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Pathway for the Movement of Water and Cryoprotectants in Bovine Oocytes and Embryos[1]

Bo Jin,[3] Yasunori Kawai,[3] Takao Hara,[3] Shoko Takeda,[3] Shinsuke Seki,[3] Yu-ichi Nakata,[3] Kazutsugu Matsukawa,[3] Chihiro Koshimoto,[4] Magosaburo Kasai,[3] and Keisuke Edashige[2,3]

[3]*Laboratory of Animal Science, College of Agriculture, Kochi University, Nankoku, Kochi 783-8502*
[4]*Frontier Science Research Center, University of Miyazaki, Kiyotake, Miyazaki 889-1692, Japan.*

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Summary sentence: Water, ethylene glycol and glycerol permeate bovine morulae predominantly through aquaporin 3.

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[2]Correspondence. FAX: 81 88 864 5200;
e-mail: keisuke@cc.kochi-u.ac.jp

ABSTRACT
The permeability of cells is important for cryopreservation. Previously, we showed in mice that the permeability to water and cryoprotectants of oocytes and embryos at early cleavage stages (early embryos) is low because these molecules move across the plasma membrane predominantly by simple diffusion through the lipid bilayer, whereas that of morulae and blastocysts is high because of a water channel, aquaporin 3 (AQP3). In this study, we examined the pathway for the movement of water and cryoprotectants in bovine oocytes/embryos and the role of AQP3 in the movement, by determining permeability, first in intact bovine oocytes/embryos, then in bovine morulae with suppressed AQP3-expression, and finally in mouse oocytes expressing bovine AQP3. The results suggest that water moves through bovine oocytes and early embryos slowly by simple diffusion, as in mice, although channel processes are also involved in the movement. On the other hand, water appears to move through morulae and blastocysts predominantly by facilitated diffusion via channels as in mice. Like water, cryoprotectants appear to move through bovine oocytes/early embryos mostly by simple diffusion, but channel processes would also be involved in the movement of glycerol and ethylene glycol unlike in mice. In bovine morulae, though glycerol and ethylene glycol would move predominantly by facilitated diffusion mostly through AQP3 as in mice, dimethylsulfoxide appears to move predominantly by simple diffusion unlike in mice. These results indicate that permeability-related properties of bovine oocytes/embryos are similar to those of mouse oocytes/embryos but species-specific differences do exist.

INTRODUCTION

Recently, vitrification has become popular for preservation embryos/oocytes because they can be cryopreserved simply and instantly [1]. In this method, a very high concentration of cryoprotectant is used. The permeability of the plasma membrane is an important factor determining the suitable condition for vitrification of oocytes/embryos because it affects major forms of cell injury during vitrification, i.e., damage from intracellular ice, the toxicity of the cryoprotectant, and osmotic swelling during removal of the cryoprotectant [2]. In the mouse, permeability to water and cryoprotectants is low in oocytes and embryos at early developmental stages (early embryos), but increases greatly in morulae [3-5]. This suggests that the pathway for the movement of water and cryoprotectants changes during development. In addition, the permeability differs for each cryoprotectant [3, 5]

There are two pathways for the movement of water and cryoprotectants across the plasma membrane, simple diffusion through the lipid bilayer and facilitated diffusion through channels. With simple diffusion, the permeability to water (hydraulic conductivity, L_P) or a cryoprotectant (P_S) is low, but the activation energy (E_a), the temperature dependency of the permeability, is high. Therefore, a longer exposure to the cryopreservation solution is necessary to dehydrate the cell and allow the cryoprotectant to penetrate. In addition, the period of exposure to the cryoprotectant would vary depending on the temperature. On the other hand, when water and cryoprotectant(s) permeate the plasma membrane by facilitated diffusion through channels, the permeability would be high and its E_a would be low. In this case, therefore, the period of exposure to the cryopreservation solution needs to be limited to prevent damage from the chemical toxicity of the cryoprotectant. The temperature at which the cell is exposed to the solution before cooling and the permeated cryoprotectant is removed from the cell after warming would be less important than for simple diffusion, because facilitated diffusion through channels is less affected by temperature.

Verkman et al. [6] suggested that an L_P higher than 4.5 μm min^{-1} atm^{-1} and an E_a for the L_P lower than 6 kcal mol^{-1} are suggestive of the movement of water across the plasma membrane principally through water channels, and that a low L_P with an E_a higher than 10 kcal mol^{-1} is suggestive of movement principally by simple diffusion. However, they pointed out that there appears to be no a priori theoretical basis for such values. Thus, their criterion for deducing the predominant pathway for the movement of water in cells is not a strict one. Although no such criterion has been reported for the movement of cryoprotectants across the plasma membrane, it is reasonable to consider that a high P_S with a low E_a is suggestive of movement predominantly via channels, and a low P_S with a high E_a is suggestive of movement predominantly by simple diffusion across the plasma membrane.

Based on this criterion, we deduced the pathway for the movement of water and cryoprotectants in mouse oocytes and embryos at various stages [4, 5]. We suggested from the permeability and its E_a, that water and cryoprotectants move across the plasma membrane in mouse oocytes and early embryos slowly, principally by simple diffusion, but that water, ethylene glycol, glycerol, dimethylsulfoxide (DMSO) and acetamide move through morulae rapidly, principally by facilitated diffusion via channels. We also showed that aquaporin 3 (AQP3), an aquaglyceroporin [7], plays an important role in the rapid movement of water, ethylene glycol and glycerol but not DMSO and acetamide. We also showed that the permeability of mouse morulae to water in the presence of a cryoprotectant is complex presumably because of interaction. For instance, in the presence of ethylene glycol, water would not permeate AQP3, and in the presence of propylene glycol, most water molecules and propylene glycol molecules would not move through AQP3. The difference in the pathway for the movement of water and cryoprotectants would affect conditions suitable for the vitrification of mouse embryos at different developmental stages; for example, mouse morulae can be vitrified successfully by a simple one-step method using an ethylene glycol-based solution [8], but pretreatment with a lower concentration of ethylene glycol is necessary to vitrify early mouse embryos [9].

Conditions for the vitrification of mammalian oocytes and embryos differ not only among developmental stages. In the human, the survival of morulae vitrified by a similar method to the one used for mouse morulae [8] appears to be lower than that of mouse morulae [10]. On the other hand, bovine blastocysts can be vitrified with high viability by a similar method to the one used for mouse blastocysts [11]. However, bovine oocytes and early embryos have low tolerance to vitrification [12]. Therefore, the permeability of the plasma membrane and/or other cryobiological properties would also be different among species.

In the bovine, the permeability of oocytes to water and cryoprotectants is low [13, 14], but Széll et al. (1989) suggested that the permeability of bovine morulae and blastocysts to glycerol and ethylene glycol was higher [15]. It has also been suggested that the permeability of the plasma membrane to water and cryoprotectants affected the survival of bovine blastocysts after vitrification [16, 17]. In a preliminary experiment, we showed in bovine oocytes and embryos that the permeability to cryoprotectants changes during development, and suggested that the pattern is similar to that for mouse embryos [18]. Therefore, it is plausible that water and cryoprotectants move through bovine oocytes and embryos via similar pathways as in mouse oocytes and embryos.

The aim of this study is to examine whether the pathway for the movement of water and cryoprotectants through bovine oocytes/embryos is similar to that in the mouse, with special reference to the role of AQP3. First, we examined the L_P, P_S and the E_a for the permeability of bovine oocytes and embryos at various stages to water in a hypertonic sucrose solution and to cryoprotectants and water in various cryoprotectant solutions, to deduce whether movement across the plasma membrane is predominantly by simple diffusion or facilitated diffusion. Then, we examined the role of AQP3 in the movement of water and cryoprotectants in bovine morulae by suppressing its expression. Finally, we examined permeability of the mouse oocyte, in which AQP3 protein is not expressed virtually [4], and in which bovine AQP3 is expressed.

MATERIALS AND METHODS

Collection of Oocytes and Embryos

To obtain bovine oocytes and embryos, ovaries from Japanese Brown and Japanese Black heifers were obtained at a local slaughter house. Cumulus-oocyte complexes (COCs) were collected by aspirating follicles 2-5 mm in diameter using a 5 ml syringe with a 19-gage needle. COCs were cultured for 22 h in 200 μl drops of TCM-199 (Invitrogen Corp., Carlsbad, CA) supplemented with 5% fetal calf serum covered with paraffin oil at 38.5 ^{o}C in humidified atmosphere with 5% CO_2, 5% O_2, and 90% N_2, and were fertilized *in vitro* as described below. Some bovine oocytes were matured in the same conditions except for atmosphere (5% CO_2 in air), and denuded of cumulus cells by pipetting with a fine pipette in PB1 medium containing 0.2% hyaluronidase and washed 3 times with PB1 medium for determining the permeability of mature oocytes (at the metaphase II stage) to water and cryoprotectants. We considered oocytes to be mature if they had a polar body. Maturation rates of bovine oocytes in the two culture conditions were not significantly different (data not shown).

To obtain immature mouse oocytes, mature female ICR mice (CLEA Japan, Tokyo, Japan) were injected with 5 iu of equine chorionic gonadotrophin (eCG). Forty-eight hours later, oocytes at the germinal vesicle stage were collected from ovaries. The oocytes were denuded of cumulus cells using a fine pipette, then, induced to mature to the metaphase II stage, as described below. The experiments were approved by the Animal Care and Use Committee of Kochi University.

In vitro Fertilization and in vitro Culture

Frozen/thawed semen of a Japanese Brown bull in a 0.5 ml straw was thawed in water at 37^{o}C, then diluted with a sperm washing medium comprising Brackett and Oliphant (BO) medium [19] supplemented with 4 mg/ml caffeine and 4 U/ml heparin (Novo-Heparin Injection 1000, Aventis Pharma LTD, Tokyo, Japan), and washed twice by centrifugation. The sperm were re-suspended in BO medium containing 2 mg/ml caffeine, 2 U/ml heparin and 10 mg/ml bovine serum albumin (BSA) at a concentration of ~6 x 10^6 cells/ml, and 100-μl drops of this suspension were prepared under paraffin oil in a culture dish (fertilization droplets). After being cultured for 22 h, COCs were washed three times with BO medium containing 10 mg/ml BSA, introduced into fertilization droplets (20 COCs/droplet), and cultured at 38.5 ^{o}C for 5 h in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2. Then, oocytes were denuded of cumulus cells and sperm by gentle pipetting with a fine glass pipette, and transferred to 200 μl droplets (~20 oocytes/droplet) of a culture medium comprising modified SOF medium [20] supplemented with 5% fetal calf serum, under paraffin oil, and the oocytes were cultured at 38.5 ^{o}C in humidified atmosphere with 5% CO_2, 5% O_2, and 90% N_2 for 4 days to obtain 16 cell-embryos, to obtain morulae for 5 days, and to obtain blastocysts for 6-8 days. To measure total cell volume, each blastocyst in PB1 medium was held with a holding pipette connected to a micromanipulator on an inverted microscope and pierced once or twice with a microneedle connected with another micromanipulator to shrink their blastocoele. Blastocysts with a shrunken blastocoele were incubated in the culture medium in a CO_2 incubator for 4-5 min, and those without reexpanded blastocoele were used for experiments.

Determining the Permeability to Water and Cryoprotectants of Bovine Oocytes and Embryos at Various Developmental Stages

The permeability to water and cryoprotectants of bovine oocytes, 16-cell embryos, morulae, and blastocysts was determined from changes in volume during suspension in PB1 medium containing 0.43 M sucrose (n = 15, 9, 27, and 20, respectively), 10% (vol/vol) glycerol (n = 6, 4, 11, and 6, respectively), 8% (vol/vol) ethylene glycol (n = 6, 7, 9, and 9, respectively), 9.5% (vol/vol) DMSO (n = 6, 8, 11, and 4, respectively), or 10% (vol/vol) propylene glycol (n = 6, 7, 11, and 6, respectively) for 5 or 20 min at 25 ^{o}C and then at 15 ^{o}C, as described previously [3-5, 21, 22]. The concentrations of cryoprotectants were varied to prepare solutions with similar osmolality. The osmolality of sucrose, ethylene glycol, glycerol and propylene glycol was calculated from published data about colligative properties in aqueous solutions [23, 24]. The osmolality of DMSO in aqueous solutions was measured with a vapor pressure osmometer (Vapro 5520; Wescor Inc., Logan, UT) [5]. The osmolality of PB1 medium (isotonic buffer) was measured with a freezing point depression osmometer (OM801, Vogel, Giessen, Germany). The osmolality of each solution used is shown in Table 1.

Each oocyte or embryo was placed in a 100-μl drop of PB1 medium covered with paraffin oil in a Petri dish (90 x 10 mm) at 25 ^{o}C, and held by a holding pipette (outer diameter, 100-140 μm) connected to a micromanipulator on an inverted microscope. The inner diameter of the holding pipette was small enough not to distort the oocyte or embryo. The temperature of the paraffin oil covering the solution was considered to be the temperature of the solutions, and was kept at 25 ± 1 or 15 ± 1 ^{o}C by controlling the temperature of the room. An oocyte or embryo held by the holding pipette was kept in PB1 medium for 5 min, and then covered with a covering pipette with a larger inner diameter (about 200 μm) connected to another micromanipulator. Then, by sliding the dish, the oocyte or embryo was introduced into a drop of PB1 medium containing sucrose (sucrose solution) or a cryoprotectant (cryoprotectant solution). By removing the covering pipette, the oocyte/embryo was abruptly exposed to the solution. Microscopic images of the oocyte/embryo during exposure to the solution were recorded with a time-lapse videotape recorder (ETV-820, Sony, Tokyo, Japan) every 0.5 sec for 5-20 min. The cross-sectional area of the oocyte/embryo was measured using an image analyzer (VM-50, Olympus, Tokyo, Japan). It was expressed as the relative cross sectional area, S , by dividing it by the area of the same oocyte/embryo in isotonic PB1 medium. Relative volume was obtained from $V = S^{3/2}$. The permeability of the oocyte/embryo was determined by fitting the movement of water and cryoprotectants using a two-parameter formalism as described previously [21, 22]. The related constants and parameters used are listed in Table 2.

For each oocyte/embryo, osmotically inactive volume (V_b), L_P in sucrose solution, P_S for a cryoprotectant, and L_P in the presence of the cryoprotectant were examined at 25^{o}C and then at 15^{o}C, although the V_b value was determined only at 25^{o}C.

To determine V_b, an oocyte/embryo was exposed first to PB1 medium containing 0.43 M sucrose for 10 min, and then to PB1 medium containing 0.18 M sucrose for 5 min at 25 ^{o}C. From the relative volumes of the oocyte /embryo after exposure, a V_b value was obtained with a Boyl-van't Hoff plot [4]. The V_b value was used to determine the L_P and P_S for each oocyte/embryo. From changes in volume of the oocyte/embryo in PB1 medium containing 0.43 M sucrose, L_P in the absence of cryoprotectant was determined.

Then the oocyte/embryo was returned to isotonic PB1 medium at 25 ^{o}C. After 10 min, it was exposed to PB1 medium containing a cryoprotectant for 20 min (for oocyte) or 10min (for embryo) at 25 ^{o}C to examine the Ps and the Lp in the presence of the cryoprotectant. To remove cryoprotectant from the cell, the oocyte/embryo was transferred to PB1 medium containing 0.5 M sucrose at 25^{o}C, kept there for 10 min, and then equilibrated with fresh PB1 medium at 25^{o}C. After 10 min, it was transferred to PB1 medium at 15^{o}C, kept for 5 min, and suspended in PB1 medium containing 0.43 M sucrose at 15 ^{o}C for 10 min. The oocyte/embryo was suspended in isotonic PB1 medium for 10 min at 15 ^{o}C, and exposed to PB1 medium containing a cryoprotectant for 20 min (for oocyte) or 10 min (for embryo) at 15 ^{o}C. From the changes in volume of oocytes and embryos, we determined the L_P in a sucrose solution, P_S and L_P in the presence of the cryoprotectant at 25 and 15 $^{\circ}$C using the V_b value for each oocyte/embryo. From the L_P and P_S values at 25 and 15 $^{\circ}$C, E_a values were obtained.

Measurement of the Permeability to Water and Cryoprotectants of Bovine Morulae in which the Expression of AQP3 was Suppressed

The role of AQP3 in the movement of water and cryoprotectants in bovine morulae was examined by measuring the L_P and P_S of morulae in which the expression of AQP3 was suppressed by injecting double-stranded (ds) RNA of bovine *AQP3* at the 1-cell stage, as in the mouse [5]. The cDNA of bovine *AQP3* was cloned from bovine kidney cDNA by the polymerase chain reaction (PCR): the sense strand was 5'-GGATCCTAATACGACTCACTATAGGCGCCTGCCCGCCCGAAGC-3' and the antisense strand was 5'-GGATCCTAATACGACTCACTATAGGGCCCCCGCCCATTCACATC-3' (underlined sequences indicate T7 promoter sequences). These primers were derived from the bovine *AQP3* sequence (GenBank accession number, BC123791). The PCR was conducted with the following profile: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec for 30 cycles. The PCR product contained the open reading frame of bovine *AQP3*. The dsRNA of *AQP3* was synthesized with the T7 RiboMAX Express RNAi System (Promega Corporation, Madison, WI) using the PCR product, according to the manufacturer's instructions.

To suppress the expression of AQP3 in morulae, a bovine 1-cell zygote was held with a holding pipette connected to a micromanipulator on an inverted microscope. The embryo was injected with 2-10 pl of dsRNA of bovine *AQP3* (1 pg/pl) with an injection needle connected to another micromanipulator. As a control, we used non-injected morulae (intact morulae) because, in preliminary experiments, the permeability of intact morulae to water was not significantly different from that of water-injected morulae as in the mouse [5] (data not shown). The embryos were transferred to 200 μl droplets of culture medium under paraffin oil, and cultured at 38.5 $^{\circ}$C for 5 days to develop into morulae. Only compacted morulae were used in the experiment.
The permeability of intact oocytes, intact morulae, and dsRNA-injected morulae was measured in PB1 medium containing sucrose (n = 20, 19, and 20, respectively), glycerol (n = 5, 8, and 5, respectively), ethylene glycol (n = 5, 5, and 5, respectively), DMSO (n = 5, 5, and 5, respectively), or propylene glycol (n = 5, 5, and 5, respectively) as described above, except that measurement was only performed at 25°C.

To confirm the suppression of AQP3-expression in bovine morulae, we tested for the presence of the protein by immunofluorescent-staining using a commercially available anti-rat AQP3 rabbit antibody, which cross-reacts with bovine AQP3 [25]. The L_P of bovine morulae which had been injected or not injected (intact) with dsRNA of bovine *AQP3* and bovine oocytes at the metaphase II stage was determined at 25 $^{\circ}$C as the same method described above. Then, the samples were fixed with a 2% paraformaldehyde solution containing 0.01 M sodium metaperiodate, 0.1 M lysine, and 0.1 M phosphate buffer (pH 7.4) at 4°C for 60 min, washed with Dulbecco's Ca^{2+} free-PBS (PBS(-)) containing 5 mg/ml BSA, and treated with PBS(-) containing 0.02% Triton X-100, 0.1 M lysine, and 10% non-immune goat serum (Biosource, Camarillo, CA) at room temperature for 30 min. The sample were then incubated in blocking solution; PBS (-) containing 5% non-immune goat serum and 5 mg/ml BSA at 25 $^{\circ}$C for 60 min, washed, and incubated in an anti-rat AQP3 rabbit antibody (Chemicon International Inc., Temecula, CA) solution diluted with blocking solution (1/400) at 4°C for 60 min. After being washed, the oocytes and morulae were incubated in a diluted fluorescein isothiocyanate (FITC) -conjugated anti-rabbit immunoglobulin goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA) solution diluted with blocking solution (1/1500) at 25 ^{o}C for 2 h, washed again, and observed under a fluorescence microscope.

Measurements of the Permeability to Water and Cryoprotectants of Mouse Oocytes Expressing Bovine AQP3

We determined the permeability-related properties of bovine AQP3 by measuring the permeability of mouse oocytes in which bovine AQP3 was exogenously expressed by a method described previously [5, 21]. One reason for using mouse oocytes rather than bovine oocytes was that it was shown that water/cryoprotectant channels are virtually no expressed in the plasma membrane of mouse oocytes [4, 5]. Another reason was that the membrane-permeability of bovine oocytes and temperature dependency in the present study (Tables 3 and 4) suggest that mature bovine oocytes express water/cryoprotectant channels. Thus, mouse oocytes expressing bovine AQP3 would be a model for mammalian cells abundantly expressing AQP3 like bovine morulae.

Bovine *AQP3* cRNA was synthesized essentially the same way as rat *Aqp3* cRNA [5, 21, 26]. Briefly, cDNA of bovine *AQP3* was cloned from bovine kidney cDNA by PCR; the sense strand was 5'- CG<u>GAATTC</u>CGCCTGCCCGCCCGAAGC-3', and the antisense strand was 5'-GC<u>TCTAGA</u>GCCCCCGCCCATTCACATC-3' (underlined sequences indicate inserted EcoRI and XbaI sites, respectively). These primers were derived from the bovine *AQP3* sequence (GenBank accession number, BC123791). The PCR cycle had the following profile; 94 ^{o}C for 30 sec, 55 ^{o}C for 30 sec, and 72 ^{o}C for 60 sec for 30 cycles. The PCR product contained the open reading frame of bovine *AQP3*. The EcoRI/XbaI fragment of the PCR product was subcloned into the EcoRI/XbaI site of the pTnT Vector (Promega Corporation, Madison, WI), an *in vitro* transcription/translation plasmid. After digestion of the construct by BamHI, capped cRNA of *AQP3* was synthesized using SP6 polymerase (New England BioLabs Inc., Ipswich, MA). BamHI, EcoRI, and XbaI were obtained from Takara (Otsu, Japan).

To express bovine AQP3, immature mouse oocytes at the germinal vesicle stage were injected with the cRNA of bovine *AQP3* as described in our previous studies [5, 21]. Briefly, an immature oocyte was held with a holding pipette connected to a micromanipulator on an inverted microscope. The oocyte was injected with 2-10 pl of cRNA of bovine *AQP3* (1 pg/pl) with an injection needle connected to another micromanipulator. As s control, non-injected oocytes (intact oocytes) were used. The oocytes were cultured in a humidified CO_2 incubator at 37^{o}C for 12-15 h in MEM supplemented with 10% fetal calf serum, 50 μg/ml sodium pyruvate, 2 mM glutamine, 60 μg/ml penicillin G, and 50 μg/ml streptomycin. Only oocytes that had a polar body after being cultured were considered to have matured, and used in the experiment.
To detect the expression of bovine AQP3, the mouse oocytes were fixed, stained and observed as described above.

The permeability of intact oocytes and cRNA-injected oocytes in PB1 medium containing sucrose (n = 5 and 5, respectively), glycerol (n = 5 and 5, respectively), ethylene glycol (n = 5 and 8, respectively), DMSO (n = 5 and 8, respectively), or propylene glycol (n = 5 and 5, respectively) was measured as described above, except that the measurement which only made at 25°C.

Statistical Analysis

The significant differences in the L_P and P_S of intact oocytes and intact embryos at various stages (Tables 3 and 4) and of intact oocytes, intact morulae and *AQP3* dsRNA-injected morulae (Tables 5 and 6) were analyzed with one-way ANOVA using Graphpad Software's Instat, V. 3.02 followed by Tukey-Kramaer Multiple comparison Test. The significant differences in the L_P and P_S of intact oocytes and *AQP3* cRNA-injected oocytes were analyzed with two-tailed t-test using the same Software (Tables 7 and 8). $P < 0.05$ was considered significant.

RESULTS

Permeability of Bovine Oocytes and Embryos at Various Developmental Stages to Water and Cryoprotectants and its Temperature Dependency

To deduce the major pathway for the movement of water and cryoprotectants across the plasma membrane at various developmental stages, bovine oocytes and embryos were suspended in hypertonic sucrose solutions and then various cryoprotectant solutions at 25 and 15°C. From the changes in volume, we obtained first V_b, then L_P in the absence of cryoprotectant, finally P_S and L_P in the presence of cryoprotectants. From the permeability at different temperatures, the E_a for the permeability was determined.

The V_b values of oocytes, 16-cell embryos, morulae, and blastocysts were 0.25 ± 0.10 (n = 24), 0.40 ± 0.08 (n = 26), 0.31 ± 0.08 (n = 42), and 0.25 ± 0.10 (n = 25), respectively, which were used to determine the L_P and P_S of intact oocytes and embryos in a sucrose solution and cryoprotectant solutions (Figs. 1-3, Tables 3 and 4).

In a hypertonic solution containing 0.43 M sucrose at 25°C, oocytes shrunk to 69% in 20 sec, whereas at 15°C, they shrunk to 78% in 20 sec (Fig. 1 A), indicating that oocytes shrunk slowly at 25°C and more slowly at 15°C. The L_P of oocytes at 25°C was relatively low (1.8 $\mu m\ min^{-1} atm^{-1}$), and the E_a for the L_P was high (9 $kcal\ mol^{-1}$) (Table 3). Similar results were obtained in 16-cell embryos (Fig. 1B) (L_P at 25°C being 1.2 $\mu m\ min^{-1} atm^{-1}$, and E_a for the L_P being 9 $kcal\ mol^{-1}$) (Table 3). On the other hand, morulae and blastocysts shrunk rapidly at 25°C (72-73% in 10 sec). They also shrunk rapidly at 15°C (75-76% in 10 sec) but slightly more slowly than at 25°C (Figs. 1C and 1D). The L_P of morulae and blastocysts at 25°C was significantly high (2.6-3.2 $\mu m\ min^{-1} atm^{-1}$) and the E_a for the L_P was quite low (3 $kcal\ mol^{-1}$) (Table 3).

In a glycerol solution at 25 °C, oocytes shrunk markedly (46% at 30 sec) and regained their volume slowly (66% in 5 min) (Fig. 2A). At 15°C, on the other hand, they shrunk more slowly (48% in 1 min) and regained their volume slowly (64% in 5 min) (Fig. 3A). Similar results were obtained in 16-cell embryos; they shrunk slowly (62% in 1 min) and regained their volume slightly (75% in 5 min), whereas at 15°C, they shrunk more slowly (61% in 2 min) and regained their volume more slowly (69% in 5 min). These results indicate that oocytes and 16-cell embryos shrunk slowly and regained their volume slightly at 25°C and that the volume change was more slowly at 15°C. On the other hand, morulae shrunk less (70% in 20 sec) and regained their volume rapidly (89-94% in 3 min) at 25°C (Fig. 2A). At 15°C, they shrunk and swelled similarly (63% in 20 sec and 88% in 3 min) (Fig. 3A). In blastocysts, similar results were obtained. The P_{Gly} at 25°C of oocytes and 16-cell embryos was relatively low (0.5 x10^{-3}cm/min), while that of morulae and blastocysts was markedly higher (2.8-3.8 x10^{-3}cm/min) (Table 4). The E_a for the P_{Gly} of oocytes and 16-cell embryos was high (11-17 kcal mol^{-1}), whereas that of morulae and blastocysts was low (4-6 kcal mol-^{1}). The L_P of oocytes and 16-cell embryos in the presence of glycerol at 25°C was relatively low (0.7-1.0 μm $min^{-1}atm^{-1}$), while that of morulae was slightly but significantly higher (1.8 μm $min^{-1}atm^{-1}$) (Table 3). The E_a for the L_P of oocytes and 16-cell embryos (8 kcal mol^{-1}) was relatively high but that of morulae was substantially low (2 kcal mol^{-1}). The E_a for the L_P of blastocysts (3 kcal mol^{-1}) was also low as in morulae, although the L_P at 25°C was relatively low (1.1 μm $min^{-1}atm^{-1}$).

In an ethylene glycol solution at 25°C, oocytes and 16-cell embryos shrunk to 71-77% in 30 sec and regained their volume slowly (86% in 3 min) (Fig. 2B). At 15°C, on the other hand, they shrunk and regained their volume more slowly (71-75% in 1 min and 78% in 3 min) (Fig 3B). On the other hand, morulae and blastocysts shrunk a little and regained their volume rapidly at 25°C (86-91% in 20 sec and 96-97% in 2 min), and they shrunk and regained their volume slightly more slowly at 15°C (88-94 in 20 sec and 97-98% in 3 min).The P_{EG} of oocytes and 16-cell embryos at 25°C was relatively low (2.0-3.5x10^{-3}cm min^{-1}) but that of morulae and blastocysts was remarkably high (10.9-12.3 x10^{-3}cm min^{-1}) (Table 4). The E_a for the P_{EG} of oocytes and 16-cell embryos was relatively high (10-15 kcal mol^{-1}) whereas that of morulae and blastocysts was low (2-4 kcal mol^{-1}) (Table 4). However, the L_P of oocytes and embryos at various developmental stages in the presence of ethylene glycol at 25°C was low (0.6-0.9 μm $min^{-1}atm^{-1}$) and the E_a for the L_P was high (8-14 kcal mol^{-1}) (Table 3), although a large number of water channels would be expressed in morulae and blastocysts.

In a DMSO solution at 25°C, oocytes and embryos at various stages shrunk slowly (58-72% in 30 sec) and regained their volume relatively slowly (89-91% in 5 min) (Fig. 2C). At 15°C, they shrunk and regained their volume more slowly (57-71% in 30 sec and 71-92% in 5 min) (Fig. 3C). The P_{DMSO} of oocytes and 16-cell embryos at 25°C was relatively low (1.5-1.6 x10^{-3}cm min^{-1}) and similar to that of morulae and blastocysts (1.7-2.1 x10^{-3}cm min^{-1}) (Table 4). The E_a for the P_{DMSO} of oocytes and embryos was high (13-21 kcal mol^{-1}) regardless of the developmental stage. The L_P of oocytes and embryos at various developmental stages in the presence of DMSO at 25°C was relatively low (0.8-1.4 μm $min^{-1}atm^{-1}$) (Table 3). However, the E_a for the L_P of oocytes and 16-cell embryos was relatively high (9-11 kcal mol^{-1}), but that of morulae and blastocysts was quite low (2-4 kcal mol^{-1}).

In a propylene glycol solution at 25 °C, oocytes and embryos at various developmental stages shrunk slowly (68-78% in 30 sec) and regained their volume slowly (94-98% in 5 min) (Fig. 2D), regardless of developmental stage. At 15 °C , they shrunk and regained their volume more slowly (65-76% in 1 min, 81-85% in 5 min) (Fig. 3D).Therefore, the P_{PG} of oocytes at 25°C (2.6 x10^{-3}cm min^{-1}) was similar to that of embryos at various developmental stages (2.5-3.4 x10^{-3}cm min^{-1}) (Table 4), and the E_a for the P_{PG} was very high in both oocytes (17 kcal mol^{-1}) and embryos (20-21 kcal mol^{-1}) (Table 4). In the presence of PG, the L_P of oocytes and embryos at 25°C was low (0.8-1.1 μm $min^{-1}atm^{-1}$) (Table 3), and the E_a for the L_P of oocytes and embryos was high (8-12 kcal mol^{-1}), regardless of the developmental stage.

Permeability to Water and Cryoprotectants of Bovine Morulae in which the Expression of AQP3 was Suppressed

To elucidate the involvement of AQP3 in the movement of water and cryoprotectants in bovine morulae, we tried to suppress the expression of AQP3 in morulae by injecting dsRNA of bovine *AQP3* in 1-cell embryos. Fig. 4 shows the expression of AQP3 in bovine morulae and oocytes. Although we could confirm the expression of AQP3 protein in the cytoplasm, we could not clearly confirm to be in the plasma membrane because a large amount of cytoplasmic lipid droplets in morulae and oocytes unevenly shaded the fluorescence of AQP3 protein. Intact morulae expressed AQP3 abundantly, but dsRNA-injected morulae expressed much less AQP3. This shows that the injection of dsRNA of AQP3 suppressed the expression of AQP3 markedly. In intact bovine oocytes, however, the expression of AQP3 was substantially lower than in intact morulae, but appeared to be higher than in dsRNA-injected morulae. Therefore, bovine oocytes expressed a small amount of AQP3.

Figure 5 shows changes in volume of bovine morulae and oocytes in a hypertonic sucrose solution. Intact morulae shrunk rapidly (71% in 10 sec), whereas dsRNA-injected morulae shrunk slowly (74% in 1 min). Oocytes also shrunk more rapidly than dsRNA-injected morulae but more slowly than intact oocytes (68% in 20 sec). The L_P of dsRNA-injected morulae (0.6 μm $min^{-1}atm^{-1}$) was much lower that that of intact morulae (3.3 μm $min^{-1}atm^{-1}$) and intact oocytes (1.9 μm $min^{-1}atm^{-1}$) (Table 5). These results indicate that AQP3 protein is located in the apical side of the plasma membrane of bovine morulae, and that AQP3 plays a major role in the movement of water in bovine morulae.

Figure 6 shows changes in volume of bovine intact oocytes and *AQP3* dsRNA-injected and intact morulae. In a glycerol solution, intact morulae shrunk rapidly (71% in 10 sec) and regained their volume rapidly (93% in 2 min), whereas dsRNA-injected morulae shrunk slowly (59% in 1 min) and regained their volume slowly (73% in 10 min) (Fig. 6A). Oocytes shrunk markedly (47% in 30 sec) and regained their volume more slowly than intact morulae but faster than dsRNA-injected morulae (80% in 10 min). The P_{Gly} and L_P of dsRNA-injected morulae (0.2 $x10^{-3}$cm min^{-1} and 0.6 μm $min^{-1}atm^{-1}$, respectively) were much lower than those of intact morulae (5.9 $x10^{-3}$cm min^{-1} and 2.5 μm $min^{-1}atm^{-1}$, respectively) (Tables 5 and 6). The L_P of dsRNA-injected morulae (0.6 μm $min^{-1}atm^{-1}$) was even lower than those of oocytes (1.3 μm $min^{-1}atm^{-1}$). The P_{Gly} of dsRNA-injected morulae (0.2 $x10^{-3}$cm min^{-1}) was also lower than that of intact oocytes (0.7 $x10^{-3}$cm min^{-1}) but was not significant.

In an ethylene glycol solution, intact morulae shrunk only slightly (99% in 5 sec) and regained their volume (100% in 10 sec), whereas dsRNA-injected morulae shrunk slowly (80% in 1 min) and regained their volume quite slowly (86% in 3 min) (Fig. 6B). Oocytes shrunk extensively (67% in 30 sec) and regained their volume slowly (84% in 3 min). The P_{EG} of dsRNA-injected morulae (1.5 $x10^{-3}$cm min^{-1}) was much lower than that of intact morulae (17.6 $x10^{-3}$cm min^{-1}) (Table 6). The value was even lower than that of oocytes (2.5 $x10^{-3}$cm min^{-1}) but was not significant. The L_P of dsRNA-injected morulae (0.4 μm $min^{-1}atm^{-1}$) was as low as that of intact morulae and oocytes (0.9-1.1 μm $min^{-1}atm^{-1}$) (Table 5).

In a DMSO solution, intact morulae and oocytes shrunk rapidly (58-60% in 10sec) and regained their volume relatively slowly (82% in 3 min) (Fig. 6C). On the other hand, dsRNA-injected morulae shrunk markedly slowly (73% in 30 sec), but regained their volume like intact morulae and oocytes (82% in 3 min). The P_{DMSO} of dsRNA-injected morulae (0.9 x10^{-3}cm min^{-1}) was not significantly different from that of oocytes (2.0 x10^{-3}cm min^{-1}) and intact morulae (1.1 x10^{-3}cm min^{-1}) (Table 6), whereas the L_P of dsRNA-injected morulae (0.5 μm min^{-1}atm^{-1}) was significantly lower than that of oocytes and intact morulae (2.1-3.3 μm min^{-1}atm^{-1}) (Table 5). In a propylene glycol solution, dsRNA-injected morulae shrunk (77% in 30 sec) and regained their volume slowly (84% in 3 min) like intact morulae and oocytes (66-74% in 30 sec and 88-89% in 3 min) (Fig. 6D). The P_{PG} of dsRNA-injected morulae (1.8 x10^{-3}cm min^{-1}) was not significantly different from that of oocytes (3.1 x10^{-3}cm min^{-1}) and intact morulae (2.7 x10^{-3}cm min^{-1}) (Table 6). Like the L_P of morulae and oocytes, the L_P of dsRNA-injected morulae was low (0.5 μm min^{-1}atm^{-1}). The value was slightly but significantly lower than that of intact morulae and oocytes (1.0-1.2 μm min^{-1}atm^{-1}) (Table 5).

Permeability to Water and Cryoprotectants of Mouse Oocytes Expressing Bovine AQP3
The results in Figure 4, and Tables 5 and 6 show that bovine oocytes expressed endogenous AQP3, which would be involved in the movement of water and cryoprotectants to some extent. In addition, other water channels and cryoprotectant channels could be involved in the relatively high membrane-permeability of bovine oocytes. On the other hand, mouse oocytes do not express a functional level of water channels and cryoprotectant channels [4, 5], and thus would not express these channels virtually. Therefore, we considered that mouse oocytes are more suitable cells than bovine oocytes that were expressing bovine AQP3 exogenously in order to examine the transporting property of bovine AQP3. We injected bovine *AQP3* cRNA into mouse oocytes at the germinal vesicle stage and matured to the metaphase II stage to express bovine AQP3 protein. Figure 7 shows that bovine AQP3 protein was expressed in mouse oocytes and is located in the cytoplasm and at around the plasma membrane. Although we can confirm the expression near the plasma membrane, we could not clearly confirm the expression in the plasma membrane. In a hypertonic sucrose solution, intact mouse oocytes shrunk quite slowly (59% in 1 min), whereas cRNA-injected oocytes shrunk very rapidly (59% in 10 sec) (Fig. 8). Consequently, the L_P of cRNA-injected oocytes was high (4.0 μm min^{-1}atm^{-1}), whereas that of intact mouse oocytes was low (0.7 μm min^{-1}atm^{-1}) (Table 7). These results indicate that bovine AQP3 protein was located in the plasma membrane. Therefore, we considered mouse oocytes expressing bovine AQP3 to be a good model for bovine morulae.

In a glycerol solution, cRNA-injected oocytes shrunk very rapidly (58% in 5 sec) and regained their volume very rapidly (94% in 2 min), whereas intact oocytes shrunk extensively (36% in 1 min) and regained their volume very slowly (41% in 10 min) (Fig. 9A). The P_{GLY} and L_P of cRNA-injected oocytes were markedly high (4.6 x10^{-3}cm min^{-1} and 2.8 μm min^{-1}atm^{-1}, respectively), whereas those of intact oocytes were quite low (0.02 x10^{-3}cm min^{-1} and 0.6 μm min^{-1}atm^{-1}, respectively) (Tables 7 and 8).

In an ethylene glycol solution, cRNA-injected oocytes shrunk only a little (93% in 10 sec) and regained the volume soon (99% in 2 min), whereas that of intact oocytes shrunk greatly (55% in 30 sec) and regained their volume slowly (95% in 10 min) (Fig. 9B). The P_{EG} of cRNA-injected oocytes (12.6 x10^{-3}cm min^{-1}) was 13 times higher than that of intact oocytes (1.0 x10^{-3}cm min^{-1}) (Table 8). On the other hand, the L_P of cRNA-injected oocytes (0.7 μm min^{-1}atm^{-1}) was as low as that of intact oocytes (0.7 μm min^{-1}atm^{-1}) (Table 7).

In a DMSO solution, cRNA-injected oocytes shrunk quite rapidly (48% in 5 sec) but regained their volume slowly (90% in 5 min), whereas intact oocytes shrunk slowly (64% in 30 sec) and regained their volume slowly (92% in 5 min) (Fig. 9C). The P_{DMSO} of cRNA-injected oocytes was not high (1.4 $\times 10^{-3}$cm min^{-1}) and was similar to that of intact oocytes (1.6 $\times 10^{-3}$cm min^{-1}) (Table 8). On the other hand, the L_P of cRNA-injected oocytes (3.8 μm min^{-1}atm^{-1}) was much higher than that of intact oocytes (0.6 μm min^{-1}atm^{-1}) (Table 7).

In a propylene glycol solution, oocytes shrunk slowly (70-74% in 30 sec) and regained their volume slowly (90-92% in 3 min), regardless of the injection of *AQP3* cRNA (Fig. 9D). Therefore, the P_{PG} of cRNA-injected oocytes (3.1 $\times 10^{-3}$cm min^{-1}) was similar to that of intact oocytes (2.4 $\times 10^{-3}$cm min^{-1}) (Table 8). The L_P of cRNA-injected oocytes (1.0 μm min^{-1}atm^{-1}) was as low as that of intact oocytes (0.7 μm min^{-1}atm^{-1}) (Table 7).

DISCUSSION

The permeability of the plasma membrane is one of important cryobiological properties affecting the survival of a cell after vitrification. In previous studies, we have clarified the pathway for the movement of water and cryoprotectants in mouse oocytes and embryos at various developmental stages [4, 5]. In the present study, we deduced the pathway in bovine oocytes/embryos to know the species difference.

To determine the permeability of bovine oocytes/embryos, we first examined the V_b of the oocytes and embryos. The value was variable (0.25-0.40), but was higher than that of mouse oocytes and embryos (0.14-0.16) [4], probably because bovine oocytes/embryos contain lipid droplets abundantly. However, the V_b of bovine morulae in the present study (0.31) was much higher than that reported by Mazur and Schneider (1986) for *in vivo*-derived morulae (0.16) [27]. We used bovine embryos derived from *in vitro* fertilization and *in vitro* culture. It is known that bovine embryos derived from *in vitro* fertilization and *in vitro* culture have a larger amount of lipid droplets than those developed *in vivo*, thus, the V_b value that we obtained might be higher than that of their report.

The L_P for bovine oocytes (1.8 μm min^{-1}atm^{-1}) and 16-cell embryos (1.2 μm min^{-1}atm^{-1}) in a sucrose solution was lower than that of bovine morulae and blastocysts (2.6-3.1 μm min^{-1}atm^{-1}) (Table 3), but higher than that of mouse oocytes (0.7 μm min^{-1}atm^{-1}) (Table 7) [4, 5]. The E_a value for the L_P (9 kcal mol^{-1}) was also much higher than that of bovine morulae and blastocysts (3 kcal/mol) (Table 3), although it was lower than 10 kcal mol^{-1}. These results suggest that water molecules move through bovine oocytes and early embryos predominantly by simple diffusion, but a smaller proportion of water would move via channel processes. Since a little amount of AQP3 was expressed in bovine oocytes (Fig. 4), it would be involved in the movement of water in bovine oocytes and early embryos. On the other hand, we have already shown that mouse oocytes and early embryos do not express water channels at a functional level, and water molecules move through the plasma membrane predominantly by simple diffusion but not by channel processes [4, 5]. Therefore, bovine oocytes and early embryos have different permeability-related properties from those of mouse oocytes and early embryos.

AQP3 is an aquaglyceroporin and transport not only water but also cryoprotectants [7]. We have showed in previous studies that mouse AQP3 transported glycerol and ethylene glycol but not

DMSO and propylene glycol [4, 5]. We have also showed that mouse AQP3 transported water in a hypertonic sucrose solution, in a glycerol solution, and in a DMSO solution but not in an ethylene glycol solution and in a propylene glycol solution [4, 5]. In the present study, we showed that bovine AQP3 expressing in mouse oocytes also had the same permeability-related properties (Tables 7 and 8). Therefore, it is possible that bovine oocytes and early embryos have higher permeability to glycerol and ethylene glycol. As expected, the P_{Gly} of bovine oocytes and 16-cell embryos was 0.5 $x10^{-3}$cm min^{-1} (Tables 4 and 6), and was 17-50 time higher than that of mouse oocytes (Table 8) [5]. The P_{EG} of bovine oocytes and 16-cell embryos was 2.0-3.5 $x10^{-3}$cm min^{-1} and this value was also 3-4 times higher than that of mouse oocytes (0.4-0.8 x 10^{-3}cm min^{-1}) (Table 8) [5]. In bovine oocytes, therefore, AQP3 would play a role in the movement of glycerol and ethylene glycol unlike in mouse oocytes. On the other hand, P_{DMSO} and P_{PG} of bovine oocytes (1.5-2.0 $x10^{-3}$cm min^{-1} and 2.5-3.1 $x10^{-3}$cm min^{-1}, respectively) (Tables 4 and 6) were as low as those of mouse oocytes (1.6 and 2.4 x 10^{-3}cm min^{-1}, respectively) (Table 8), showing that bovine AQP3 and other channels did not transport these cryoprotectants.

The L_P of bovine oocytes in a glycerol solution and a DMSO solution was higher than that of mouse oocytes (Table 7) and *AQP3* dsRNA-injected bovine morulae (Table 5). However, this tendency was not observed in an ethylene glycol solution and a propylene glycol solution. Similar results were also obtained with bovine AQP3; bovine AQP3 transported water in the presence of glycerol, and DMSO, but not ethylene glycol or propylene glycol (Table 7), as mouse AQP3 [5].

Bovine morulae and blastocysts had a high L_P value (2.6-3.2 μm $min^{-1}atom^{-1}$) with a low E_a value (3 kcal mol^{-1}) (Table 3) in a sucrose solution, like mouse morulae (3.3-4.2 μm $min^{-1}atom^{-1}$ and 6 kcal mol^{-1}, respectively) [4, 5]. This suggests that water move through bovine morulae/blastocysts predominantly by facilitated diffusion via channel processes. The movement would mostly rely on AQP3 because the suppression of AQP3-expression in bovine morulae by its dsRNA strongly decreased the L_P value (from 3.3 to 0.6 μm $min^{-1}atom^{-1}$) (Table 5). The value was also lower than that of bovine oocytes (1.8-1.9 μm $min^{-1}atom^{-1}$) (Tables 3 and 5). In general, it is considered that the gene-silencing is succeeded greatly if the expression level of the target protein or target mRNA decreased to less than 10%. In the present study, the L_P of bovine morulae (3.3 μm min^{-1} atm^{-1}) decreased to 19% (0.63 μm min^{-1} atm^{-1}) by injecting *AQP3* dsRNA (Table 5) and the value was similar to that of mouse oocytes (0.65 μm min^{-1} atm^{-1}) (Table 7) in which virtually no water channels are not expressed, suggesting that water moves through dsRNA-injected bovine morulae predominantly by simple diffusion. In addition, P_{Gly} of bovine morulae decreased to 4% by injecting *AQP3* dsRNA (Table 6), although the P_{Gly} of dsRNA-injected morulae was still 12 times higher than that of mouse oocytes (Table 8). Taking account of the suppression of AQP3 protein-expression (Fig. 4) and the very low L_P and P_{Gly} of *AQP3* dsRNA-injected bovine morulae (Tables 5 and 6), we considered that the suppression of AQP3 in the apical side of the plasma membrane of bovine morulae was succeeded greatly (probably less than 10%).

P_{Gly} and P_{EG} of bovine morulae/blastocysts (2.8-3.8 and 10.1-12.3 $x10^{-3}$cm min^{-1}, respectively) were 3-7 times higher than the values for bovine oocytes/early embryos (0.5 and 2.0-3.5 $x10^{-3}$cm min^{-1}, respectively) (Table 4) as those of mouse morulae/blastocysts [4, 5], probably because bovine AQP3 transported glycerol and ethylene glycol effectively (Table 8). This was supported by the marked decrease in the P_{Gly} and P_{EG} (to 4% and 8%, respectively) of bovine morulae by injecting *AQP3* dsRNA (Table 6). On the other hand, P_{DMSO} and P_{PG} of bovine morulae did not decrease by the injection of *AQP3* dsRNA (Table 6) probably because bovine AQP3 did not transfer DMSO and propylene glycol (Table 8). Previously, we suggested that mouse morulae expressed cryoprotectant channels other than AQP3 and that the channels transported DMSO in mouse morulae [5]. In the present study, it was shown that bovine morulae and blastocysts did not express DMSO-permeable channels, because bovine morulae/blastocysts had a low P_{DMSO} and a high E_a for the P_{DMSO} as bovine oocytes and 16-cell embryos (Table 4). Therefore, the permeability-related property of bovine morulae/blastocysts was partly different from that of mouse morulae/blastocysts by the lack of channels which were permeable to DMSO.

Bovine AQP3 transported water in the presence of glycerol or DMSO, but not in the presence of ethylene glycol or propylene glycol. In a glycerol solution and a DMSO solution, L_P of bovine morulae was higher than that of bovine oocytes (Table 3), and the suppression of AQP3 expression significantly decreased the L_P (Table 5). In these solutions, therefore, bovine morulae transported water predominantly through AQP3. In the presence of ethylene glycol or propylene glycol, on the other hand, bovine AQP3 did not transport water (Table 7) as mouse AQP3, and thus the suppression of AQP3 expression in bovine morulae did not affect L_P (Table 5), as in the case of mouse morulae [5].

It has been shown that bovine oocytes are less tolerant to cryopreservation than mouse oocytes. However, the present study shows that bovine oocytes have higher membrane permeability than mouse oocytes. If the tolerance of the cell to cryopreservation was speculated only by the permeability of the plasma membrane, therefore, bovine oocytes could be vitrified more easily than mouse oocytes because higher membrane-permeability would promote dehydration and movement of cryoprotectant before cooling, and also promote removal of cryoprotectants after warming, which would prevent damages by intracellular ice formation, the toxicity of cryoprotectants, and excessive osmotic swelling. Therefore, other factors should be involved in the low tolerance of bovine oocytes to cryopreservation. Further studies are needed to clarify this.

Various cryopreservation protocols for mammalian oocytes/embryos have been developed first in the mouse [1, 28, 29] and in other species. For the modification and optimization of cryopreservation protocol in various species, empirical approach has been adopted so far. The present study shows that the difference in the important permeability-related property of oocytes/embryos exists among mammalian species, and that the difference is derived from the expression level of water-channels and cryoprotectant-channels. Pathway for the movement of water and cryoprotectants across the plasma membrane would markedly affect the condition for vitrification. If the movement of water and cryoprotectants depends predominantly on simple diffusion across the plasma membrane, the permeability to them would be low and would be markedly affected by temperature. On the other hand, if the movement depends predominantly on facilitated diffusion via channels, membrane-permeability would be markedly high and would be less affected by temperature. These properties are important for adopting a suitable condition for vitrification. Therefore, theoretical approach would be useful for developing suitable protocols of vitrification of oocytes/embryos. In the treatment of infertility and fertility preservation in gynecologic carcinoma, the cryopreservation of human oocytes with high and consistent survival has been expected. To find suitable conditions for human oocytes, theoretical approach would be useful because they could not be used for experiment.

The present study clarified the similarity and difference in the permeability-related property of oocytes and embryos in different species.

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Figure legends

FIG. 1. Changes in volume of bovine oocytes (A), 16-cell embryos (B), morulae (C), and blastocysts (D) in a hypertonic sucrose solution. Bovine oocytes and embryos were exposed to PB1 medium containing 0.43 M sucrose at 25^{o}C (open) and 15^{o}C (shaded) for 10 min. Graphs show the changes in volume during the first 2 min. Data are the means of relative volume ± SD. The numbers of oocytes, 16-cell embryos, morulae, and blastocysts were 15, 9, 27, and 20, respectively.

FIG. 2. Changes in volume of bovine oocytes (circles), 16-cell embryos (triangles), morulae (squares), and blastocysts (diamonds) in PB1 medium containing a cryoprotectant at 25^{o}C.

Bovine oocytes and embryos were exposed to PB1 medium containing 10% (vol/vol) glycerol (A), 8% ethylene glycol (B), 9.5% (vol/vol) DMSO (C), and 10% (vol/vol) propylene glycol (D) at 25^{o}C for 10 (embryos) or 20 (oocytes) min. Graphs show the changes in volume during the first 10 min (A_1-D_1) and 2 min (A_2-D_2). Data are the means of relative volume ± SD. The numbers of oocytes, 16-cell embryos, morulae, and blastocysts are as follows; in glycerol (6, 4, 11, 6), ethylene glycol at (6, 7, 9, 9), DMSO (6, 8, 11, 4), and propylene glycol (6, 7, 11, 6).

FIG 3. Changes in volume of bovine oocytes (circles), 16-cell embryos (triangles), morulae (squares), and blastocysts (diamonds) in PB1 medium containing a cryoprotectant at 15^{o}C. Bovine oocytes and embryos were exposed to PB1 medium containing 10% (vol/vol) glycerol (A), 8% ethylene glycol (B), 9.5% (vol/vol) DMSO (C), and 10% (vol/vol) propylene glycol (D) at 15^{o}C for 10 (embryos) or 20 (oocytes) min. Graphs show the changes in volume during the first 10 min (A_1-D_1) and 2 min (A_2-D_2). Data are the means of relative volume ± SD. Oocytes and embryos were the same ones as those in FIG. 2.

FIG. 4. The suppression of AQP3-expression in bovine morulae. Bovine embryos at the 1-cell stage were injected with double-stranded RNA of bovine *AQP3*, and cultured to the morula stage. The expression of AQP3 was detected by immunofluorescent-staining using anti-rat AQP3 rabbit antibody and FITC-conjugated anti-rabbit immunoglobulin goat antibody. A, an intact bovine morula; B, a double-stranded RNA-injected bovine morula; C, a mature bovine oocyte.

FIG 5. Changes in volume in PB1 medium containing sucrose of mouse morulae injected with the double-stranded RNA of bovine *AQP3* at the 1-cell stage. Mature bovine oocytes (open circles), double stranded RNA-injected morulae (closed squares) and intact morulae (open squares) were exposed to PB1 medium containing 0.43 M sucrose for 10 min 25^{o}C. Graphs show the changes in volume during the first 2 min. Data are the means of relative volume ± SD. The numbers of intact oocytes, intact morulae, and double-stranded RNA-injected morulae were 20, 19, and 20, respectively.

FIG. 6. Changes in volume in PB1 medium containing a cryoprotectant of bovine morulae injected with double-stranded RNA of bovine *AQP3* at the 1-cell stage. Bovine oocytes (open circles), double-stranded RNA-injected morulae (closed squares) and intact morulae (open squares) were exposed to PB1 medium containing 10% (vol/vol) glycerol (A), 8% (vol/vol) ethylene glycol (B), 9.5% (vol/vol) DMSO (C), or 10% (v/v) propylene glycol (D) at 25°C for 10 (morulae) or 20 (oocytes) min. Graphs show the changes in volume during the first 10 min (A_1-D_1) and 2 min (A_2-D_2). The data are the means of relative volume ± SD. The numbers of intact oocytes, intact morulae, and double-stranded RNA-injected morulae are as follows; in glycerol (5, 8, 5), ethylene glycol (5, 5, 5), DMSO (5, 5, 5), and propylene glycol (5, 5, 5).

FIG 7. The expression of bovine AQP3 in mouse oocytes. Mouse oocytes at the germinal vesicle stage were injected with cRNA of bovine *AQP3*, and cultured for 12-14 h. The expression of AQP3 was detected by immunofluorescent-staining using anti-rat AQP3 rabbit antibody and FITC-conjugated anti-rabbit immunoglobulin goat antibody. A, an intact oocyte; B, a cRNA-injected oocyte.

FIG 8. Changes in volume in a hypertonic sucrose solution of mouse oocytes at metaphase II stage that had been injected with bovine *AQP3* cRNA at the germinal vesicle stage. Intact (open) and cRNA-injected (closed) oocytes were exposed to PB1 medium containing 0.43 M sucrose at 25°C for 10 min. Graphs show the changes in volume during the first 2 min. The data are the means of relative volume ± SD. The number of intact oocytes and cRNA-injected oocytes is 5 each.

FIG. 9. Changes in volume in PB1 medium containing a cryoprotectant of mouse oocytes at the metaphase II stage that had been injected with bovine *AQP3* cRNA at the germinal vesicle stage. Intact (open) and cRNA-injected (closed) oocytes were exposed to PB1 medium containing 10% (vol/vol) glycerol (A), 8% (vol/vol) ethylene glycol (B), 9.5% (vol/vol) DMSO (C),or 10% (vol/vol) propylene glycol (D) at 25^{o}C for 20 min. Graphs show the changes in volume during the first 10 min (A_1-D_1) and 2 min (A_2-D_2). The data are the means of relative volume ± SD. The numbers of intact oocytes and cRNA-injected oocytes are as follows; in glycerol (5, 5), ethylene glycol (5, 8), DMSO (5, 7), and propylene glycol (5, 5).

TABLE 1. The osmolality of solutions

Solvent	Additive Solutes	Osmolality (Osm/kg)
Water	0.18 M sucrose	0.20[c]
	0.43 M sucrose	0.50[c]
	10% (v/v) glycerol	1.59[c]
	8% (v/v) ethylene glycol	1.61[c]
	9.5% (v/v) DMSO[b]	1.55[d]
	10% (v/v) propylene glycol	1.56[c]
PB1[a]	0.18 M sucrose	0.50
	0.43 M sucrose	0.80
	10% (v/v) glycerol	1.89
	8% (v/v) ethylene glycol	1.91
	9.5% (v/v) DMSO	1.85
	10% (v/v) propylene glycol	1.86

[a]The osmolality of PB1 medium measured with a freezing point depression osmometer was 0.3 Osm/kg.

[b]Dimethylsulfoxide.

[c]Osmolality calculated from published data on the colligative properties of the solutes in aqueous solutions [23].

[d]Osmolality measured with a vapor pressure osmometer.

TABLE 2. Constants and parameters used for fitting permeability parameters

Symbol	Meaning	Values
R	Gas constant	8.206×10^{-2} L atm $K^{-1}mol^{-1}$
T	Absolute temperature	288 K and 298 K
$\overline{V}_w$	Partial molar volume of water	0.018 L mol^{-1}
$\overline{V}_{Gly}$	Partial molar volume of glycerol[a]	0.071 L mol^{-1}
$\overline{V}_{EG}$	Partial molar volume of ethylene glycol[a]	0.054 L mol^{-1}
$\overline{V}_{DMSO}$	Partial molar volume of dimethylsulfoxide[b]	0.069 L mol^{-1}
$\overline{V}_{PG}$	Partial molar volume of propylene glycol[a]	0.070 L mol^{-1}

[a]Partial molar volumes of cryoprotectants from Wolf et al. [23].
[b]Partial molar volume of dimethylsulfoxide from Kiyohara et al. [24].

TABLE 3. Permeability to water (L_P, µm min^{-1}atm^{-1}) of bovine oocytes and embryos in PB1 medium containing sucrose or a cryoprotectant at 15°C and 25°C and the activation energy (E_a, kcal mol^{-1}) of the L_p

Cell	L_p and its E_a	Sucrose	Cryoprotectant			
			Glycerol	Ethylene glycol	DMSO	Propylene glycol
Oocyte	L_p at 15°C	1.09±0.27	0.62±0.10	0.36±0.11	0.65±0.24	0.60±0.13
	L_p at 25°C	1.81±0.45 ^{a}	0.99±0.17 ^{a}	0.84±0.29 ^{ab}	1.24±0.22 ^{ab}	1.08±0.24
	E_a	8.7	8.0	14.4	11.0	10.0
16-cell embryo	L_p at 15°C	0.70±0.19	0.45±0.20	0.29±0.08	0.46±0.19	0.37±0.06
	L_p at 25°C	1.16±0.29 ^{a}	0.71±0.15 ^{a}	0.58±0.19 ^{ab}	0.79±0.40 ^{a}	0.76±0.10
	E_a	8.6	7.8	11.8	9.2	12.3
Morula	L_p at 15°C	2.62±0.73	1.57±0.19	0.30±0.10	1.21±0.49	0.46±0.20
	L_p at 25°C	3.15±0.96 ^{b}	1.76±0.48 ^{b}	0.58±0.30 ^{a}	1.37±0.50 ^{b}	0.76±0.33
	E_a	3.1	1.9	11.2	2.1	8.6
Blastocyst	L_p at 15°C	2.26±0.73	0.95±0.36	0.59±0.07	0.97±0.49	0.51±0.06
	L_p at 25°C	2.62±0.64 ^{b}	1.12±0.46 ^{a}	0.93±0.25 ^{b}	1.18±0.35 ^{ab}	0.83±0.52
	E_a	2.5	2.8	7.8	4.4	8.3

Values are expressed as means±SD.

$^{a, b}$ Values with different superscripts were significantly different ($P < 0.05$) in the L_P at 25°C as a function of the developmental stage, and there is no significant difference in the L_P at 25°C in the experimental group of propylene glycol.

Values were determined from the data shown in Figs. 1-3.

TABLE 4. Permeability to cryoprotectants (P_s) (x10^{-3} cm min^{-1}) of bovine oocytes and embryos in PB1 medium containing a cryoprotectant at 15°C and 25°C and the activation energy (E_a, kcal mol^{-1}) of the P_s

Cell	P_s and its E_a	Cryoprotectant			
		Glycerol	Ethylene glycol	DMSO	Propylene glycol
Oocyte					
	P_s at 15°C	0.19±0.09	1.46±0.68	0.69±0.24	0.95±0.14
	P_s at 25°C	0.51±0.29 [a]	3.50±0.63 [a]	1.49±0.22	2.55±0.90
	E_a	17.1	14.9	13.2	16.9
16-cell embryo					
	P_s at 15°C	0.26±0.24	1.12±1.00	0.48±0.21	0.73±0.21
	P_s at 25°C	0.48±0.25 [a]	2.04±1.39 [a]	1.63±0.51	2.48±0.70
	E_a	10.6	10.3	20.7	20.9
Morula					
	P_s at 15°C	2.57±0.73	11.13±2.94	0.48±0.21	0.83±0.19
	P_s at 25°C	3.75±0.87 [b]	12.34±4.76 [b]	1.67±0.84	2.75±0.47
	E_a	6.4	1.8	21.1	20.4
Blastocyst					
	P_s at 15°C	2.21±0.69	8.62±4.01	0.68±0.42	1.02±0.53
	P_s at 25°C	2.76±0.99 [b]	10.85±2.74 [b]	2.07±0.78	3.36±2.11
	E_a	3.8	3.9	19.0	20.3

Values are expressed as means±SD.

[a, b] Values with different superscripts were significantly different ($P < 0.05$) in the P_S at 25°C as a function of the developmental stage, and there is no significant difference in the P_S at 25°C in the experimental group of DMSO and propylene glycol.

Values were determined from the data shown in Figs. 2 and 3.

TABLE 5. Hydraulic conductivity (L_P, μm min^{-1}atm^{-1}) of bovine oocytes and morulae injected with dsRNA of *AQP3* measured in PB1 medium containing sucrose or a cryoprotectant at 25°C

Cell	Sucrose	Cryoprotectant			
		Glycerol	Ethylene glycol	DMSO	Propylene glycol
Intact oocyte	1.93±0.62 ^{a}	1.28±0.23 ^{a}	0.90±0.28	2.14±0.83 ^{a}	1.15±0.08 ^{a}
Intact morula	3.30±0.70 ^{b}	2.48±0.54 ^{b}	1.11±0.91	3.34±0.84 ^{b}	0.95±0.24 ^{a}
dsRNA-injected morula	0.63±0.27 ^{c}	0.55±0.16 ^{c}	0.38±0.06	0.53±0.08 ^{c}	0.54±0.15 ^{b}

Values are expressed as means±SD.

$^{a\text{-}c}$ Values with different superscripts were significantly different ($P < 0.05$) as a function of the cell type, and there is no significant difference in the experimental group of ethylene glycol.

Values were determined from the data shown in Figs. 5 and 6.

TABLE 6. Permeability to cryoprotectants (P_s, x10^{-3} cm min^{-1}) of bovine oocytes and morulae injected with *AQP3* dsRNA measured in PB1 medium containing a cryoprotectant at 25^{o}C

Cell	Cryoprotectant			
	Glycerol	Ethylene glycol	DMSO	Propylene glycol
Intact oocyte	0.69±0.47 [a]	2.50±1.32 [a]	1.98±1.04	3.08±0.86
Intact morula	5.91±2.28 [b]	17.58±10.74 [b]	1.07±0.41	2.73±0.97
dsRNA-injected morula	0.24±0.15 [a]	1.47±0.89 [a]	0.86±0.37	1.80±1.20

Values are expressed as means±SD.

[a, b] Values with different superscripts were significantly different ($P < 0.05$) as a function of the cell type, and there is no significant difference in the experimental group of DMSO and propylene glycol.

Values were determined from the data shown in Fig. 6.

TABLE 7. Hydraulic conductivity (L_P, μm $mim^{-1}atm^{-1}$) of mouse oocytes injected with bovine *AQP3* cRNA measured in PB1 medium containing sucrose or a cryoprotectant at 25°C

Treatment	Sucrose	Cryoprotectant			
		Glycerol	Ethylene glycol	DMSO	Propylene glycol
Intact	0.65±0.16	0.62±0.17	0.68±0.14	0.62±0.08	0.71±0.11
cRNA-injected	4.02±1.80*	2.80±0.53*	0.66±0.26	3.83±1.16*	1.01±0.28

Values are expressed as means±SD.

*Significantly different from the value for intact oocytes ($P < 0.05$).

Values were determined from the data shown in Figs. 8 and 9.

TABLE 8. Permeability to cryoprotectants (P_S, x10^{-3} cm min^{-1}) of mouse oocytes injected with bovine *AQP3* cRNA in PB1 medium containing a cryoprotectant at 25^{o}C

Treatment	Cryoprotectant			
	Glycerol	Ethylene glycol	DMSO	Propylene glycol
Intact	0.02±0.0	0.99±0.30	1.56±0.29	2.41±0.78
cRNA-injected	4.59±1.09*	12.64±8.26*	1.42±0.72	3.07±0.85

Values are expressed as means±SD.
*Significantly different from the value for intact oocytes ($P < 0.05$).
Values were determined from the data shown in Fig. 9.

FIG. 1

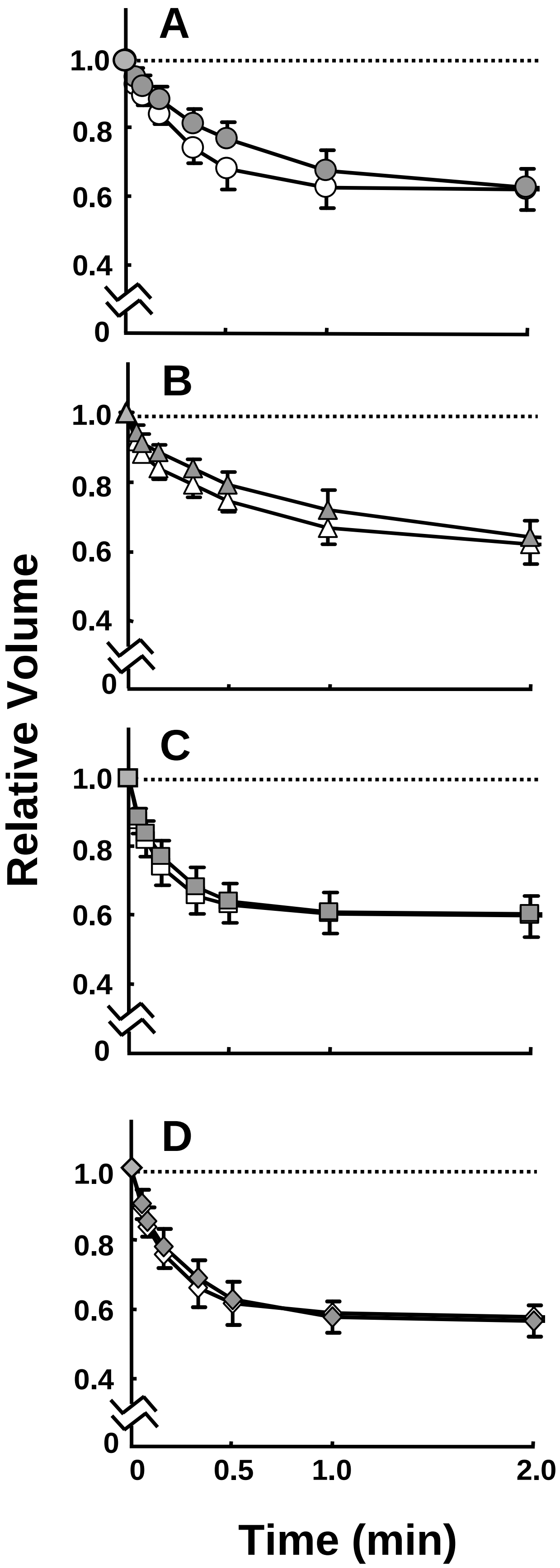
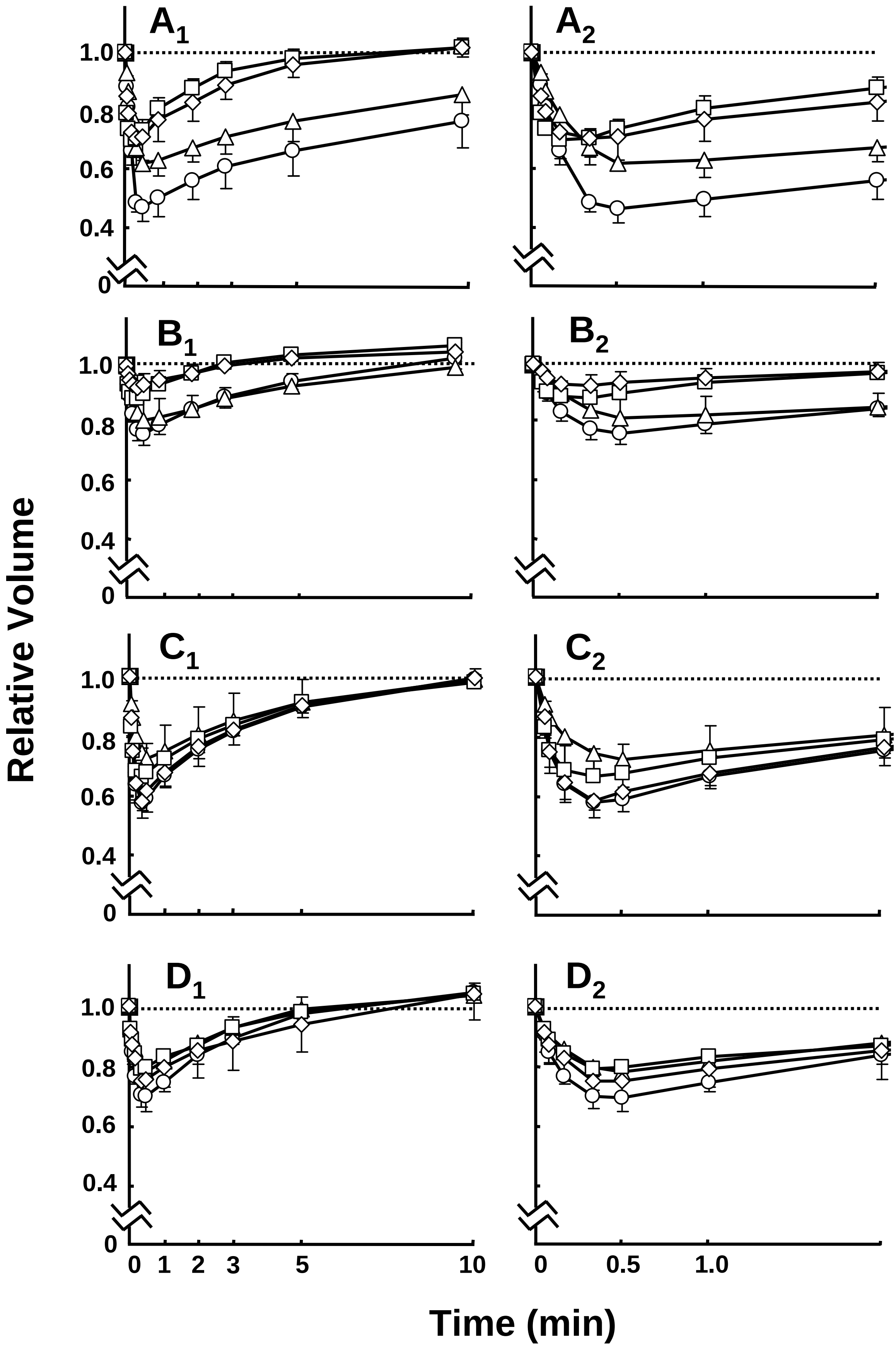


FIG. 2


A1
A2
B1
B2
C1
C2
D1
D2
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0.8
0.6
0.4
0
0 1 2 3 5 10
0 0.5 1.0
Relative Volume
Time (min)

FIG. 3

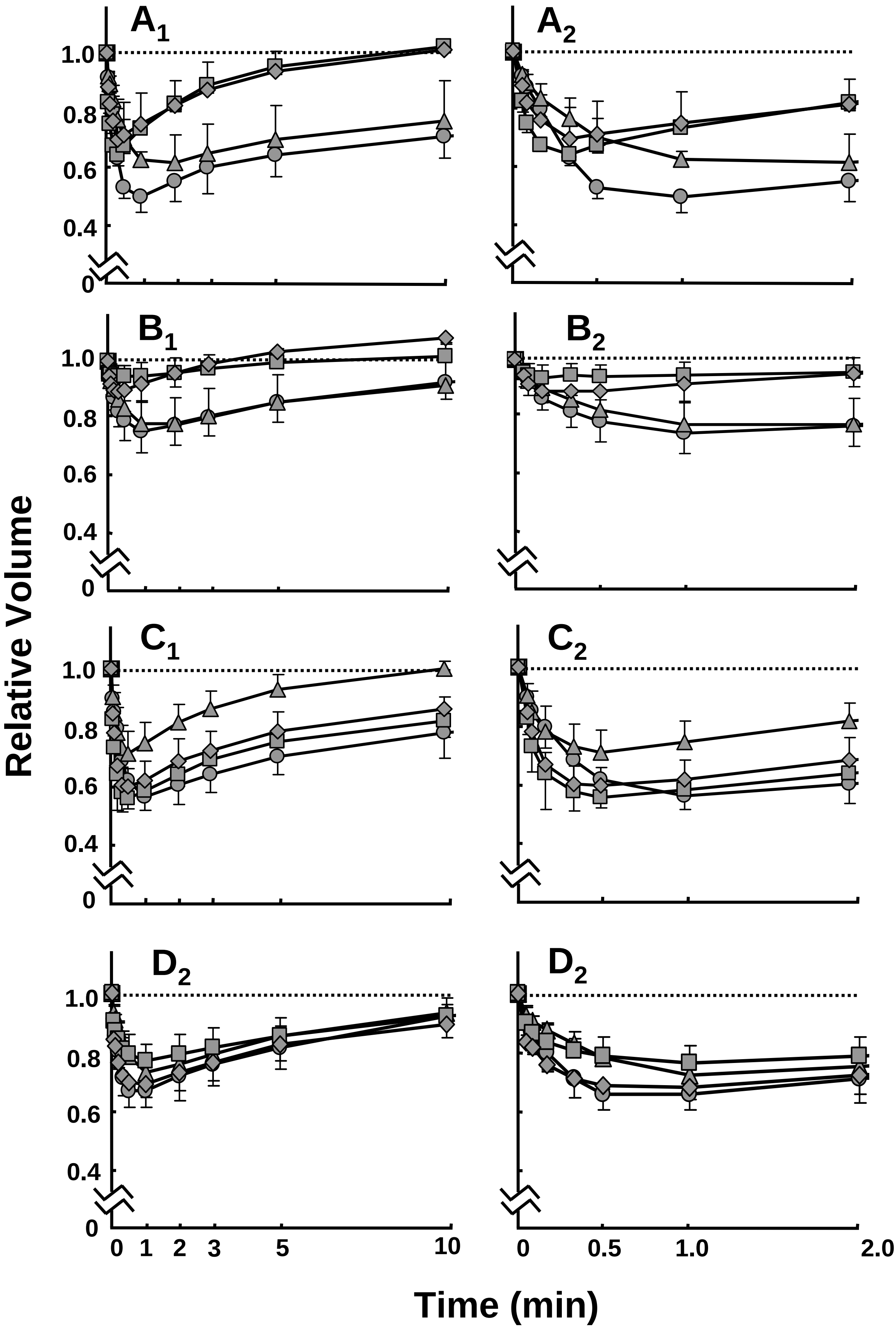

A1
A2
B1
B2
C1
C2
D2
D2
Relative Volume
1.0
0.8
0.6
0.4
0
0 1 2 3 5 10
0 0.5 1.0 2.0
Time (min)

FIG. 4

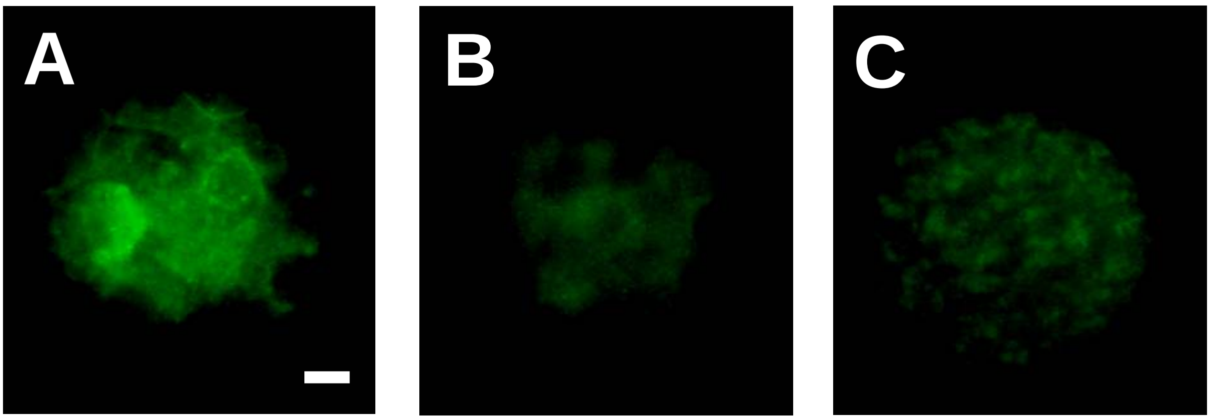


FIG. 5

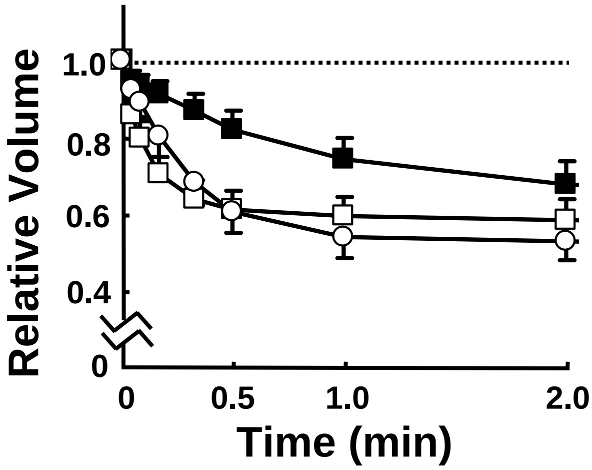
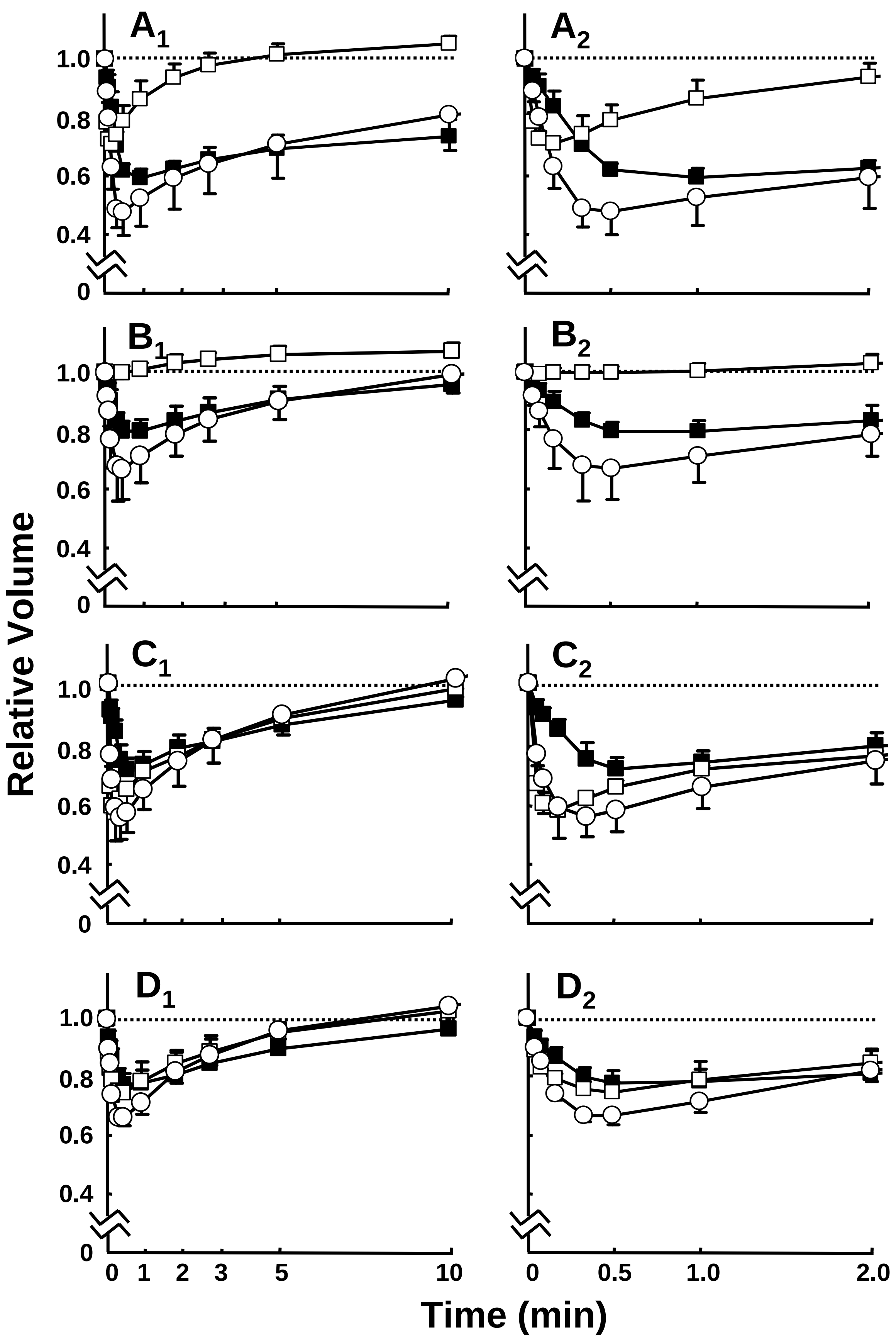


FIG. 6



A1
A2
B1
B2
C1
C2
D1
D2
Relative Volume
1.0
0.8
0.6
0.4
0
0
1
2
3
5
10
0.5
1.0
2.0
Time (min)

FIG. 7

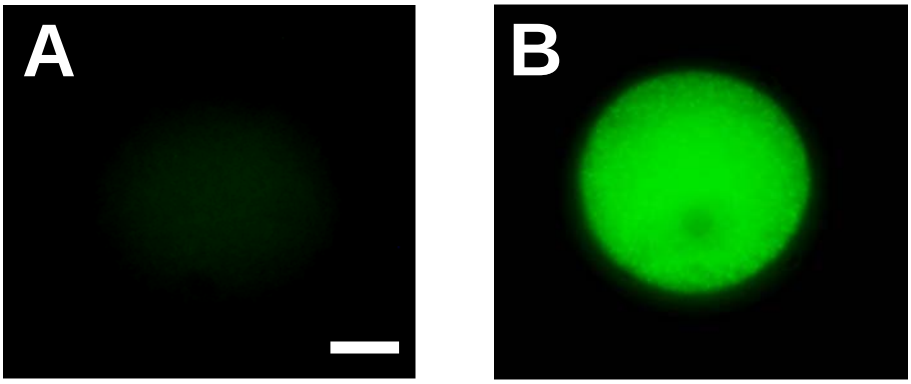


FIG. 8

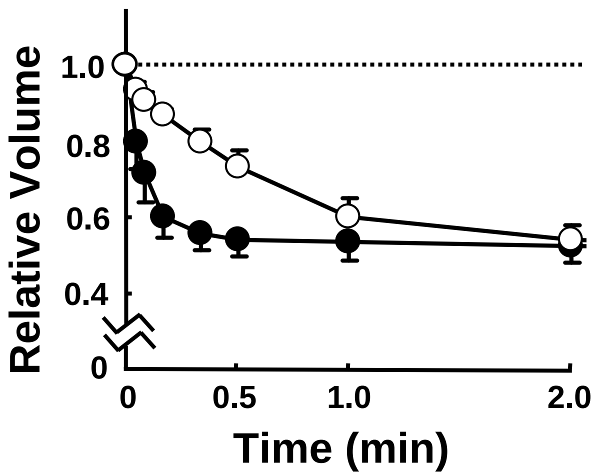
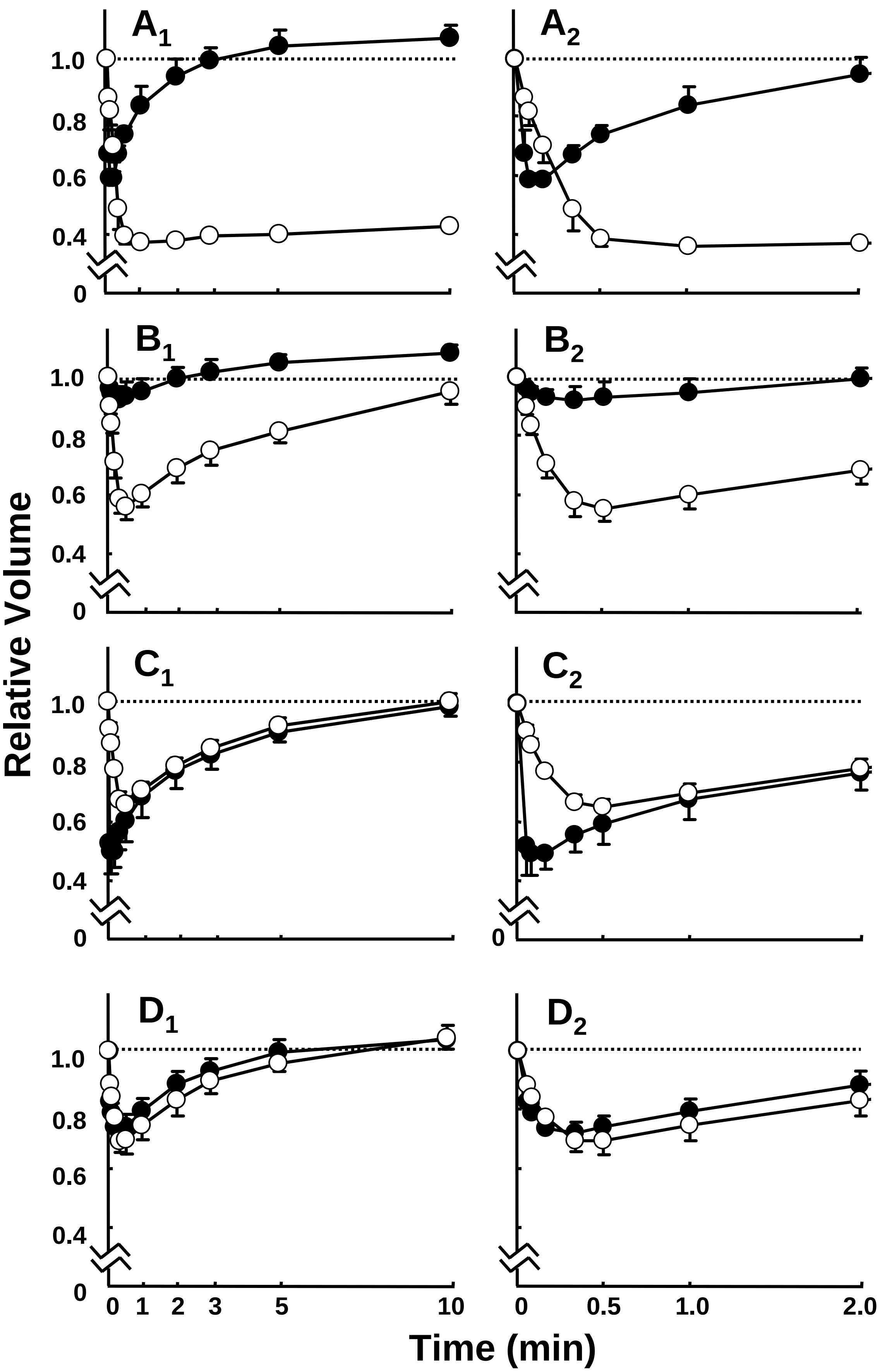


FIG. 9



A1
A2
B1
B2
C1
C2
D1
D2
1.0
0.8
0.6
0.4
0
Relative Volume
0 1 2 3 5 10
0 0.5 1.0 2.0
Time (min)