE), was obtained from Eastman (Rochester, NY, U.S.A.). Egg yolk L-α-phosphatidylcholine (PC, type XI-E), egg yolk L-α-phosphatidylethanolamine (PE, type III) and asolectin (soy bean phospholipid, type II-S) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Asolectin was partially purified by washing with acetone [7]. Paramagnetic broadening reagent, potassium tris(oxalato)chromate (III) trihydrate ((oxalato)chromate) was synthesized according to Bailar and Jones [8]. Potassium ferricyanide was purchased from Wako Pure Chemical Industries (Osaka, Japan) and purified by recrystalization.

Membrane preparations. Phospholipid vesicles were prepared by a sonication method. Namely, a phospholipid suspension in a buffer solution was sonicated with a bath-type sonicator, model NS50-05U of Nihon Seiki (Tokyo, Japan), under a nitrogen atmosphere. It became almost clear in 5 min. A light fraction of sarcoplasmic reticulum vesicles was prepared from rabbit white skeletal muscle as described previously [9]. The protein concentration was determined by the biuret method.

Apparatus. Two models of X-band ESR spectrometers with a 100 kHz field modulation, PE-1X and FE-3X of JEOL (Tokyo, Japan), were used. The latter was equipped with the FS 10B rapid scan unit and the 9835B data acquisition system of JEOL. The constitution and performance of the stopped-flow system were as follows: the flow of sample solutions was controlled with a stop-valve located downstream of the observation cell. When it was open, solutions in two 15-ml sample reservoirs were delivered by the pressure of nitrogen gas into a Diflon mixing chamber (a modification of model 03, Union Giken, Hirakata, Japan) coupled with an observation cell (thick-walled quartz capillary of 1 mm inner diameter). The nitrogen gas pressure usually applied was 4 kg/cm^2, with which the flow rate of 3.3 ml/s was obtained. The open-valve period was set such that 100 μl of a solution was delivered each time. The observation cell was positioned in a JEOL cylindrical TE_{011} mode cavity. The dead volume, that from the mixing point to the observation point, was estimated to be about 50 μl, which gave a dead time of 15 ms. A mixing controller (Union Giken model RA-416) controlled the opening and closing of the stop valve through release or delivery of pressurized nitrogen gas and also triggered the rapid scan unit, which in turn enabled the data acquisition system. The overall performance of the present stopped-flow system coupled with the ESR spectrometer FE-3X is similar to that reported by Jacobs et al. [10].

ESR measurement. Two isotonic solutions, A and B, were placed in the sample reservoirs of the stopped-flow apparatus. Solution A contained membrane vesicles, TEMPONE, appropriate salts and buffers while solution B contained the broadening agent, salts and buffers. The osmotic pressure of solutions was measured with a model 3WII osmometer of Advanced Instruments, Inc. (Needham Heights, MS, U.S.A.) For conventional

recordings, the ESR magnetic field sweep rate was usually 20 G/min unless otherwise stated. Sweeping was started from a field just below the position of a signal peak at the time of the mixing of the two solutions so that the signal peak was recorded 5–10 s after the mixing. For the measurement of internal volumes of sarcoplasmic reticulum vesicles, the composition of solutions A and B was as follows: when (oxalato)chromate was used as a broadening agent, solution A contained 4.55 mg sarcoplasmic reticulum protein/ml, 0.909 mM TEMPONE, 130 mM potassium gluconate, 9.09 mM Mops-KOH, 4.55 mM $MgCl_2$ and 0.455 mM Ca^{2+}-EGTA (molar ratio 1 : 1), pH 7.0. Solution B for the resting state of sarcoplasmic reticulum contained 90.9 mM (oxalato)chromate, 9.09 mM Mops-KOH (pH 7.0), 4.55 mM $MgCl_2$ and 0.455 mM Ca-EGTA (1 : 1). In solution B for the active state of sarcoplasmic reticulum vesicle, 3.64 mM ATP replaced 4.55 mM $MgCl_2$. When ferricyanide was used as a broadening agent, solution A contained 5.00 mg sarcoplasmic reticulum protein/ml, 1.82 mM TEMPONE, 396 mM potassium gluconate, 9.09 mM Mops-KOH (pH 7.0), 4.55 mM $MgCl_2$, 0.455 mM Ca^{2+}-EGTA (1 : 1). Solution B for the resting state of sarcoplasmic reticulum vesicle contained 273 mM potassium ferricyanide, 9.09 mM Mops-KOH (pH 7.0), 0.455 mM Ca^{2+}-EGTA (1 : 1) and 4.55 mM $MgCl_2$. In solution B for the active state of sarcoplasmic reticulum vesicle, 4.55 mM $MgCl_2$ was replaced with 3.64 mM ATP.

Measurement of ATPase activity. The rate of ATP hydrolysis was followed at 25 °C by measuring the production of inorganic phosphate. To measure the 'total' ATPase activity in the presence of Ca^{2+} and (oxalato)chromate, the reaction mixture contained finally 0.227 mg sarcoplasmic reticulum protein/ml, 1.82 mM ATP, 2.27 mM $MgCl_2$, 9.09 mM Mops-KOH (pH 7.0), 0.455 mM Ca^{2+}-EGTA (1 : 1), 45.4 mM $K_3Cr(C_2O_4)_3$ and 168 mM sucrose. To measure the 'total' ATPase activity in the absence of (oxalato)chromate, 45.4 mM $K_3Cr(C_2O_4)_3$ and 168 mM sucrose were replaced with 90 mM KCl and 136 mM sucrose to maintain the osmolarity. For determination of the 'total' ATPase activity in the presence of ferricyanide, the reaction mixture contained 0.227 mg sarcoplasmic reticulum protein/ml, 1.82 mM ATP, 2.27 mM $MgCl_2$, 9.09 mM Mops-KOH (pH 7.0), 0.455 mM Ca^{2+}-EGTA (1 : 1), 136 mM $K_3Fe(CN)_3$ and 198 mM potassium gluconate. The corresponding control experiment was performed by omitting ferricyanide and increasing the concentration of potassium gluconate to 396 mM to maintain the osmolarity. Ca^{2+}-EGTA was replaced with 4.55 mM EGTA to measure Ca^{2+}-independent ATPase activity ('basic' activity). The 'net' Ca^{2+}-ATPase activity was expressed as the difference between 'total' and 'basic' ATPase activity. The reaction was started by the addition of ATP and stopped after appropriate periods of time by adding trichloroacetic acid (final concentration of 5%). After removal of the protein by centrifugation, the liberated inorganic phosphate was determined according to Martin and Doty [11].

Results and Discussion

ESR line broadening effect of potassium tris(oxalato)chromate and potassium ferricyanide

The broadening effects of the two metal complexes on an ESR signal of 0.5 mM TEMPONE in an aqueous solution were examined. The signal peak height was measured at field modulation width of 0.5 G with the addition of various concentrations of the broadening agent. Peak heights expressed in percent relative to the control (at varying concentrations of (oxalato)chromate in mM) were 1.83 (10), 0.38 (20), 0.32 (30) and nearly zero (40). When ferricyanide was used as a broadening agent, relative peak heights (with ferricyanide concentrations in mM) were 2.71 (25), 0.61 (50), 0.21 (75) and nearly zero (100). These results are in good agreement with those of previous studies [4,5]. Nickel dichloride induced aggregation of membrane vesicles containing acidic phospholipids and thereby could not be used as a broadening agent. In the following experiments we used either 40–50 mM (oxalato)chromate or about 100 mM ferricyanide as a broadening agent.

Rapid permeation of TEMPONE and slow permeation of broadening agents through the sarcoplasmic reticulum membrane

A suspension of sarcoplasmic reticulum vesicles (5 mg protein/ml) in a medium containing 0.3 M

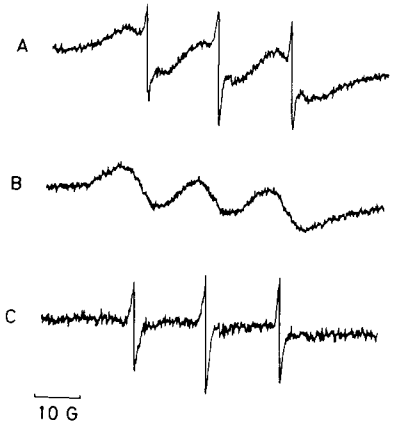

Fig. 1. ESR spectra obtained with the ESR-stopped flow system coupled with a rapid scan unit and a data acquisition system. The field scan rate was about 0.6 G/ms and each scan was completed in 100 ms. Data are the average of 250 scans. See Materials and Methods for experimental details. (A) A spectrum from the 1:1 mixture of a solution containing sarcoplasmic reticulum vesicles and TEMPONE and an isotonic solution of potassium tris(oxalato)chromate. The field scan and the data acquisition were started 0.1 s after the two sample solutions were admixed. (B) A spectrum from the 1:1 mixture of a solution of TEMPONE and a solution of tris(oxalato)chromate. (C) A difference spectrum produced by subtraction of the signal in Fig. 1B from those in Fig. 1A.

sucrose, 10 mM Mops (pH 7.0) and 1 mM TEMPONE was admixed with an equal volume of an isotonic solution containing 0.1 M (oxalato)chromate, 10 mM Mops (pH 7.0) and 69 mM sucrose. An ESR spectrum obtained 0.1 s after the mixing, using the rapid scan unit and data acquisition system, consisted of sharp and broad triplets as shown in Fig. 1A. When sarcoplasmic reticulum vesicle was omitted from the solution, only the broad triplet was observed as shown in Fig. 1B. Subtraction of the data in Fig. 1B from those in Fig. 1A resulted in a sharp triplet shown in Fig. 1C. These results clearly indicate that the triplet signal comes from TEMPONE inside the vesicles while the broad triplet is due to TEMPONE interacting magnetically with paramagnetic tris(oxalato)chromate ions outside the vesicles.

The characteristics of the spectrum shown in Fig. 1A were identical when data acquisition was started 0.1 s, 1 s, or 10 s after the mixing. Neither did the spectrum depend on whether TEMPONE was included in a solution containing sarcoplasmic reticulum vesicles or in a solution of (oxalato)chromate. This implies that the permeation of TEMPONE through the sarcoplasmic reticulum membrane is very rapid. The distribution equilibrium could be established within 0.1 s. Since TEMPONE is a neutral spin probe, it can be assumed to distribute homogeneously in the aqueous space inside and outside the vesicles. These two features, homogeneous and rapid distribution, provide the present method with a basis for the rapid and simple determination of the internal volumes of vesicles.

The signal intensity of the sharp triplet was found to decrease slowly in the minutes immediately after the mixing of the two solutions as shown in Fig. 2. Spectra in Fig. 2 were not taken using the rapid scan unit but with a conventional recording system. Quite a similar phenomenon was observed when ferricyanide was used as a broadening agent. This time-dependent decrease in the signal intensity suggests that these paramagnetic complex ions permeate the sarcoplasmic reticulum membrane in minutes. This necessitates ESR measurements with a higher time-resolution and hence the use of the stopped-flow apparatus is indispensable for the present experiments. Without

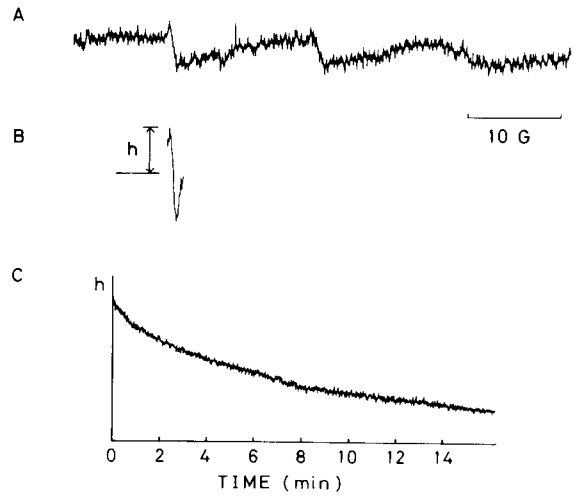

Fig. 2. (A) An ESR spectrum recorded with the conventional recording system. The field scan was started from the lowest point just after the mixing of a solution containing sarcoplasmic reticulum vesicles and TEMPONE with a solution of (oxalato)chromate. The scan rate was 3.3 G/min. (B) The low-field line recorded within 10 s after the mixing of the two solutions. (C) Time course of the peak height of the low-field line. Time zero was set at the mixing of the two solutions.

such equipment it would take at least a few minutes after mixing the two solutions to record the ESR spectrum and hence a considerably decayed signal could be recorded. However, the slow permeation of the broadening agent in the time scale of a few minutes also indicates that the use of a rapid scan system is not essential. Measurements using rapid scan are disadvantageous since they require a large number of repetitive measurements and the accumulation of much data to overcome a poor signal-to-noise ratio, thus consuming a large volume of sample solutions. Therefore, data were subsequently obtained with a conventional signal recording system.

Intravesicular volumes of sonicated phospholipid vesicles

We estimated first the internal volumes of sonicated phospholipid vesicles in order to evaluate the feasibility of the present method. Reproduced in Fig. 3A is an ESR spectrum obtained by 2-fold dilution with 143 mM KCl and 6.45 mM Mops-KOH (pH 7.0) of a solution containing sonicated egg PC vesicles (20 mg lipid/ml), 2 mM TEMPONE, 143 mM KCl and 6.45 mM Mops-KOH (pH 7.0). When this solution was admixed, using the stopped-flow apparatus, with an equal volume of an isotonic solution containing 0.1 M (oxalato)chromate and 10 mM Mops-KOH (pH 7.0). The resultant mixture gave the spectrum shown in Fig. 3B. This did not change within 30 min, in agreement with the observation by Vistnes and Puskin [5], indicating that phospholipid membranes are relatively impermeable to paramagnetic ions compared with the sarcoplasmic reticulum membrne. The spectrum consisted of three superimposed signals: a broad triplet of TEMPONE interacting (oxalato)chromate ions in aqueous phase outside vesicles and two sharp triplets originating from spin labels in the aqueous space inside vesicles and in membrane lipid. Sharp triplets due to the spin labels in the aqueous space inside the vesicles (W) and in the lipid phase (L) are resolved at the high-field peaks marked W and L in Fig. 3B, respectively. These assignments are made based on the solvent effect on the nitrogen hyperfine coupling constants [12]: about 16.5 G for spin labels in aqueous phase and about 14.5 G for labels in membrane. The signal due to spin

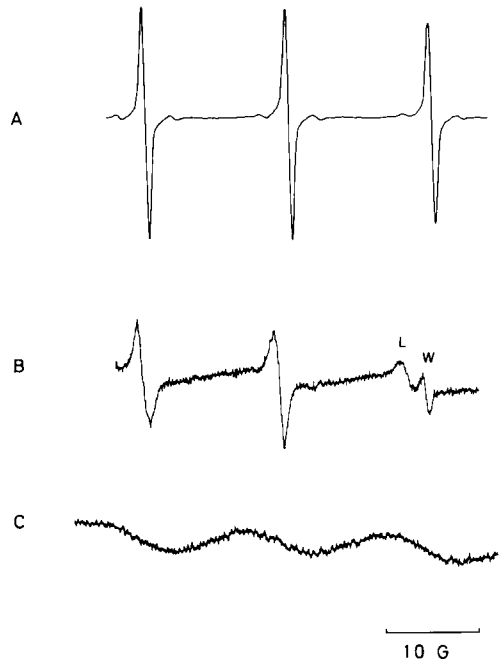

Fig. 3. (A) Spectrum of a solution containing sonicated egg PC vesicles (20 mg lipid/ml), 2 mM TEMPONE, 143 mM KCl and 6.5 mM Mops-KOH (pH 7.0). (B) Spectrum of a solution containing sonicated egg PC vesicles (10 mg lipid/ml), 1 mM TEMPONE, 50 mM $K_3Cr(C_2O_4)_3$, 72 mM KCl and 8.3 mM Mops-KOH (pH 7.0). (C) Spectrum from a solution of 1 mM TEMPONE, 50 mM $K_3Cr(C_2O_4)_3$, 72 mM KCl and 8.3 mM Mops-KOH (pH 7.0).

labels in the membrane lipid, which was not observable in the case of sarcoplasmic reticulum vesicles (see Fig. 1A and below), was observed in the case of phospholipid vesicles since the volume of the lipid phase was comparable with that of the aqueous phase inside the vesicles. Fig. 3C shows a broadened spectrum from a solution of identical composition to that employed in Fig. 2B but with vesicles omitted.

The intravesicular volume (V_{in} in μl/mg lipid) can be calculated according to the following equation.

$$V_{in} = 1000c^{-1}(h_{BW}/G_B)/(h_A/G_A)\ (\mu l/\text{mg lipid}) \qquad (1)$$

where h_{BW} refers to the peak height of signal W in Fig. 3B recorded with amplifier gain G_B, h_A to the height of the high-field peak in Fig. 3A recorded with amplifier gain G_A and c is the concentration of phospholipid vesicles (mg/ml). The

TABLE I

INTERNAL VOLUME OF SONICATED PHOSPHOLIPID VESICLES

Each value corresponds to a different preparation of sonicated vesicles. The values are averages of at least five determinations which did not vary more than 10%. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PE : PC (7 : 3), mixture of PE and PC with weight ratio of 7 : 3.

Phospholipid	V_{in} (μl/mg lipid)
Egg PC	0.35, 0.43
Asolectin	0.21, 0.24, 0.38
PE : PC (7 : 3)	0.44

results obtained with this method for some of sonicated phospholipid vesicles are shown in Table I. The scatter in the results seems to reflect the variance within the vesicle preparations rather than that in the measurement of intravesicular volume.

Sonicated phospholipid vesicles are known to be 25–30 nm in diameter and their lipid bilayers are about 5 nm thick [13,14]. If the specific gravity of hydrated phospholipid is assumed to be unity, the intravesicular volume of sonicated vesicles can be estimated at 0.28–0.42 μl/mg lipid. The results shown in Table I are in good agreement with these calculated values.

Internal volumes of sarcoplasmic reticulum vesicles in resting and active states

A solution containing sarcoplasmic reticulum vesicles and TEMPONE was admixed with a solution of spin broadening agent ± ATP. Each of the three peaks of the ESR spectrum was recorded 5–10 s after the mixing. For the measurement of V_{in} of sarcoplasmic reticulum vesicles in the resting state with use of (oxalato)chromate as a broadening agent, the composition of the mixed solution was 2.28 mg protein/ml, 0.45 mM TEMPONE. 45.1 mM (oxalato)chromate, 65.0 mM potassium gluconate, 9.09 mM Mops-KOH (pH 7.0), 4.55 mM $MgCl_2$ and 0.455 mM Ca-EGTA (1 : 1). An ESR spectrum obtained under these conditions is presented in Fig. 4. The sharp triplet is clearly assigned, based on the value of nitrogen hyperfine coupling constant, to the spin labels in aqueous space inside vesicles. To measure V_{in} of sarcoplasmic reticulum vesicles during Ca^{2+} uptake (in the active state), the composition of the mixed solution was 2.28 mg protein/ml, 0.45 mM TEMPONE, 45.1 mM (oxalato)chromate, 65.0 mM potassium gluconate, 9.09 mM Mops-KOH (pH 7.0), 2.28 mM $MgCl_2$, 0.455 mM Ca-EGTA (1 : 1) and 1.82 mM ATP. From the peak heights of these signals, the intravesicular volume of sarcoplasmic reticulum vesicles in the resting state and active state has been determined to be 2.4 and 2.1 μl/mg protein, respectively. These values are in the range of previously reported values for V_{in} of sarcoplasmic reticulum vesicles, 2–7 μl/mg protein depending on ion concentrations in the medium, which were deduced by Kasai [15] from [^{14}C]inulin exclusion volumes and the specific volume of the membrane.

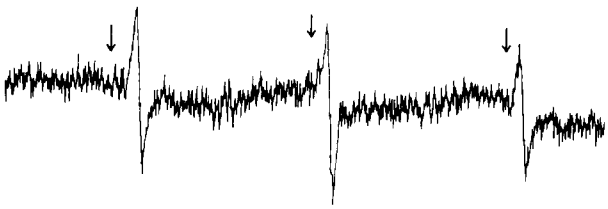

Fig. 4. A spectrum of 0.5 mM TEMPONE in a suspension of sarcoplasmic reticulum vesicles (in the resting state, 2.28 mg protein/ml) mixed with a solution containing (oxalato)chromate. With continuous scanning of magnetic field at a rate of 20 G/min, the stopped flow apparatus was operated at the points marked by the arrow in the figure so that each of the triplet lines was recorded about 10 s after the mixing of the two solutions. See Materials and Methods for experimental details.

An approximately 10% decrease in intravesicular volume was observed during Ca^{2+} uptake. Since the internal volume of sarcoplasmic reticulum vesicles can be determined with an error of 3–5%, this decrease is significant. Similar results were obtained when ferricyanide was used as a broadening agent (see Table II.). The agreement in absolute values derived from the two samples using ferricyanide as a broadening agent seems somewhat poor. This variance probably originates mainly from errors in the protein determination. On the other hand, a 9–14% decrease in the internal volume upon the activation of Ca^{2+} pumps was reproducibly observed as long as the measurements for sarcoplasmic reticulum vesicles in both resting and active states were carried out with the same sample preparation.

In order to check the Ca^{2+} transport activity of

TABLE II

INTERNAL VOLUME OF SARCOPLASMIC RETICULUM VESICLES

The values are mean ± S.D. Figures in parentheses refer to the number of ESR measurements. Results in different rows were derived from different preparations of sample solutions. Values using (oxalato)chromate (Cr(III)) and ferricyanide (Fe(III)) were obtained with different sarcoplasmic reticulum vesicle preparations. See Materials and Methods for experimental details.

Broadening agent	V_{in} (μl/mg protein)		Ratio (active)/(resting)
	resting state	active state	
Cr(III)	2.39 ± 0.06 (5)	–	–
	2.41 ± 0.09 (7)	2.11 ± 0.10 (14)	0.88
Fe(III)	2.59 ± 0.08 (9)	2.23 ± 0.10 (6)	0.86
	2.21 ± 0.07 (13)	2.02 ± 0.07 (11)	0.91

sarcoplasmic reticulum vesicle Ca^{2+}-ATPase in the presence of high concentrations of paramagnetic ions, Ca^{2+}-ATPase activity was measured and the results are summarized in Table III. Clearly Ca^{2+}-ATPase activity was not inhibited by either (oxalato)chromate or ferricyanide.

A change in the volume of sarcoplasmic reticulum vesicle upon Ca^{2+} uptake has been reported by other investigators [16,17] using the light scattering method. The decrease in sarcoplasmic reticulum vesicle volume under conditions similar to those used in the present study has also been described [16]. However, the technique of light scattering cannot determine the absolute values of the intravesicular volume but can only detect volume changes. In contrast, the present method can determine absolute values and their small changes. Many lines of evidence have accumulated to suggest that Ca^{2+} trnasport via Ca^{2+}-ATPase is electrogenic [16,18–22]. If this is the case, positive charges brought into the sarcoplasmic reticulum lumen by the uptake of Ca^{2+} ions should be compensated by Cl^- influx or by K^+ efflux. The contribution of K^+ influx may be predominant because the sarcoplasmic reticulum membrane is thought to contain a larger number of monovalent cation channels of higher conductance than anion channels [23].

TABLE III

ATPase ACTIVITY OF SARCOPLASMIC RETICULUM VESICLES

Total ATPase activity was measured in the presence of 0.455 mM Ca-EGTA (mixture with molar ratio of 1:1) while basic ATPase activity was obtained in the presence of 4.55 mM EGTA instead. Other constituents in the reaction mixture were the same as those for the determination of V_{in} shown in Table II, except for the protein concentration, which was changed to about 1/10 (0.227 mg/ml from 2.27 or 2.5).

Sample	ATPase activity (nmol · mg^{-1} · min^{-1})		
	total	basic	Ca^{2+}-ATPase
SR(1)	578	102	476
SR(1) + Cr(III)	612	–	510 [a]
SR(2)	348	18	330
SR(2) + Fe(III)	550	70	480

[a] The basic activity in the absence of (oxalato)chromate was subtracted from the total ATPase activity.

In the present study, the volume measurements of sarcoplasmic reticulum vesicles during the Ca^{2+} uptake were carried out under conditions in which the reaction mixture contained high concentrations of potassium gluconate but few chloride ions. It has been reported that the gluconate ion is not trnasported during active Ca^{2+} transport by sarcoplasmic reticulum vesicles [16]. Therefore, the influx of positive charges accompanying the Ca^{2+} uptake should be compensated almost solely by K^+ efflux. Assuming that the influx of one Ca^{2+} induces the efflux of two K^+ and that the sarcoplasmic reticulum vesicle takes up 100 nmol of Ca^{2+}/mg protein, an approximately 10% decrease in intravesicular volume could be expected to maintain the osmolarity inside the sarcoplasmic reticulum vesicle. Although this is only an estimate, it yields values close to those actually observed (Table II).

In conclusion, the present investigation has demonstrated that the ESR-stopped flow technique together with the combination of TEMPONE and (oxalato)chromate or ferricyanide is widely applicable to the simple and rapid determination of internal volumes of liposomes and biomembrane vesicles.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Special Project Research (No. 61107006,

No. 61215024 and No. 62617510) from The Japanese Ministry of Education, Science and Culture.

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