such that water molecules with greater ease of evaporation have higher $T_2$ values.

In contrast, for hydration water that has low mobility and shows Gaussian decay, $T_2$ was found not to correlate with ease of evaporation under nonisothermal conditions, suggesting that molecular motion that determines the ease of evaporation is not reflected in $T_2$; in this case, $T_2$ cannot be used as a parameter to indicate molecular mobility.

The water molecules in the API hydrates studied were found to have wide-ranging molecular mobilities, from low molecular mobility that could not be evaluated by NMR relaxation times, such as the water molecules in pipemidic acid hydrate, to high molecular mobility that could be evaluated by NMR relaxation times, such as the water molecules in ceftazidime hydrate.

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# Effect of sugars on storage stability of lyophilized liposome/DNA complexes with high transfection efficiency

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**Abstract**

Cationic lipid-based gene delivery systems have shown promise in transfecting cells in vitro and in vivo. However, liposome/DNA complexes tend to form aggregates after preparation. Lyophilization of these systems, therefore, has become of increasing interest. In this study, we investigated the feasibility of preserving complexes as a dried preparation using a modified dehydration rehydration vesicle (DRV) method as a convenient and reliable procedure. We also studied storage stability of a lyophilized novel cationic gene delivery system incorporating sucrose, isomaltose and isomaltotriose. Liposomes were composed of 3β-[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and L-dioleoylphosphatidylethanolamine (DOPE), plus sucrose, isomaltose or isomaltotriose. Lyophilized liposome/DNA complexes were stored at −20, 25, 40 and 50 °C and their stability was followed for 50 days. Liposome/DNA complexes with sucrose could be stored even at 50 °C without large loss of transfection efficiency. The transfection efficiency of formulations stored at various temperatures indicated that the stabilizing effect of sugars on plasmid DNA was higher in the following order: isomaltotriose < isomaltose < sucrose, which was inverse to the order of their glass transition temperature ($T_g$) values. It was concluded that we could prepare novel lyophilized liposome/DNA complexes with high transfection efficiency and stability, which might be concerned that sucrose stabilized plasmid DNA in liposomes by directly interacting with plasmid DNA rather than by vitrifying to a high $T_g$ solid.


*Keywords:* Cationic liposome; Sucrose; Dehydration rehydration vesicle; Transfection; Storage stability; Lyophilization

## 1. Introduction

Cationic liposome-mediated transfer of DNA is a promising approach, because of low immunogenicity and toxicity, ease of preparation, and potential applications for active targeting. The disadvantages include poor efficiency of transfection in vivo. Therefore, many cationic lipid-based transfection reagents have been developed for the efficient delivery of DNA into cells (Gao and Huang, 1991; Vigneron et al., 1996). Commercially available cationic liposomes or particles are mixed with plasmid DNA, and tend to form large liposome/DNA aggregates in solution, especially at high DNA concentrations. They form as a result of electrostatic binding between cationic liposomes and negatively charged DNA, and are inherently difficult to manipulate, resulting in a decrease of transfection (Sternberg et al., 1994; Lai and van Zanten, 2002). Because of this problem, cationic liposome/DNA complexes have to be freshly prepared when they are used. This would make it demanding to prepare them, and make quality control very difficult due to the fact that preparation of cationic liposome/DNA complexes is a process that is poorly defined and difficult to control.

To produce stable gene delivery systems that avoid these problems, lyophilization is suitable for long-term storage. There are many studies about lyophilization of liposome vectors using sugars (Anchordoquy et al., 1997; Li et al., 2000; Molina et al., 2004). Disaccharides were used in most studies. Especially, sucrose, which has a high glass transition temperature ($T_g$), is known to be effective to maintain the stability of liposomes, presumably by forming glasses under the typical freezing conditions used for lyophilization (Molina et al., 2001). To develop lyophilized liposome complexes with plasmid DNA vector, we used a modified dehydration rehydration vesicle (DRV) method as a convenient and reliable procedure (Perrie and Gregoriadis, 2000). The technique of the DRV method, employing sucrose at

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the lyophilization stage, has been evaluated for a range of solutes (Zadi and Gregoriadis, 2000; Kawano et al., 2003) and plasmid DNA (Perrie et al., 2004). The effects of sugars on the stability of lyophilized liposomes, sizes of liposomes and entrapment efficiency of solutes using DRV methods have been reported (Zadi and Gregoriadis, 2000; Kawano et al., 2003), but there have been a few reports about the effect of sugars on the stability of plasmid DNA in liposomes during storage (Li et al., 2000).

In this study, we examined stability of lyophilized liposome/DNA complexes with sucrose, isomaltose or isomaltotriose at different temperatures over 50 days, and determined which sugars could inhibit aggregation and maintain the transfection activity of plasmid DNA during preservation at temperatures above $T_g$. We found that DRV/DNA complexes with sucrose could be stored even at 50 °C without a large loss of transfection activity. Isomaltose and isomaltotriose were selected as excipients because their $T_g$ values were higher than that of sucrose and therefore, they were expected to exhibit a greater stabilizing effect.

## 2. Materials and methods

### 2.1. Materials

3β-[*N*-(*N*′,*N*′-Dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and L-dioleoylphosphatidylethanolamine (DOPE) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Lipofectamine 2000 was purchased from Invitrogen Corp. (Carlsbad, CA, USA). The Pica gene luciferase assay kit was purchased from Toyo Ink Mfg. Co. Ltd. (Tokyo, Japan). BCA protein assay reagent was purchased from Pierce (Rockford, IL, USA). All other chemicals used were of reagent grade. The plasmid DNA encoding the luciferase marker gene (pAAV-CMV-Luc) was supplied by Dr. S. Tanaka in Mt. Sinai School of Medicine (NY, USA). All reagents were of analytical grade. RPMI1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Grand Island, NY, USA).

### 2.2. Preparation of DRV

The preparation method has been reported previously (Perrie and Gregoriadis, 2000). Briefly, lipids (e.g., DC-Chol:DOPE = 3:2 and 1:2 mol/mol) were dissolved in chloroform and a dried film was formed by rotary-evaporation. The preparation was hydrated with filtered water and vortexed at room temperature. The resulting multilamellar vesicle (MLV) suspension was extruded through a series of polycarbonate membranes with pore sizes of 0.6 and 0.2 μm (Millipore, Billerica, MA) to yield about 200-nm-sized vesicles. A sugar/total lipid (w/w) of 5, and 12.5–100 μg of plasmid DNA at a charge ratio of (+/−) of 2 and 16 were carefully added to the vesicle suspension, and the mixture was transferred to polypropylene tubes (10 mm in diameter and 40 mm in length), frozen by immersing in liquid nitrogen for 10 min, and lyophilized (DRVs) using a Freezevac C-1 lyophilizer (Tozai Tsusho Co., Tokyo, Japan) at a vacuum level below 5 Pa. Shelf temperature was controlled at −40 °C for 12 h, at −20 °C for 12 h, at 0 °C for 8 h, at 20 °C for 4 h, and at 30 °C for 4 h. After lyophilization, dry nitrogen was introduced in the drying chamber, and vials of DRVs were sealed with screw caps in a nitrogen atmosphere. Water contents of formulations obtained were less than 0.5%, as determined by the Karl Fischer method.

Prior to the measurement of transfection efficiency, the dry cake of DRVs was rehydrated with milli-Q water (1 ml of water per vial) and ultracentrifuged at 45,000 rpm for 45 min to partition sugars from the liposome suspension. The supernatant was collected and then milli-Q water was added to the DRV pellets to achieve 100 μg DNA/ml (DRV pellet suspension).

### 2.3. Measurement of size

The mean particle size of the DRVs suspended in water was determined using a light scattering instrument (DLS-7000, Otsuka Electronics Co. Ltd., Osaka, Japan) by a dynamic laser light scattering method at 25 ± 1 °C. The reported particle size was the average value of two measurements.

### 2.4. Stability test

Vials of DRVs were transferred to vessels containing $P_2O_5$ and were stored at −20, 25, 40 and 50 °C for 50 days.

### 2.5. Measurement of $T_g$

A $T_g$ of DRV formulation was measured by using a model 2920 differential scanning calorimeter (DSC) with a refrigerator cooling system (TA Instruments, Newcastle, DE, USA). Approximately 3 mg of DRV cake was put in an aluminum sample pan, dried in vacuum at 25 °C for 16 h and sealed hermetically in a nitrogen atmosphere in order to prevent water sorption during sample preparation. DSC traces were measured at a heating rate of 20 °C/min. An empty pan was used as a reference sample. Temperature calibration of the instrument was carried out using indium. $T_g$ values reported were obtained for first heating scan. The $T_g$ values and changes in the heat capacity at $T_g$ of stored samples were similar to those before storage, indicating that crystallization of amorphous excipient in the formulations did not occur during stability studies.

### 2.6. Entrapment efficiency of plasmid DNA in DRV

The plasmid DNA in the supernatant after ultracentrifugation of the rehydrated DRV suspension at 45,000 rpm for 45 min was measured as free plasmid DNA using a PicoGreen dsDNA Quantitation Kit *200-2000 assays* (Molecular Probes, Inc., OR, USA).

### 2.7. Cell culture

Human cervical carcinoma HeLa cells were kindly provided by Toyobo Co. Ltd. (Osaka, Japan) and grown in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% $CO_2$

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atmosphere. Cell cultures were prepared by plating cells in 35-mm culture dishes 24 h prior to each experiment.

### 2.8. Transfection of cells

The DRV pellet suspension prepared as described was diluted with medium without FBS to a final concentration of 2 μg plasmid DNA per well. After transfection in the medium without FBS for 3 h, 1 ml of the growth medium containing 10% FBS was added to the wells and culturing was continued for an additional 21 h.

### 2.9. Expression assays

Luciferase expression was measured using the luciferase assay system. Incubation was terminated by washing the plates three times with cold phosphate-buffered saline (pH 7.4, PBS). Cell lysis solution (Pica gene) was added to the cell monolayers, which were then subjected to freezing (−80 °C) and thawing at 37 °C, followed by centrifugation at 13,000 rpm for 10 min. The supernatants were frozen and stored at −80 °C until the assays. Aliquots of 10 μl of the supernatants were mixed with 100 μl of luciferin solution (Pica gene) and counts per second (cps) were measured with a chemoluminometer (Wallac ARVO SX 1420 Multilabel Counter, Perkin Elmer Life Science, Japan, Co. Ltd., Kanagawa, Japan). The protein concentration of the supernatants was determined with BCA reagent, using bovine serum albumin as a standard, and cps/μg protein was calculated.

### 2.10. Statistical analysis

Statistical significance of the data was evaluated by Student's *t*-test. A $p$ value of 0.05 or less was considered significant. All experiments were repeated at least two times. Duplicate determinations of luciferase expression values typically differed by less than 10%.

## 3. Results and discussion

Notably, liposomes composed of DC-Chol together with DOPE (DC-Chol/DOPE liposomes) have been classified as one of the most efficient vectors for the transfection of plasmid DNA into cells (Zhou and Huang, 1994; Farhood et al., 1994, 1995) and in clinical trials (Nabel et al., 1993, 1994). It has been demonstrated that a 3:2 or 1:1 molar ratio of DC-Chol/DOPE in liposomes results in high transfection efficiency (Farhood et al., 1995). Recently, we reported that DC-Chol/DOPE liposomes with molar ratio 1:2 showed more efficient transfection than those with molar ratio 3:2 or 1:1 in medium with FBS, having transfection efficiency comparable to that of Lipofectamine 2000, a commercial transfection reagent. Also, these lipoplexes showed a maximum at (+/−) 2:1 of transfection efficiency (Maitani et al., 2007). Therefore, we selected two kinds of DRV formulations: the conventional one (DC-Chol/DOPE = 3:2, A1–A3) and the novel one (DC-Chol/DOPE = 1:2, B1–B6), and prepared DRV/DNA complexes at sugar/total lipid (w/w) of 5, and charge ratio (+/−) of cationic lipid (DC-Chol) to DNA of 2 or 16 (Table 1).

### 3.1. DRVs without sugars

In preliminary experiments of the preparation of DRVs without sugars, the size was increased to over 1 μm. For the process of freeze-drying, fast freezing and addition of sugars in the freezing state resulted in less aggregation. This finding agreed with that reported by Molina et al. (2001). Moreover, in this study, since some preparations of dry cakes of DRVs after lyophilization were needed to perform the subsequent procedures, sugars were added to the suspension of liposomes and plasmid DNA at a weight ratio of 5:1 (sugar:total lipids) and mixed before freezing.

### 3.2. $T_g$ measurement of DRVs

Fig. 1 shows representative DSC traces of DRV formulations containing plasmid DNA (formulations B1–B3) and lyophilized sugar. The formulations were considered to be amorphous because they exhibited base line shifts due to the glass transition. The $T_g$ values of the lyophilized sugar ($T_g^s$) were higher in the order: sucrose < isomaltose < isomaltotriose. The $T_g$ values of DRV formulations increased in the order: B1 < B2 < B3, indicating that the molecular mobility of the formulation containing sucrose was higher than that of the formulations containing isomaltose or isomaltotriose. The $T_g$ values of DRV formulations and lyophilized sugars with and without liposomes are summarized in Table 2. The $T_g$ values of lyophilized sugars with cationic liposomes (DRV formulation without DNA) were slightly lower than those of the corresponding lyophilized sugars. In contrast, a

Table 1
Composition of DRV formulation

| Formulation | DC-Chol:DOPE (mol:mol) | Charge ratio, lipid:DNA (+/−) | Sugar |
|---|---|---|---|
| A1 | 3:2 | 16:1 | Sucrose |
| A2 | 3:2 | 16:1 | Isomaltose |
| A3 | 3:2 | 16:1 | Isomaltotriose |
| B1 | 1:2 | 2:1 | Sucrose |
| B2 | 1:2 | 2:1 | Isomaltose |
| B3 | 1:2 | 2:1 | Isomaltotriose |
| B4 | 1:2 | 16:1 | Sucrose |
| B5 | 1:2 | 16:1 | Isomaltose |
| B6 | 1:2 | 16:1 | Isomaltotriose |

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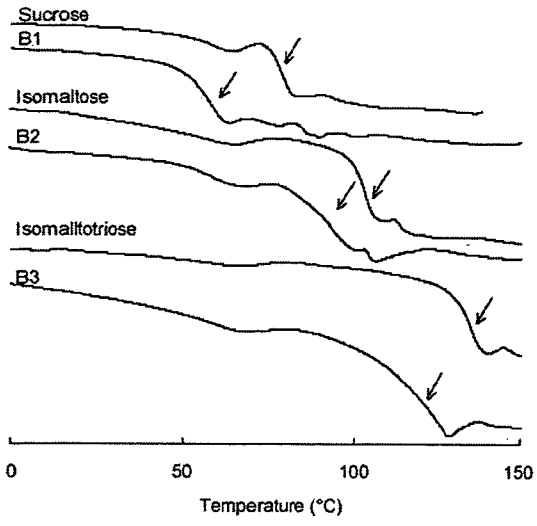


Fig. 1. Representative DSC traces of DRV formulations and sugars for first heating scan. Arrows in the figure represent $T_g$.

large decrease in $T_g$ of the DRV/DNA complexes was observed, as indicated by the $T_g$ values of formulations B1–B3, as shown in Fig. 2. The $T_g$ values of the formulations can be expressed by the Gordon–Taylor equation (Eq. (1)) assuming that miscibility of the sugars and the liposome/plasmid DNA complex is complete:

$$T_g = \frac{kW_c T_g^c + W_s T_g^s}{W_s + kW_c} \quad (1)$$

where $W_s$, $W_c$, $T_g^s$, and $T_g^c$ are the weight fraction and $T_g$ of sugar and complex, respectively, and $k$ is a constant. Differences in the

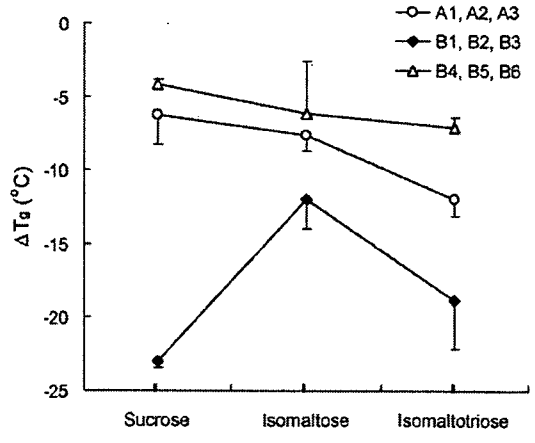


Fig. 2. Difference in $T_g^{midpoint}$ between DRV formulations and corresponding lyophilized sugars in Table 2. Error bars represent standard deviation ($n = 3$).

$T_g$ between the formulations and the corresponding sugars ($\Delta T_g$) are expressed by the following equation:

$$\Delta T_g = T_g - T_g^s = -\frac{kW_c(T_g^s - T_g^c)}{W_s + kW_c} \quad (2)$$

$T_g^c$ (not determined) is expected to be lower than $T_g^s$, since $T_g$ values of the formulations studied were lower than those of corresponding lyophilized sugar. For formulations B1–B3, the $T_g^c$ value is considered to be the same, since the formulations contain the same amount of plasmid DNA in liposomes. A smaller $T_g^s - T_g^c$ value is consequently expected for the formulation containing the sugar with lower $T_g$. Assuming that $k$ is not largely different between sugars, the smallest difference in $T_g$ between a formulation and the corresponding lyophilized sugar should

Table 2
$T_g$ of DRV formulations and lyophilized sugars

| | $T_g^{midpoint}$ (°C) | S.D. | $T_g^{onset}$ (°C) | S.D. |
|---|---|---|---|---|
| DRV formulation[a] | | | | |
| A1 (sucrose (DC/DOPE = 3/2)) | 72.0 | 2.1 | 68.8 | 2.6 |
| A2 (isomaltose (DC/DOPE = 3/2)) | 94.4 | 1.1 | 87.3 | 4.9 |
| A3 (isomaltotriose (DC/DOPE = 3/2)) | 121.1 | 1.2 | 108.2 | 1.6 |
| B1 (sucrose (DC/DOPE = 1/2)) | 55.2 | 0.5 | 50.4 | 0.5 |
| B2 (isomaltose (DC/DOPE = 1/2)) | 90.7 | 0.5 | 83.1 | 1.6 |
| B3 (isomaltotriose (DC/DOPE = 1/2)) | 114.2 | 3.3 | 104.6 | 2.7 |
| B4 (sucrose (DC/DOPE = 1/2)) | 74.0 | 0.4 | 71.1 | 0.5 |
| B5 (isomaltose (DC/DOPE = 1/2)) | 96.6 | 3.6 | 86.9 | 1.4 |
| B6 (isomaltotriose (DC/DOPE = 1/2)) | 125.9 | 0.6 | 118.3 | 0.9 |
| Lyophilized sugar with cationic liposomes | | | | |
| Sucrose + (DC/DOPE = 3/2) | 75.1 | 0.1 | 73.7 | 0.8 |
| Isomaltose + (DC/DOPE = 3/2) | 98.1 | 0.8 | 97.5 | 1.0 |
| Isomaltotriose + (DC/DOPE = 3/2) | 129.5 | 2.0 | 123.3 | 2.7 |
| Sucrose + (DC/DOPE = 1/2) | 77.8 | 0.8 | 72.5 | 0.5 |
| Isomaltose + (DC/DOPE = 1/2) | 101.8 | 0.1 | 94.2 | 3.1 |
| Isomaltotriose + (DC/DOPE = 1/2) | 129.1 | 0.1 | 123.9 | 0.5 |
| Lyophilized sugar | | | | |
| Sucrose | 78.2 | 0.2 | 74.5 | 0.2 |
| Isomaltose | 102.7 | 0.6 | 98.6 | 0.2 |
| Isomaltotriose | 133.0 | 0.8 | 128.8 | 0.2 |

$T_g$ values reported were obtained for first heating scan ($n = 3$).
[a] Sugar, liposome and plasmid DNA used in Table 1.

Table 3
Particle size of liposomes after rehydration of DRVs stored at −20 °C for 50 days

| Formulation | Diameter (nm) | Dispersion (%) | Diameter (nm) | Dispersion (%) |
|---|---|---|---|---|
| A1 | 179 | 62 | 1274 | 38 |
| A2 | 178 | 69 | 1269 | 31 |
| A3 | 191 | 70 | 1558 | 30 |
| B1 | 448 | 10 | 3495 | 90 |
| B2 | 364 | 8 | 3826 | 92 |
| B3 | 487 | 11 | 3161 | 88 |
| B4 | 238 | 30 | 1451 | 70 |
| B5 | 256 | 32 | 1591 | 68 |
| B6 | 205 | 32 | 1130 | 68 |

be observed for sucrose-based formulations. Formulation B1, however, exhibited a larger difference in $T_g$ than formulation B2 or B3 (Fig. 2), suggesting that the Gordon–Taylor equation was not applicable to formulation B1. This deviation from the Gordon–Taylor equation suggests that plasmid DNA may interact with sucrose more strongly than with isomaltose or isomaltotriose.

### 3.3. Size distribution of DRV suspension after rehydration and entrapment efficiency

DRVs stored at −20 °C for 50 days were rehydrated. The size distribution of DRV/DNA complex suspensions was heterogeneous, depending on the charge ratio (+/−), but not depending on sugars (Table 3). The DC-Chol/DOPE = 3/2 complex (formulations A1–A3) showed the smallest size. In the DC-Chol/DOPE = 1/2 complex, formulations B1–B3 with low cationic charge (+/−) of 2 showed larger size than formulations B4–B6 with that of 16. It seemed that the charge of DRVs was critical in determining sizes because the electric repulsion of the complex opposed aggregation. Also sugar amount might affect size of DRV. Molina et al. (2001) reported that formulations with high sucrose/DNA ratios are capable of maintaining particle size during the freezing step, and suggested that the separation of individual particles within sugar matrices is responsible for the protection of cationic lipid DOTAP/DOPE liposome vectors during the freezing step of a typical lyophilization protocol.

DRV methods in which lyophilization is performed after the addition of sucrose to the liposome suspension increase the entrapment efficiency of DNA in DRVs. Crowe and Crowe (1993) reported that trehalose outside and inside liposomes prevented the aggregation of liposomes and stabilized liposomes to entrap solutes. Zadi and Gregoriadis (2000) reported that a small amount of sugar outside liposomes disturbs the liposomal membrane and makes solute outside liposomes enter them. In our case, free plasmid DNA was not detected in the supernatant of any DRV suspensions using a PicoGreen Kit. The cationic charge of DRVs was so high that whether plasmid DNA was entrapped and/or adsorbed on DRVs was not clear.

### 3.4. Effect of sugars on transfection efficiency (TE)

In preliminary experiments, when lyophilized formulations B1–B3 of DRV/DNA complexes were preserved at room temperature for 24 h and then rehydrated, they showed similar TE values with nonlyophilized ones. To investigate the effect of sugars on lyophilization of the DRV/DNA complexes, the stability of plasmid DNA was evaluated by measuring TE after preservation at various temperatures.

At −20 and 25 °C, formulations B1–B3 showed the highest TE, and then formulations B4–B6 showed intermediate TE higher than those of formulations A1–A3 (Fig. 3). This finding indicated that TE was affected by the cationic lipid ratio in liposomes, and by the charge ratio (+/−) of cationic lipid to plasmid DNA, more than by the sugar. Molina et al. (2004) reported that progressive degradation of DOTAP lipoplex in terms of TE was observed during storage in the dried state at −20 °C, and the presence of DOPE enhanced degradation under these conditions. To the contrary, formulations B1–B3 with rich DOPE and the low charge ratio (+/−), exhibited high TE. This difference may be due to difference of cationic lipids.

The TE of formulations at various temperatures indicated that the stabilizing effect of sugars on DNA was higher in the following order: isomaltotriose < isomaltose < sucrose, except for formulation B1. This order of the stabilizing effects of sugars was inverse to the order of their $T_g$ values. About the effects of the glassy state on liposome in the freeze-drying state, it was reported that the solute retention in dry liposomes may be prolonged by increasing the $T_g$ of the dry liposome preparation (Sun et al., 1996; Crowe et al., 1997; van Winden and Crommelin, 1999). On the other hand, in cationic lipid/DNA complexes during lyophilization, sample vitrification did not correlate with maintenance of transfection efficiency (Allison and Anchordoquy, 2000). It was likely that sucrose might stabilize plasmid DNA by directly interacting with plasmid DNA rather than by vitrifying to a high $T_g$ solid. Supporting our inference, it was reported that the efficacy of transfection of lipoplexes was enhanced by mixing medium and disaccharides (Tseng et al., 2007).

Formulation B1 maintained a high TE value even during storage at 50 °C for 50 days, having about one-fourth of the TE of a commercially available transfection reagent, Lipofectamine 2000. We examined the cytotoxicity of the DRV/DNA complex, as indicated by the protein concentration after transfection (data not shown). All formulations showed low cytotoxicity compared with Lipofectamine 2000.

Entrapment of plasmid DNA inside DRVs, rather than a greater association at the surface of liposomes, may offer a more

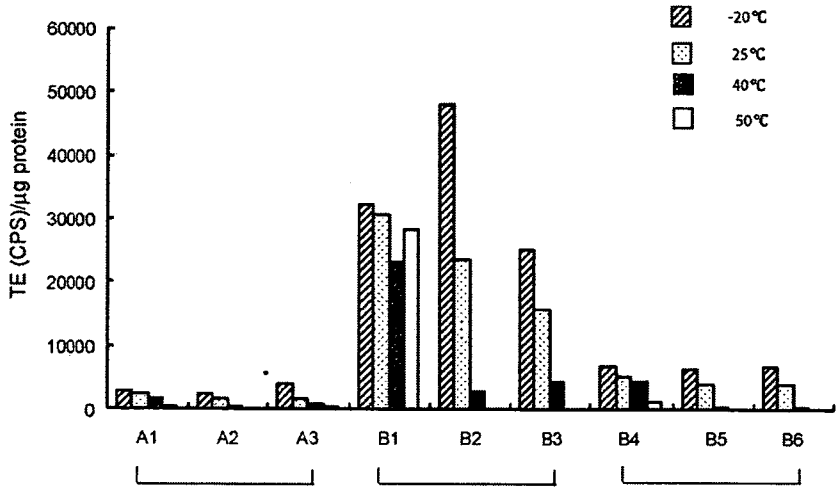


Fig. 3. Transfection efficiency in HeLa cells transfected with DRV/DNA complex with sugars after storage at various temperatures for 50 days. Each DRV/DNA complex was diluted with medium without FBS to a final concentration of 2 μg of plasmid DNA in 1 ml of medium per well. Each column represents the mean ($n = 2$). TE of B5 and B6 at 50 °C was not detected.

controlled approach to vesicle formation. This cryo-protective effect may be desirable when preparing DRV/DNA complexes without aggregation and with entrapment of DNA.

## 4. Conclusion

We developed lyophilized formulations of liposome (DRV)/plasmid DNA complex vectors prepared with the DRV method. DRV/DNA complexes lyophilized with sucrose could be stored even at 50 °C for 50 days without a large loss of transfection efficiency. This finding suggests that sucrose might stabilize plasmid DNA by directly interacting with plasmid DNA rather than by vitrifying to a high $T_g$ solid. Further long-term stability studies will be required to determine the shelf life of lyophilized liposome/DNA complexes at room temperature. These findings provide new information about the effects of physicochemical changes of nonviral vectors during lyophilization.

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——資　　料——

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# 平成18年度「日本薬局方の試験法に関する研究」研究報告
# 水分吸着等温線の解析による局方収載添加剤の吸湿性に関する研究**

吉岡　澄江，阿曽　幸男，川西　　徹*

## 1. はじめに

前年度の当該研究では，高分子添加剤の吸湿性を評価するための有用な試験法として，真空水分吸脱着測定装置を用いて測定される吸着等温線に基づく方法（吸着等温線法）を検討した．すなわち，日本薬局方（JP）各条の性状の項に「吸湿性」と記載するときの判断基準として通常用いられている「25℃，75%相対湿度（RH）の条件に7日間保存したときに観察される水分吸着量が3.0%以上かそれ未満か」という基準を，高分子添加剤に適用するときの問題点を明らかにし，特異的な水分吸着特性を示す高分子添加剤にも適用できる試験法として，吸着等温線に基づく方法を提案した[1]．

今年度は，添加剤に含まれる水の量を規定する「水分」及び「乾燥減量」の規格に関する問題点を，添加剤の吸着等温線の解析に基づいて考察する．「乾燥減量」は歴史的に簡便な方法としてJP各条に広く設定されてきた．一方，カールフィッシャー法で測定される「水分」は水和物に原則として設定されるほか，乾燥条件下で分解が起こり「乾燥減量」が適用できない品目に設定されてきた．しかし，JP収載品目の中には，「水分」と「乾燥減量」の関係が曖昧なものがある．例えば，乳糖水和物については，国際調和案では「水分」だけが規定されているのに対し，日本薬局方では「水分」のほかに「乾燥減量」も規定されている．本研究では糖類及び水溶性高分子の代表的な添加剤について吸着等温線を測定し，その解析に基いて「水分」及び「乾燥減量」のそれぞれの必要性を考察する．

今年度は更に，JP各条における水分に関する問題として，「乾燥した試料」及び「乾燥物（脱水物）換算」の妥当性についても考察する．すなわち，水分を含有する品目について「定量」や「純度試験」を実施するときに，乾燥した試料を用いる方法と，秤量した試料の重量を「水分」あるいは「乾燥減量」で測定した水分量に基づいて換算する方法があるが，いずれの方法を適用すべきかを考察する．通常，水分量を換算する方法の方が，あらかじめ試料を乾燥させる操作を行わなくてもよいことから，より簡便であると考えられており，非水滴定法による含量試験など，水が試験を妨害することがない限り，「乾燥物（脱水物）換算」が採用される場合が多い．しかし，JPに複数収載されている糖類においても，品目によって異なる方法が適用されており，その妥当性が明確でない場合もあるため，糖類及び高分子添加剤の「乾燥した試料」と「乾燥物（脱水物）換算」の問題点についても，吸着等温線の解析に基づいて考察する．

## 2. 実験方法

### 2.1 吸着等温線の測定

乳糖水和物，乳糖水和物（造粒），無水乳糖，乳糖凍結乾燥品，マルトース水和物について，吸着等温線を測定した．試料を真空水分吸脱着測定装置に入れたのち，60℃で0.0% RHに相当する水蒸気圧まで減圧にして乾燥させた後，25℃で，湿度を10～95% RHの範囲で10%間隔で上昇させ，更に95～0% RHの範囲で下降させて，水分吸着量及び脱

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着量を測定した．終点は重量増加あるいは減少が10分間で1 μg以下になった時点とした．D-マンニトール，D-ソルビトール，果糖についても同様に吸着等温線を測定したが，初期の乾燥は25℃で行った．

前年度に測定しなかった高分子添加剤として，デキストラン（dextran 40k）及びヒプロメロースフタール酸エステル（HPMCP）の吸着等温線を10～80% RHの湿度領域で測定した（60℃初期乾燥）．更に，高分子添加剤の吸着パターンと比較する目的で，ピペミン酸水和物の吸着等温線を10～90% RHの湿度領域で測定した（60℃初期乾燥）．

### 2.2 「乾燥減量」及び「水分」の測定

乳糖水和物，乳糖水和物（造粒），無水乳糖，マルトース水和物，D-マンニトール，D-ソルビトール，果糖について，試料を25℃，58% RH下に1週間放置後，JPの各モノグラフに記載されている条件で「乾燥減量」を測定した．乾燥条件はTable 1に記した．乳糖凍結乾燥品については25℃，33% RH下に1週間放置した試料を用いた．更に無水乳糖については，58% RH及び75% RHにおいて，経時的に「乾燥減量」及び「水分」を測定した．

デキストラン，ポビドン，コポビドン，クロスポビドン，ヒプロメロース及びヒプロメロースフタール酸エステルは，減圧下で乾燥した後，25℃，33% RH下に1週間放置した試料を用い，Table 2に記した乾燥条件で測定した．それぞれの試料について「乾燥減量」と同時にカールフィッシャー法による「水分」測定を行った．

### 2.3 水分吸着速度の測定

結晶セルロース（MCC），粉末セルロース（Powdered cellulose），メチルセルロース（MC），ヒドロキシプロピルセルロース（HPC），低置換度ヒドロキシプロピルセルロース（L-HPC），ヒプロメロース（HPMC），カルメロース（CMC），カルメロースカルシウム（CMC-Ca），カルメロースナトリ

**Table 1　糖類の「乾燥減量」及び「水分」**

| | 乾燥減量 (%) | sd | 水分 (%) | sd |
|---|---|---|---|---|
| 乳糖水和物 | 0.01 (80℃, 2時間) | 0.00 | 4.79 | 0.08 |
| 乳糖水和物（造粒） | 0.11 (80℃, 2時間) | 0.01 | 4.68 | 0.38 |
| 乳糖水和物（凍結乾燥品）* | 5.45 (80℃, 2時間) | 0.12 | 5.95 | 0.24 |
| 無水乳糖 | 0.07 (80℃, 2時間) | 0.02 | 0.45 | 0.03 |
| マルトース | 0.03 (80℃, 2時間) | 0.01 | 4.75 | 0.06 |
| 果糖 | 0.17 (減圧, シリカゲル, 3時間) | 0.01 | 0.15 | 0.02 |
| D-ソルビトール | 0.90 (減圧, 80℃, 酸化リン(V), 3時間) | 0.02 | 0.86 | 0.03 |
| D-マンニトール | nd (105℃, 4時間) | | nd | |

25℃, 58% RH, 1週間保存
nd: 0.01% 未満
*: 25℃, 33% RH, 1週間保存

**Table 2　高分子添加剤の「乾燥減量」及び「水分」**

| | 乾燥減量 (%) | sd | 水分 (%) | sd |
|---|---|---|---|---|
| ポビドン | 10.55 (105℃, 3時間) | 0.68 | 9.53 | 0.76 |
| コポビドン | 4.66 (105℃, 3時間) | 0.25 | 4.39 | 0.26 |
| クロスポビドン | 10.31 (105℃, 3時間) | 0.74 | 10.63 | 0.08 |
| ヒプロメロース | 2.48 (105℃, 1時間) | 0.13 | 2.26 | 0.08 |
| ヒプロメロースフタール酸エステル | 2.42 (105℃, 1時間) | 0.05 | 2.27 | 0.12 |
| デキストラン | 10.62(105℃, 6時間) | 0.02 | 10.08 | 0.15 |

減圧乾燥後, 33% RH, 1週間保存

ウム（CMC-Na），クロスカルメロースナトリウム（cros-CMC-Na），トウモロコシデンプン（Corn starch），バレイショデンプン（Potato starch），デキストリン（Dextrin），ポビドンK90（PVP），プルラン（Pullulan），カルボキシメチルスターチナトリウム（CMS-Na），アルファ化トウモロコシデンプン（α-Corn starch），アルファ化バレイショデンプン（α-Potato starch），クロスポビドン（cros-PVP），コポビドン（PVP/VA），及び酵素分解デキストリン（Dextrin(enz)）の計21品目について，乾燥試料が水分を吸着するときの速度を測定した．試料を真空水分吸脱着測定装置に入れたのち，60℃，減圧で乾燥させ，水蒸気圧を0.0% RHに相当する圧力まで低下させた後，25℃で相対湿度を一気に60%に上昇させ，吸着量の経時的変化を測定した．

### 2.4 試　料

実験に用いた乳糖水和物，無水乳糖，デキストラン，ポビドン及びピペミン酸水和物は㈱シグマから購入したが，それ以外の試料は日本医薬品添加剤協会から提供を受けた．乳糖凍結乾燥品は乳糖水和物を10%水溶液から凍結乾燥して調製した（棚温度35℃，96時間）．

## 3. 実験結果

### 3.1 糖類の水分吸着特性

乳糖水和物，乳糖水和物（造粒），無水乳糖，乳糖凍結乾燥品について測定した吸脱着等温線をFig.1に示す．乳糖水和物は60℃，減圧条件でも水和水の脱着が見られず，25℃，0% RH～95% RHの湿度領域で一水和水が安定に維持され，それ以上の吸着も起こらないことが示された（Fig. 1A）．造粒した試料についても同様の吸脱着等温線が得られた（Fig. 1B）．

凍結乾燥した乳糖は，湿度の上昇とともに水分吸

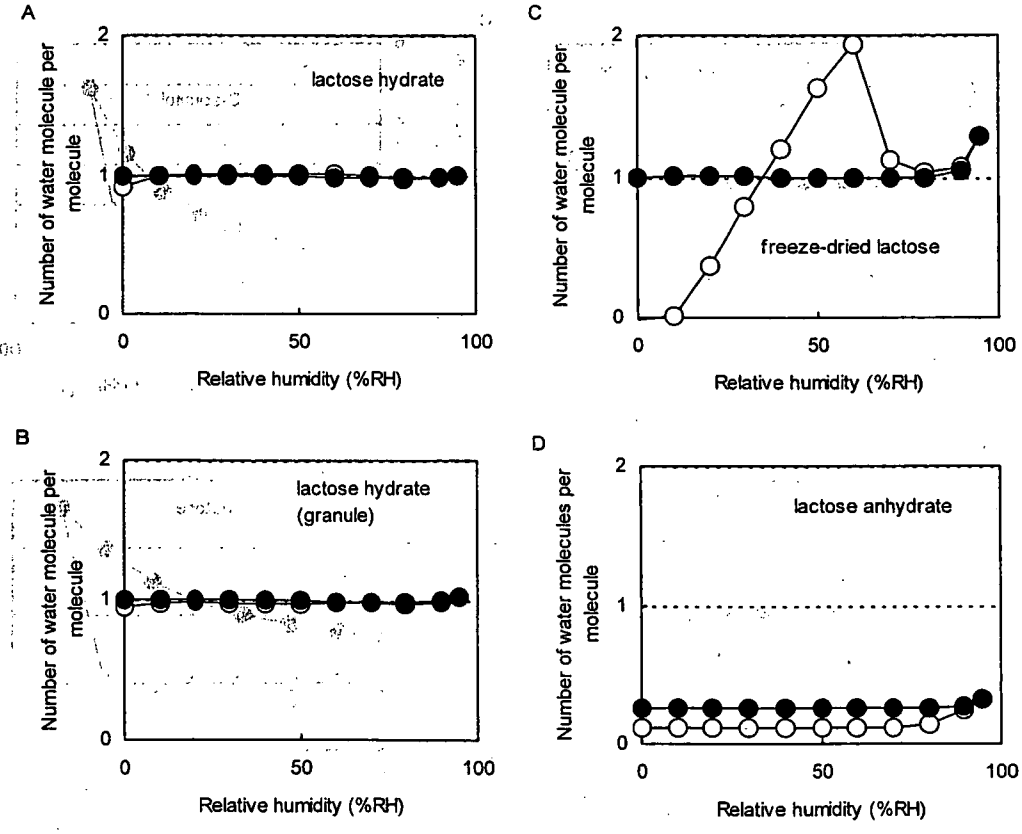


**Fig. 1**　乳糖の水分吸着等温線

着量が増加し，60% RH 付近で急激に吸着量の減少がみられ，非晶質から一水和物へ結晶化したと考えられる（Fig. 1C）．乳糖水和物は 95% RH 下でも水分吸着が見られないのに対して，乳糖凍結乾燥品では 95% RH において吸着量の増大がみられ，乳糖非晶質から水和物への結晶化は完全ではなく，一部，非晶質が残存していると考えられる．

無水乳糖は 60℃，減圧条件でも脱着しない水が存在したことから，吸着した水の一部が水和物として存在することが分かった（Fig. 1D）．90% RH を超えると無水物にも水が吸着して水和物に変化するために，脱着過程における水分量が吸着過程よりも高くなったと考えられる．

乳糖以外の糖類の吸着等温線を Fig. 2 に示す．マルトース水和物は 60℃，減圧条件でも水和水を脱着せず，更に 25℃，0% RH の低湿度条件においても脱着がみられないことから，水和水は非常に安定な結晶状態にあることが分かった（Fig. 2A）．D-マンニトールは 95% RH でも水分をほとんど吸着せず，吸湿性が低いことが示された（Fig. 2B）．D-ソルビトール及び果糖は，それぞれ 70% RH 及び 60% RH を越えると急激に水分を吸着するが，湿度の低下とともにそれらの吸着水は脱着することが分かった（Fig. 2C 及び 2D）．

### 3.2　高分子添加剤及びピペミン酸の水分吸着特性

デキストラン及びヒプロメロースフタール酸エステルの吸着等温線を，前年度に報告したポビドン，クロスポビドン，コポビドン及びヒプロメロースと比較して Fig. 3 に示す．デキストランはポビドンより吸着量は低いが，低湿度領域で吸着量が比較的高い吸着パターンを示した．ヒプロメロースフタール酸エステルはヒプロメロースとほぼ同様の吸着パターンを示した．

ピペミン酸は Fig. 4 に示すように，70% RH 付

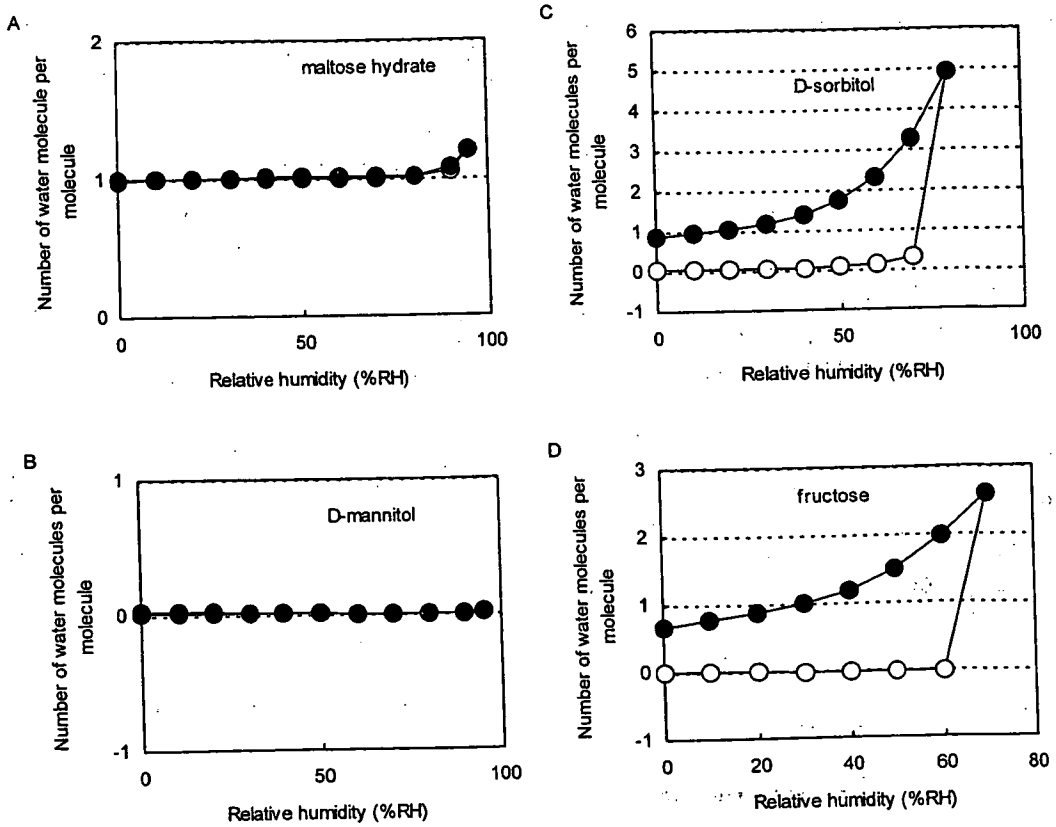


Fig. 2　糖類の水分吸着等温線

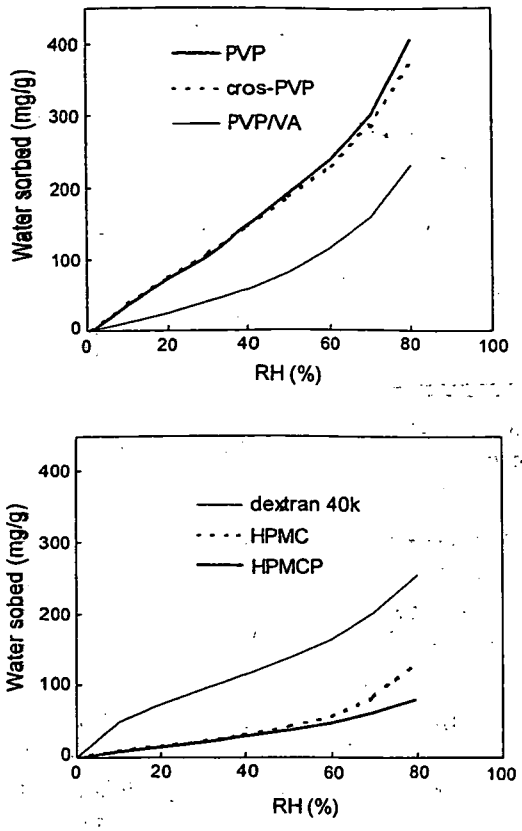


Fig. 3　高分子添加剤の水分吸着等温線

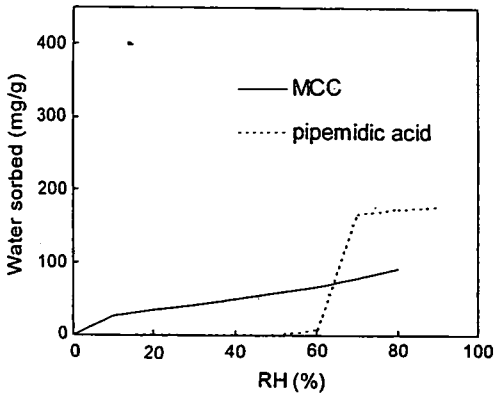


Fig. 4　ピペミン酸及び結晶セルロースの水分吸着等温線

近で結晶水として取り込むまではほとんど水分を吸着せず，高分子添加剤とは異なるパターンを示した．

### 3.3　糖類及び高分子添加剤の「乾燥減量」及び「水分」

糖類について測定された「乾燥減量」及び「水分」の結果を，それぞれ Table 1 に示す．乳糖水和物は「水分」のほとんどが「乾燥減量」では測定されず，存在する水のほとんどが脱着しにくい水，すなわち水和水であることが示された．一方，無水乳糖では約 0.5%の「水分」が測定されたが，「乾燥減量」は「水分」より低い値を示し，すべての水が脱着しやすい水（付着水）ではなく，結晶水も混在することが分かった．Fig. 5 に示すように，無水乳糖では 75% RH 条件下において「乾燥減量」は経時的な変化を示さないのに対して，「水分」は経時的に増大し，無水乳糖から乳糖水和物への変化が確認された．その変化は 58% RH では顕著ではなかった．

乳糖水和物（造粒）は，「乾燥減量」が乳糖水和物よりわずかに高く，付着水が比較的多いことが示された（Table 1）．これは非晶質が混在するためであると考えられる．非晶質の混在は，乳糖凍結乾燥品で「水分」に相当する量の「乾燥減量」が測定されたことから裏付けられる．

マルトース水和物は，乳糖水和物と同様に，「水分」のほとんどが「乾燥減量」では測定されず，存在する水のほとんどが水和水であることが示された．D-マンニトールは，「水分」及び「乾燥減量」のいずれも検出限界以下であった．D-ソルビトール及び果糖は，「乾燥減量」及び「水分」の測定値が一致し，存在する水はすべて付着水であることが分かった．

高分子添加剤について測定された「乾燥減量」及び「水分」の結果を，それぞれ Table 2 に示す．デキストラン，ヒプロメロース，ヒプロメロースフタール酸エステル，ポビドン，クロスポビドン，コポビドンのいずれも「乾燥減量」及び「水分」の測定値がほぼ一致し，付着水であることが示された．

### 3.4　添加剤の水分吸着速度

前年度に吸着等温線を測定した各添加剤について，湿度が 0% RH から 60% RH に急激に変化したときの水分吸着量の時間的変化を測定することによって，乾燥試料が水分を吸着する速度を評価した．Fig. 6 における ABCDE グループ分けは吸着速度に基づいており，A グループが最も吸着速度が小さ

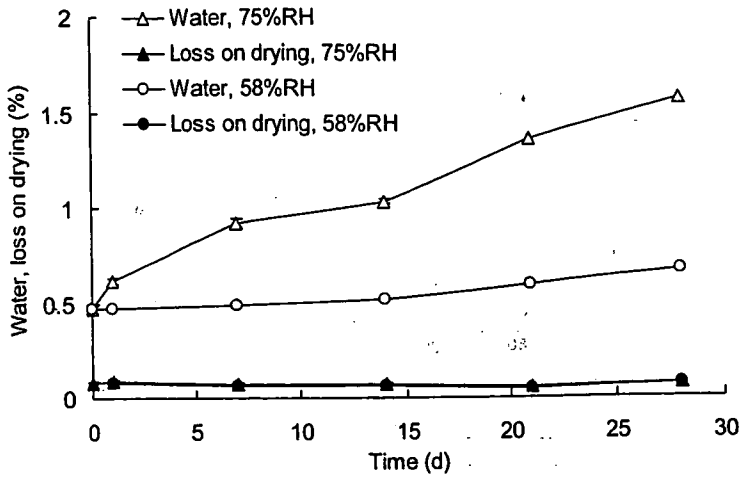


Fig. 5　無水乳糖の「乾燥減量」及び「水分」の経時的変化

いグループであり，E グループが最も吸着速度が大きいグループである．図中の実線は一次速度式への回帰線を表す．ここで回帰は誤差が大きいと考えられる 3 分以内のデータは削除して行った．

クロスポビドン，クロスカルメロースナトリウム，ヒプロメロースは一次式から僅かに外れる吸着パターンを示したが，その他の添加剤の吸着パターンは一次速度式で表すことができた．ヒプロメロース等の添加剤では複数の異なる吸着サイトを有するか，あるいは水分吸着に伴い物理的状態変化が起こるため，一次式では表すことができないものと考えられる．曲線回帰によって得られた吸着の一次速度定数を Fig. 7 に示す．吸着速度は添加剤によって大きく異なった．吸着速度の大きさは，Fig. 8 に示した 60% RH における水分吸着量の大きさと関連しなかった．

## 4．考　察

### 4.1 「乾燥減量」及び「水分」の必要性

乳糖水和物については，「水分」の規格が米国薬局方（USP），ヨーロッパ薬局方（EP），JP ともに 4.5～5.5%（造粒した粉末は 4.0～5.5%）と国際調和されているが，JP では「水分」に加えて「乾燥減量」が 0.5% 以下（80℃，2 h）と規定されている．乳糖水和物中の水は，Fig. 1A に示されるように，60℃，減圧条件でも水和水の脱着が見られず，また 25℃，0% RH の低湿度条件においても脱着しないことから，非常に安定な結晶状態にあると考えられる．これは，Table 1 に示されるように，「水分」のほとんどが「乾燥減量」では測定されないという結果からも裏付けられる．

乳糖水和物の結晶は 95% RH の高湿度下でも水の吸着は起こらず（Fig. 1），不純物のない乳糖水和物であれば，「乾燥減量」は 0 という結果が得られることになる．しかし，Fig. 1C に示されるように，非晶質状態にある乳糖凍結乾燥品は水を吸着しやすいことから，非晶質状態の乳糖の混在を試験するために乳糖水和物の規格として「水分」及び「乾燥減量」の両者を設定することは妥当であると考える．

無水乳糖は USP，EP，JP ともに「水分」1.0% 以下，「乾燥減量」0.5% 以下（80℃，2 h）と規定されている．今回用いた試料では，Fig. 1D に示すように，60℃，減圧条件でも脱着しない水が存在し，一部，水和物が混在していると考えられる．無水乳糖の結晶も 70% RH までは水の吸着が起こらないことから，「乾燥減量」は 0 という結果が得られることになる．しかし，Table 1 に示すように，「水分」のうち「乾燥減量」で測定される付着水がある比率で存在することが示され，非晶質状態の乳糖もわずかに混在していると考えられる．また，Fig. 5 に示すように，高湿度条件に保存すると「水分」が経時的に上昇し，乳糖水和物が生成されることが示唆された．これらの結果から，無水乳糖は，乳糖水

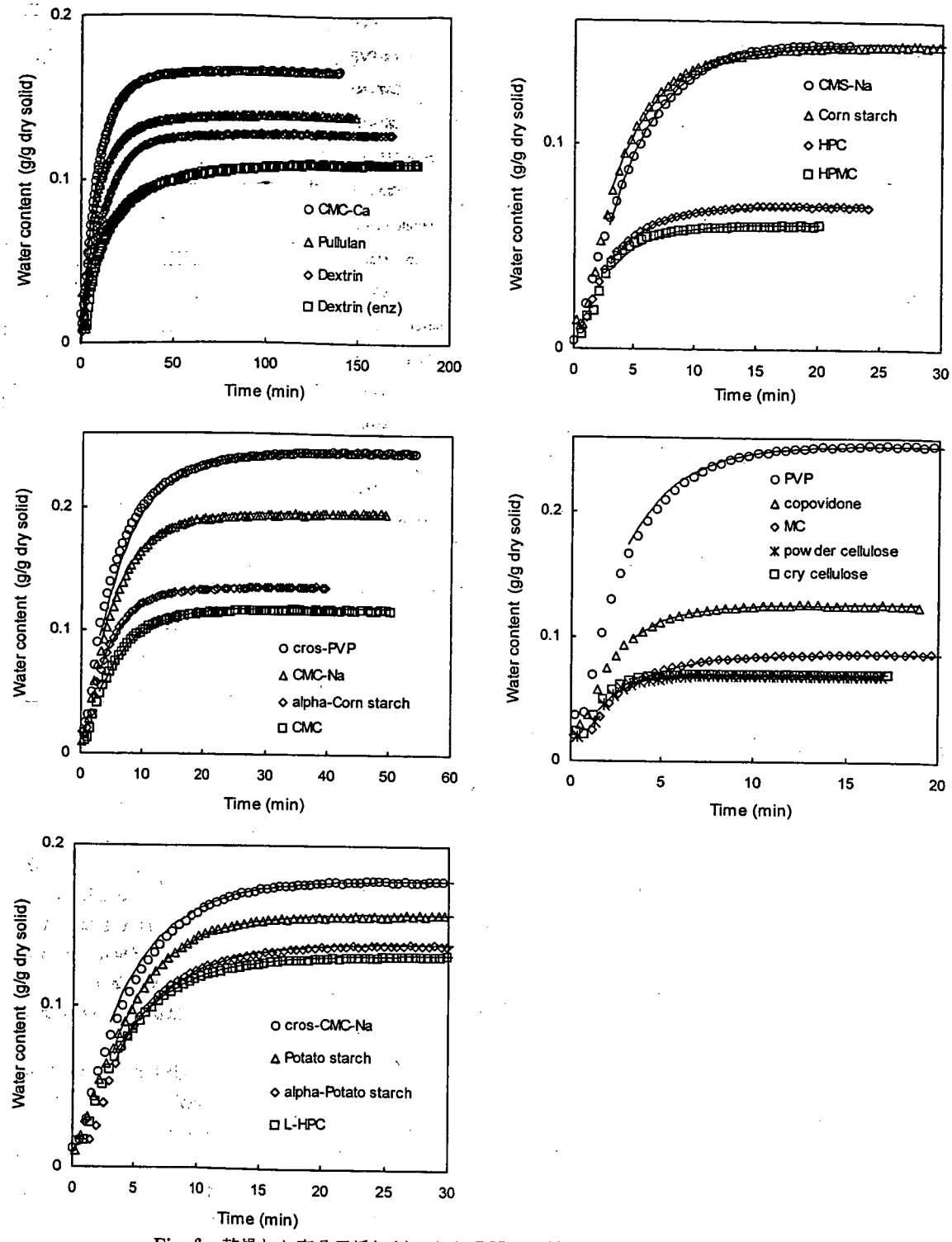


**Fig. 6**　乾燥した高分子添加剤の 60% RH における水分吸着のタイムコース

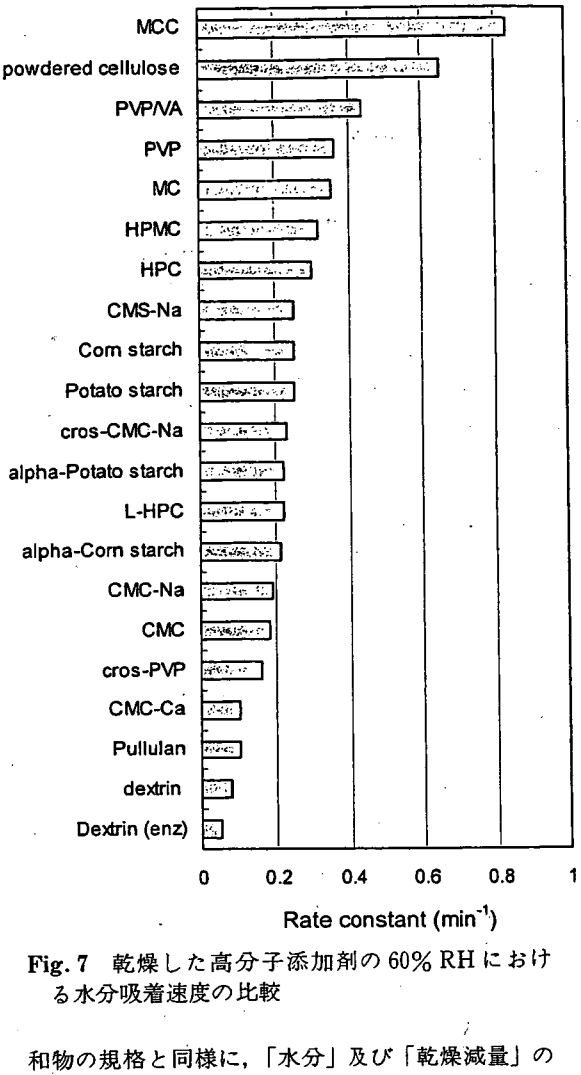


**Fig. 7**　乾燥した高分子添加剤の 60% RH における水分吸着速度の比較

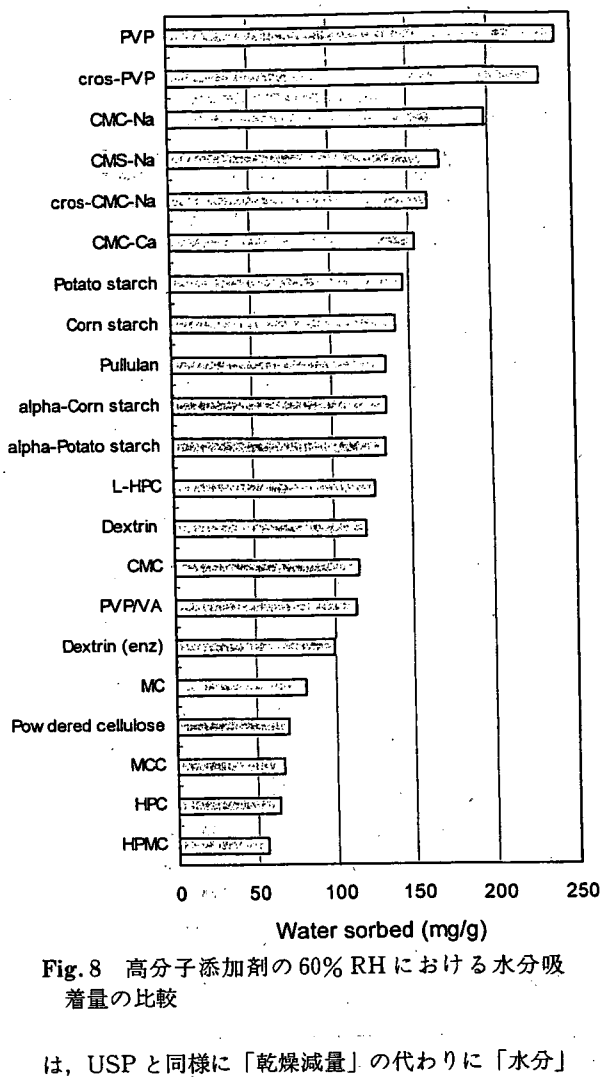


**Fig. 8**　高分子添加剤の 60% RH における水分吸着量の比較

和物の規格と同様に，「水分」及び「乾燥減量」の両者を設定することが必要であると考える．

マルトース水和物は，JP が「乾燥減量」0.5%以下（80℃，4 h）を規定しているのに対して，USP は「水分」5.0～6.5%を規定している．マルトース水和物中の水は，Fig. 2 に示されるように，乳糖水和物と同様に，60℃，減圧条件でも水和水の脱着が見られず，また 25℃，0% RH の低湿度条件においても脱着しないこと，また Table 1 に示されるように，「水分」のほとんどが「乾燥減量」では測定されないことから，非常に安定な結晶状態にあると考えられる．USP が「水分」を規定しているのは，同一のモノグラフに無水物と水和物の両者を記載しているためと考えられるが，結晶水の確認のために は，USP と同様に「乾燥減量」の代わりに「水分」を規定する方がよいと考えられる．Fig. 2 に示すように，マルトース水和物は 95% RH でもほとんど水は吸着せずに安定に存在することから，「乾燥減量」は 0 という結果が得られることになり，水分を規定すれば「乾燥減量」の規定は必要ないと考えられる．

D-マンニトールは，JP と USP が「乾燥減量」0.30%以下（105℃，4 h）を規定しているのに対して，EP は「水分」0.5%以下を規定している．Fig. 2 及び Table 1 に示すように，D-マンニトールは高湿度条件下でもほとんど水の吸着がみられず，安定に存在することから，「水分」及び「乾燥減量」ともに 0 という結果が得られることになる．不純物

の混在を試験するために，「水分」あるいは「乾燥減量」のいずれかによって，水がほとんど存在しないことを確認することで十分であると考えられる．

D-ソルビトールは，JPが「乾燥減量」2.0%以下（減圧五酸化リン80℃，3 h）を規定しているのに対して，EP及びUSPは「水分」1.5%以下を規定している．また，果糖は，JP及びUSPが「乾燥減量」0.5%以下（JP：減圧シリカゲル3 h；USP減圧70℃，4 h）を規定しているが，EPは「水分」0.5%以下を規定している．Fig. 2に示すように，D-ソルビトール及び果糖は，それぞれ70% RH及び60% RHを越えると急激に水分が吸着するが，Table 1に示されるように，「水分」と「乾燥減量」の結果が一致することから，どちらか一方の方法で吸着水の量を規定すれば十分と考えられる．

デキストランは，USP，EP，JPともに「乾燥減量」を規定している（JP 5.0%以下（105℃，6 h），USP及びEPは7.0%以下（105℃，5 h））．ヒプロメロースは，USP，EP，JPともに「乾燥減量」を規定しているのに対し（5.0%以下（105℃，1 h）），ヒプロメロースフタル酸エステルは，USP，EP，JPともに「水分」5.0%以下を規定している．ポビドンは，USP，EP，JPともに「水分」5.0%以下を規定しているのに対して，コポビドンは，USP，EP，JPともに「乾燥減量」5.0%以下（105℃，3 h）を規定している．

これらの高分子添加剤では，Table 2に示すように，「乾燥減量」と「水分」の測定結果がほぼ一致しており，水はすべて脱着しやすい水として存在することが示された．したがって，「水分」あるいは「乾燥減量」のいずれかによって水分量を測定すれば十分であると考えられる．

### 4.2 「乾燥した試料」及び「乾燥物（脱水物）換算」の妥当性

現行のJPは，乳糖水和物及び無水乳糖の旋光度試験をUSP及びEPと同様に「脱水物換算」で行うことになっているが，マルトース水和物及びD-マンニトールの定量及び旋光度試験やD-ソルビトール及び果糖の定量においては「乾燥した試料」を用いることになっている．それに対してEP及びUSPではいずれの試験も「脱水物換算」で行われる．

乳糖はFig. 1及びTable 1に示されるように，結晶水に加えて脱着しやすい水も存在するので，「乾燥した試料」ではなく，「脱水物換算」で行う必要がある．マルトース水和物中の水は，Fig. 1及びFig. 2に示されるように，結晶水として安定しており，通常の条件下では脱着しない．また高湿度条件下でもさらなる水分の吸着はみられないことから，「乾燥した試料」でも「脱水物換算」でも適用できると考えられる．D-ソルビトール及び果糖は，脱着しやすい水のみが存在するので，「乾燥した試料」でも「乾燥物換算」でもどちらでも適用できると考えられる．しかし，それぞれ約70% RH及び60% RH以上で急激に吸湿するので，「乾燥した試料」を適用する場合には，試験操作中の吸湿に注意が必要である．

ポビドン，結晶セルロース，メチルセルロース，コポビドン，ヒプロメロースは，定量法や粘度の試験にすべて「乾燥物（脱水物）換算」が適用されている．これらの高分子は，Fig. 3に示すように，ピペミン酸（Fig. 4）のようにある湿度で急激に水分を吸着し水和物となるのとは異なり，湿度の上昇とともに水分量が徐々に増大するうえ，Fig. 8に示すように吸着速度が高いので，乾燥した試料を使う方法では試験結果が変動する可能性が大きいと考えられることから，「乾燥物（脱水物）換算」を適用すべきであると思われる．もちろん「乾燥物（脱水物）換算」では，換算を適用する試験に用いる試料が「水分」あるいは「乾燥減量」試験に用いた試料と同一であることが前提となる．

デキストランは定量法，粘度及び還元性物質の純度試験に「乾燥した試料」が適用されているが，Fig. 3に示すようにポビドンと同様の吸着等温線を示すので，「乾燥物換算」を適用すべきであると思われる．特に，ポビドン，クロスポビドン，デキストランでは，低湿度で大量の水を吸着するので，「乾燥した試料」の誤差が大きくなると考えられる．ヒプロメロースフタル酸エステルは，粘度の測定に「乾燥した試料」が適用されているが，これはメタノールとジクロロメタンを溶媒として用いることなどを踏まえて，その妥当性を考察する必要があると考える．

## 5. まとめ

日本薬局方（JP）各条の水分に関する規格項目

としては，「水分」と「乾燥減量」があり，定量や純度試験の試料としては「乾燥した試料」と「乾燥物（脱水物）換算」のいずれかの記載が用いられているが，糖類及び高分子添加剤の中には，特異的な水分吸着性を示すものがあり注意を要する．これらの試験項目や試験法の選択に，より理論的に適正な根拠を与えるべく吸着等温線及び吸着速度を実測し，JP 規格の妥当性について検証した．

その結果，「水分」と「乾燥減量」に関して，現行 JP はほぼ妥当な設定がなされていると思われるが，定量等の試験試料に関しては，例えばデキストランのように，「乾燥した試料」ではなく「乾燥物換算」の適用を考慮すべきであると思われるもの，D-ソルビトールや果糖のように「乾燥した試料」を用いる試験操作中の吸湿に注意が必要なものなどが認められた．更に，無水乳糖では高湿度条件下での保存によって乳糖水和物に変化することが明らかになり，保存条件に注意を要することが示された．

## 謝　辞

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## 文　献

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Research Paper

# Significance of Local Mobility in Aggregation of β-Galactosidase Lyophilized with Trehalose, Sucrose or Stachyose

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***Purpose.*** The purpose of this study is to compare the effects of global mobility, as reflected by glass transition temperature ($T_g$) and local mobility, as reflected by rotating-frame spin-lattice relaxation time ($T_{1\rho}$) on aggregation during storage of lyophilized β-galactosidase (β-GA).

***Materials and Methods.*** The storage stability of β-GA lyophilized with sucrose, trehalose or stachyose was investigated at 12% relative humidity and various temperatures (40–90°C). β-GA aggregation was monitored by size exclusion chromatography (SEC). Furthermore, the $T_{1\rho}$ of the β-GA carbonyl carbon was measured by $^{13}C$ solid-state NMR, and $T_g$ was measured by modulated temperature differential scanning calorimetry. Changes in protein structure during freeze drying were measured by solid-state FT-IR.

***Results.*** The aggregation rate of β-GA in lyophilized formulations exhibited a change in slope at around $T_g$, indicating the effect of molecular mobility on the aggregation rate. Although the $T_g$ rank order of β-GA formulations was sucrose < trehalose < stachyose, the rank order of β-GA aggregation rate at temperatures below and above $T_g$ was also sucrose < trehalose < stachyose, thus suggesting that β-GA aggregation rate is not related to ($T-T_g$). The local mobility of β-GA, as determined by the $T_{1\rho}$ of the β-GA carbonyl carbon, was more markedly decreased by the addition of sucrose than by the addition of stachyose. The effect of trehalose on $T_{1\rho}$ was intermediate when compared to those for sucrose and stachyose. These findings suggest that β-GA aggregation rate is primarily related to local mobility. Significant differences in the second derivative FT-IR spectra were not observed between the excipients, and the differences in β-GA aggregation rate observed between the excipients could not be attributed to differences in protein secondary structure.

***Conclusions.*** The aggregation rate of β-GA in lyophilized formulations unexpectedly correlated with the local mobility of β-GA, as indicated by $T_{1\rho}$, rather than with ($T-T_g$). Sucrose exhibited the most intense stabilizing effect due to the most intense ability to inhibit local protein mobility during storage.

**KEY WORDS:** β-galactosidase; global mobility; local mobility; lyophilized formulation; solid-state stability.

## INTRODUCTION

Close correlations between storage stability and molecular mobility have been demonstrated for various lyophilized formulations of peptides and proteins (1,2). Aggregation between protein molecules is a degradation pathway commonly observed in lyophilized protein formulations. The rate of protein aggregation is generally considered to depend on the translational mobility of protein molecules, which is related to structural relaxation (α-relaxation) of the formulation. Correlations between aggregation rates and structural relaxation have been shown in various protein systems in visible ways, such as enhancement of aggregation associated with decreases in glass transition temperature ($T_g$) (3–6) and changes in the temperature dependence of aggregation rates around $T_g$ (7–10). However, recent studies have suggested that molecular mobility with a length scale shorter than structural relaxation (β-relaxation or local mobility), rather than structural relaxation, is critical to protein aggregation (11,12).

The rate of protein aggregation in lyophilized formulations is also affected by the degree of change in protein conformation produced during the freeze-drying process (1). Greater changes in protein conformation are considered to lead to enhanced aggregation during subsequent storage.

In this study, the significance of local mobility in aggregation of lyophilized β-galactosidase (β-GA), a model protein, is discussed in comparison with the significance of structural relaxation and conformational changes. β-GA underwent significant inactivation during freeze drying with dextran, thus suggesting that significant conformational changes occurred during the process (13). When freeze dried with polyvinylalcohol or methylcellulose, inactivation was not observed during freeze drying (10). However, the time

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Table II. Effects of Reconstitution Medium on β-GA Aggregation Above $T_g$ for 0.33 Trehalose Formulation

| Additives in Reconstitution Medium | Peak Height for Monomeric β-GA (Relative to Solution Prior to Freeze Drying) | |
|---|---|---|
| | After Freeze Drying | After 9 h-storage at 90°C |
| None | 0.99 (0.01) | 0.74 (0.03) |
| Dextran Sulfate | 1.00 (0.00) | 0.74 (0.01) |
| 2-hydroxylpropyl-β-cyclodextrin | 0.98 (0.00) | 0.73 (0.01) |
| Poly-L-lysine | 0.98 (0.01) | 0.74 (0.01) |
| Pluronic | 1.00 (0.01) | 0.76 (0.02) |

0.5% additives
Values in brackets represent standard deviation ($n$=3)

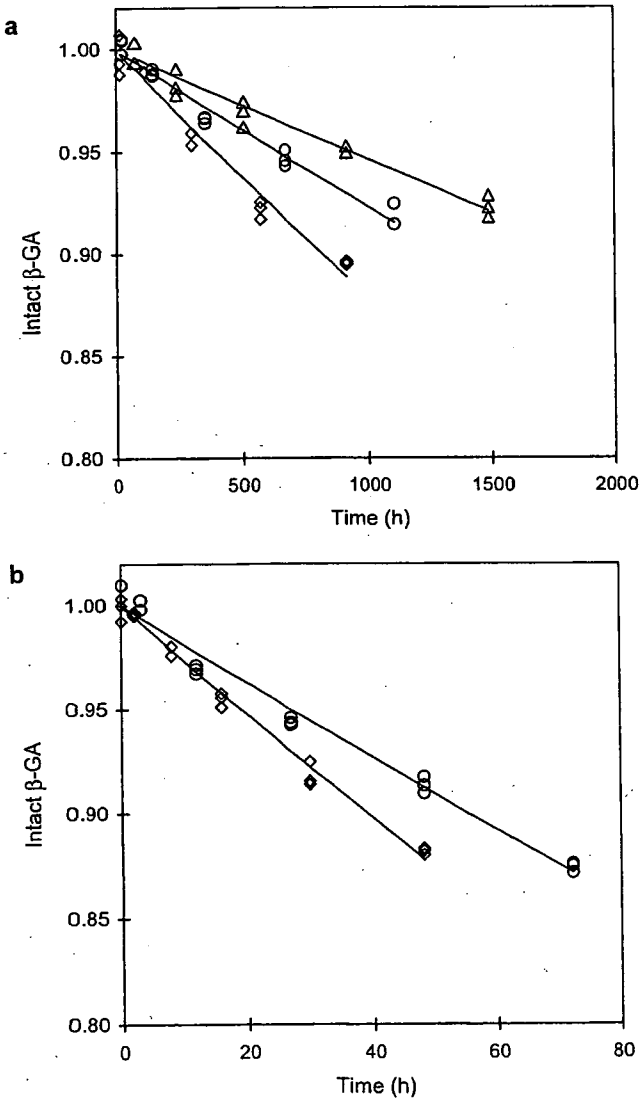


Fig. 2. Time courses of aggregation of β-GA lyophilized with sucrose (△), trehalose (O) or stachyose (◇). (a) aggregation at 50°C and excipient fraction of 0.33. (b) aggregation at 80°C and excipient fraction of 0.5.

### Excipient-Fraction Dependence of β-GA Aggregation Rate

Figure 4 shows the dependence of $t_{90}$ on the weight fraction of excipient at temperatures below $T_g$ (50°C) and above $T_g$ (80°C). As the weight fraction of excipient increased, $t_{90}$ increased for all formulations. The values of $t_{90}$ for the sucrose formulation in the amorphous state could not be determined at fractions above 0.5 at 50°C or above 0.33 at 80°C, because crystallization occurred during storage (crystallization was confirmed by the lack of crystallization peak in DSC thermograms). The $t_{90}$ observed for β-GA aggregation showed a log-linear dependence on the excipient fraction, as reported for other proteins (11,12).

Figure 5 shows the water content and $T_g$ determined for the lyophilized β-GA formulations with various weight fractions of trehalose, sucrose or stachyose. It has often been reported that lyophilized proteins without excipients do not show a distinct change in heat capacity in DSC thermograms. The $T_g$ of lyophilized β-GA alone could not be determined in the dry state, but it could be estimated at 12% RH from small changes in heat capacity (Fig. 6). The $T_g$ value determined at 12% RH depended on the excipient fraction (Fig. 5); $T_g$ decreased with increasing excipient fraction from 0 to 0.3. Only a single $T_g$ was observed in the range of excipient fractions from 0 to 0.3 for all formulations, suggesting that these formulations are a single glassy phase on levels detectable by DSC.

As shown in Figs. 4 and 5, the rank order of $t_{90}$ at a certain excipient fraction was sucrose > trehalose > stachyose, whereas that of $T_g$ of β-GA formulations was sucrose < trehalose < stachyose. The value of $t_{90}$ increased significantly with increasing excipient fraction, even at small excipient fractions, in which $T_g$ decreased significantly with increasing excipient fraction. These findings indicate that β-GA aggregation rate is not primarily related to $(T-T_g)$.

Figure 7 shows the amount of monomeric β-GA remaining after freeze drying with sucrose, trehalose or stachyose. Significant changes were observed at an excipient fraction of 0.09 for all formulations. The stachyose formulation exhibited the largest degree of β-GA aggregation during freeze drying. This finding suggests that freeze-drying processes cause changes in protein conformation at differing degrees between excipients, which in turn leads to the differences in β-GA aggregation rate observed between excipients. FT-IR is known to be useful for detecting changes in protein conformation produced during the freeze-drying process (17). Figure 8 compares the second derivative FT-IR