Investigations into the Stabilization of Drugs by Sugar Glasses: III. The Influence of Various High-pH Buffers

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Purpose. To study the effect of the high-pH buffers ammediol, borax, CHES, TRIS, and Tricine on the glass transition temperature of the freeze concentrated fraction (Tg′) of trehalose/buffer and inulin/buffer solutions at pH 6.0 and pH 9.8. Also, the glass transition temperature (Tg) of sugar glasses obtained after freeze drying of these solutions was elucidated. Additionally, the effect occurring during the freezing process on the pH of the various buffers was investigated. Furthermore, the stability of alkaline phosphatase (AP) incorporated in these sugar glasses prepared from solutions at pH 9.8 was evaluated.

Methods. The Tg′ and Tg were measured using differential scanning calorimetry (DSC), and the change of pH during freezing was estimated by using an indicator solution added to the respective solutions. The enzymatic activity of AP after freeze drying and storage at 60°C was evaluated by an enzymatic activity assay.

Results. It was found that the Tg′ and Tg of the samples investigated are strongly influenced by the presence of the buffer. On freezing, only minor changes of the pH were observed. The samples with the lowest Tg and the samples containing buffers that formed complexes with the sugars showed the poorest stability of the AP.

Conclusions. The stabilizing capacities of sugars that are currently recognized as excellent stabilizers for proteins during drying and storage can be completely lost if certain high-pH buffers such as ammediol, borax, and TRIS are used at high concentrations. Loss of stabilizing capacities can be ascribed to strong depression of the Tg′ and Tg or to complex formation.

KEY WORDS: buffers; stabilization; sugar glasses; glass transition.

INTRODUCTION

With the rapid developments in the field of biopharmaceutics, increasing attention is being paid to the development of protein-based drugs for the treatment of various disorders. During the formulation of such drugs it is necessary to ensure the stability of the protein in order to prevent degradation during production, storage, and transportation. A suitable way to stabilize proteins is to dry a solution containing the therapeutic protein and a sugar. The stabilization of the protein is achieved by the formation of a sugar glass matrix in which the protein is enclosed.

In previous studies it has been found that the disaccharide trehalose is an excellent stabilizer for proteins during freeze drying and subsequent storage (1–8). Recently, it was also shown that inulin, an oligosaccharide, can be used as a stabilizer of proteins during freeze drying (9) and subsequent compaction to tablets and storage for prolonged time (10). In those studies it was found that inulin had some advantages over trehalose as a stabilizer of alkaline phosphatase (AP).

Freeze drying of aqueous solutions of protein/sugar mixtures is generally performed in the presence of a buffer. However, the influence of buffers on the physical properties of the lyophilized samples has not been given much attention. It is generally accepted that the use of phosphate buffer should be avoided because disodium or dipotassium monohydrogen phosphate precipitates more readily during freezing than sodium or potassium dihydrogen phosphate, which leads to a strongly decreased pH of the sample (11–13). Such a dramatic pH drop is potentially detrimental to the stability of proteins and should be avoided. In a publication by Orii and Morita the change of pH during freezing of 30 different buffers was investigated (12). It was, for example, found that the pH of phosphate buffers typically dropped by more than about 2 units, TRIS-HCl and Tricine buffers showed increases of less than 1 unit, and cyclohexylaminopropanesulfonic acid buffers showed a decrease of less than 1 pH unit during freezing. It has been shown that replacing succinate buffer with glycolate buffer increases the stability of lyophilized interferon-γ. It was found that monosodium succinate crystallized during freezing, which led to a sharp drop in pH. No change of the pH was found for sodium glycolate (14). It has been found by others that when phosphate buffer pH 7.4 was frozen in the presence of a cryoprotectant (sucrose or trehalose, 0.5 and 1 M), the pH dropped by only 1 pH unit, compared to a drop of 2.4 pH units when no protectant was used (15). An explanation for this effect was, however, not given. The stability of proteins freeze dried with trehalose was previously found to be enhanced when the samples contained borax at a mole ratio borax/trehalose higher than 0.3 (16). It was suggested that the reason for the increased stability was the increased Tg caused by the specific interaction between the sugar and borax. The borax ions form covalent linkages with the hydroxyl groups of the trehalose molecule. As a result, the viscosity of the solution increased, and this has been suggested to promote glass formation during freezing. It has also been found that this diol-diol complexation is affected by the pH of the solution (16). This was also found by Pezron and coworkers, who performed ^{11}B-NMR on glycoside–borax complexes at different pHs. It was found that below pH 7.5 no signals related to borax–diol complexes were detected (17).

Except for the study of Miller *et al.* (16), as mentioned above, there are to our knowledge no detailed studies published about the effects of buffers on the glass transition temperature of the freeze-concentrated fraction (Tg′) and glass transition temperature (Tg) of sugar glasses. This is quite surprising because it is generally accepted that the Tg′ and the Tg play essential roles for the stabilization of proteins during drying (18) and storage (19–21), respectively.

In this paper we present the results of an investigation of the influence of the high-pH buffers ammediol, borax, CHES, TRIS, and Tricine on the Tg′ of trehalose and inulin in buffer solutions and the resulting Tg of freeze-dried trehalose and inulin obtained from buffer solutions. The effect of freezing

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on the pH of various buffers and sugar/buffer solutions was also evaluated. Moreover, the influence of the buffers on the stability of a protein, alkaline phosphatase (AP), that was freeze-dried with trehalose or inulin was also investigated.

EXPERIMENTAL

Materials

Trehalose, alkaline phosphatase from bovine intestinal mucosa (BIAP), 2-(N-cyclohexylamino)ethanesulfonic acid (CHES), 2-amino-2-methyl-1,3-propanediol (ammediol), 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), and *para*-nitrophenyl phosphate (pNPP) were purchased from Sigma-Aldrich (Steinheim, Germany). N-[TRIS(hydroxymethyl)methyl]glycine (Tricine) was from Fluka (Buchs, Switzerland), and sodium tetraborate (borax) was from Brocacef BV (Maarssen, The Netherlands). Inulin with a number/weight average degree of polymerization (DP_n/DP_w) of 23/26 was a gift from Sensus (Roosendaal, The Netherlands). All other chemicals were obtained from commercial suppliers and were of analytic grade. The chemical structure of ammediol, TRIS, Tricine, and CHES, respectively, are given in Fig. 1.

Freeze Drying

For the investigation of the influence of buffers on the physical properties of sugar glasses of trehalose or inulin, solutions containing 10% w/v in the respective buffers (ammediol, TRIS, Tricine, CHES, typically 0.05–0.20 M, pH 9.8) were prepared. When borax was used the concentrations were 0.005–0.060 M, pH 9.8. Also, solutions with 10% w/v sugar in buffers at pH 6.0 were freeze dried in order to evaluate the influence of pH on the Tg. To adjust to pH 9.8, 1 M NaOH was added to all buffers, except to the ammediol, which was adjusted with 1 M HCl. The solutions at pH 6.0 were prepared by addition of 1 M HCl. Before freeze drying, the Tg′ of the sample solutions was measured (see below). Samples to investigate the influence of buffers on the stability of BIAP incorporated in sugar glasses were prepared by freeze drying 2.5 or 10% w/v solutions of BIAP/sugar (1/4 and 1/9 w/w) in each buffer (pH 9.8, 0.05 M). Freeze drying was performed in a Christ Alpha 1–4 freeze dryer (Salm en Kipp, Breukelen, The Netherlands) as follows: 24 h at a shelf temperature of –35°C, a condenser temperature of –53°C, and a pressure of 0.220 mbar. The pressure was then lowered to 0.050 mbar, and the temperature was gradually increased to 20°C, which was maintained for 24 h. The dry samples were then transferred to a vacuum desiccator at room temperature, where they were kept for at least 2 days. Then half of each sample containing BIAP was transferred to an oven (Termacks, Salm & Kipp B.V., Breukelen, The Netherlands) set to 60°C for 6 days. The activities of the samples stored at room temperature and at 60°C were evaluated.

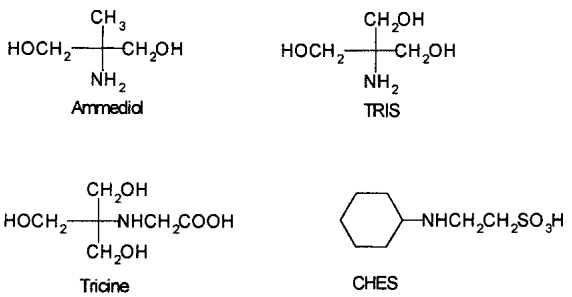

Fig. 1. The chemical structures of the various buffers used.

Differential Scanning Calorimetry

The differential scanning calorimetry (DSC) thermograms of the samples were recorded using a TA Instruments DSC 2920 (TA instruments, Ghent, Belgium), which had been calibrated with indium. The instrument was equipped with a cooling device that was supplied with a stream of helium (for Tg′) or nitrogen (for Tg) throughout the measurements. The Tg′ measurements were performed by rapid cooling to –60°C, which was held for 5 min, followed by heating at a rate of 20°C/min to 40°C. The Tg was measured by heating from 20°C (held for 2 min) to 300°C at a rate of 20°C/min. For the measurement of the Tg′, samples of about 60 mg were weighed into open aluminum pans, while the Tg was measured on samples of about 10 mg in open aluminum pans. The Tg′ and Tg values were taken as the midpoint values of the transitions measured. DSC to determine the Tg′ of the buffers (10% w/v, except borax 5% w/v, in water) were performed on a Perkin Elmer DSC 7 (Perkin Elmer, Gouda, The Netherlands). We used this DSC apparatus because it is equipped with a cooling device containing liquid nitrogen to achieve temperatures of –120°C. The sample size was about 15 mg in sealed aluminum pans. Measurements were performed from –120°C, which was held for 2 min, followed by heating to –40°C at a rate of 10°C/min.

Influence of Freezing on the pH of the Buffers

Buffer solutions (2.00 ml, 0.05 M, pH 9.8) were mixed with universal indicator (20 μl). The universal indicator contained 0.02% w/v methyl red, 0.02% w/v phenolphthalein, 0.04% w/v bromthymol blue, and 0.04% w/v thymol blue in ethanol (22). The color of the solutions before and after being frozen in liquid nitrogen was noted. Phosphate buffer (0.05 M, pH 7.3) was also investigated as a reference because the behavior of this buffer during freezing is well documented (11–13,23). Also, buffers containing 10% w/v of trehalose or inulin, respectively, were frozen with indicator solution present in order to elucidate the influence of sugars on the pH of the respective buffers during freezing.

Activity Assay of Alkaline Phosphatase

The activity of alkaline phosphatase was determined by following the enzymatic conversion of the substrate pNPP to *para*-nitrophenol using a modified version of the method described by Hinrichs *et al.* (9). The assays were performed in 96-well microplates as follows: 160 μl of 0.05 M ammediol (pH 9.8) containing 2.2 mM $MgCl_2$ was mixed with 20 μl sample; to this mixture 20 μl of pNPP (10 mg/ml in demineralized water) was added. The plate was then placed in a Benchmark Microplate reader (BioRad, Hercules, CA) set to a temperature of 37°C. To ensure good mixing the plate was shaken by the plate reader for 10 s at the start and then again after 30 min immediately before the absorbance of the samples at 405 nm was measured. A standard curve was pre-

pared by measuring the activity of BIAP in the range 0 to 5 μg/ml.

It has been reported that borax can reduce the enzymatic activity of alkaline phosphatase (24). Therefore, in the present study, the enzymatic activity assay of borax-containing samples was performed with a calibration curve generated using standard BIAP solutions containing borax. Because alkaline phosphatase is a glycoprotein, it is likely that the cause of this inhibition is the interaction between borax and the sugar groups attached to the alkaline phosphatase.

RESULTS AND DISCUSSION

Effect of Buffers on the Tg′

During freeze drying of a solution containing a protein and a sugar it is important to maintain the sample temperature below the Tg′ (18); otherwise, the freeze-concentrated fraction will be in its rubbery state. In that case, the molecular mobility is high, which might lead to degradation of the protein. Also, crystallization of the sugar may occur, by which its protective action will be completely lost. In addition, freeze drying below the Tg′ results in a porous cake, whereas a collapsed cake is obtained above the Tg′, and reconstitution of a porous cake is easier than a collapsed cake. In a previous study it has been found that the Tg′ of pure inulin is higher than that of pure trehalose (9). In this study, it was found that, in all cases, the Tg′ of the trehalose/buffer mixtures was lower than the Tg′ of the corresponding inulin/buffer mixtures. In Fig. 2, the Tg′ of each trehalose solution at pH 9.8 is given. It is evident that ammediol and TRIS have the largest influence on the Tg′, followed by Tricine and CHES; all these buffers substantially depress the Tg′ of the trehalose. Similar results were found for the inulin samples (Fig. 2). The gradual change of the Tg′ with increasing buffer/sugar ratios indicates that the composition of the freeze-concentrated fraction gradually changed. Most likely, the buffer species are incorporated together with the sugars in the freeze-concentrated fractions and form homogeneous mixtures. Ammediol and TRIS had about the same effect on the Tg′, and so did Tricine and CHES. It was expected that ammediol and TRIS have similar effects because their chemical structures are very similar (Fig. 1). Also, the Tg′ values of the pure buffer solutions were almost identical for both ammediol and TRIS (–81°C and –79°C, respectively) and for Tricine and CHES (–61°C and –58°C, respectively). For borax no Tg′ could be established. In contrast to the other buffers, the Tg′ of trehalose solutions containing borax at pH 9.8 increased with increasing borax/sugar ratios (Fig. 3). An explanation for this is the specific interaction between the borax and the hydroxyl groups of the trehalose; i.e., the borax can form covalent bonds with the hydroxyl groups, leading to a new compound with a different Tg′ than that of the pure sugar. As the amount of borax increases, so does the degree of complexation, resulting in a rise of the Tg′. This interaction is frequently mentioned in the literature and explains the increased viscosity (i.e., gelling) of polysaccharide solutions through formation of monodiol- and didiol-borax cross links (17,25). The same trend was found for the inulin samples, except that the Tg′ values were higher than those of the corresponding trehalose samples (Fig. 3). It has previously been shown that the Tg′ of oligosaccharides increases with their size (9), explaining why the Tg′ is higher for inulin than for trehalose.

In Fig. 4 it is shown that the Tg′ values of trehalose/TRIS and inulin/TRIS solutions at pH 6.0 are lower than at pH 9.8. The other buffers, except borax, demonstrated a similar decrease of the Tg′ when the pH was changed from 9.8 to 6.0 (data not shown). It was hypothesized that the change of the Tg′ is related to the change of the charge of the buffer species. The average number of ionized groups of the buffers at pH 6.0 and at pH 9.8 were calculated using the Henderson-Hasselbalch equation, ($pH = pK_a + \log [base]/[acid]$). In the case of Tricine and CHES, one charge unit is added because at the pH range under investigation the carboxylic acid and the sulfonic acid groups of the Tricine and the CHES, respectively, are fully deprotonated. As can be seen in Table I, the charge of the buffer component increases dramatically when the pH is lowered from 9.8 to 6.0 in all cases. These results, indeed, indicate that the difference in charge of the respective buffers at the two pH values are related to the different values of Tg′. In a recent study by Shalaev and co-workers (26), the influence of pH on the Tg′ of malate and citrate buffers was investigated. They found that the Tg′ decreased with increasing pH, i.e., with increasing charge, which is in concordance with our results. They suggested that the decrease in Tg′ was

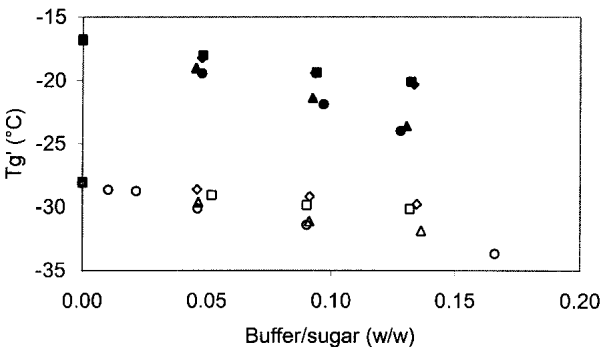

Fig. 2. The Tg′ of trehalose and inulin (10% w/v) in various buffers, pH 9.8. Inulin/Tricine (♦), inulin/CHES (■), inulin/TRIS (▲), inulin/ammediol (●), trehalose/Tricine (◇), trehalose/CHES (□), trehalose/TRIS (△), trehalose/ammediol (○). The data shown are the means of two to four measurements. The standard deviation was less than 1°C in all cases.

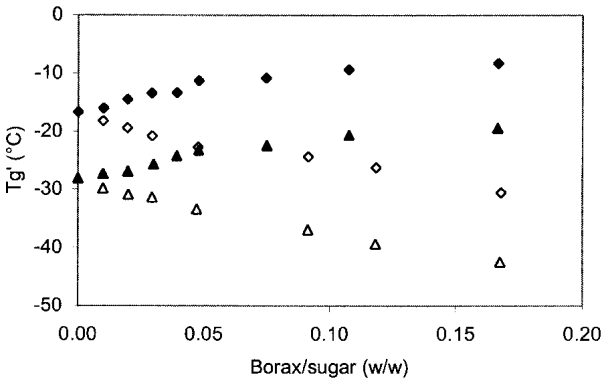

Fig. 3. The Tg′ of trehalose and inulin (10% w/v) in borax, pH 6.0 and pH 9.8. Inulin/borax pH 9.8 (♦), inulin/borax pH 6.0 (◇), trehalose/borax pH 9.8 (▲), trehalose/borax pH 6.0 (△). The data shown are the means of two to four measurements. The standard deviation was less than 1°C in all cases.

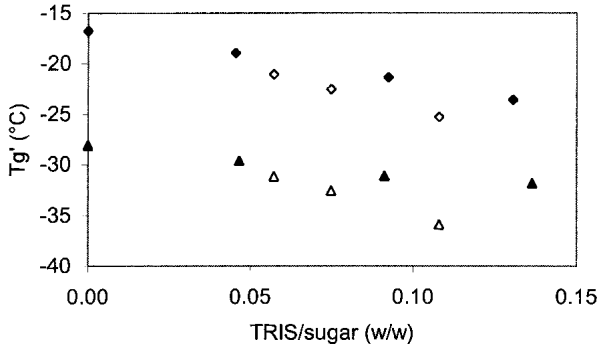

Fig. 4. The Tg′ of trehalose and inulin (10% w/v) in TRIS buffer, pH 6.0 and pH 9.8. Inulin/TRIS pH 9.8 (♦), inulin/TRIS pH 6.0 (◇), trehalose/TRIS pH 9.8 (▲), trehalose/TRIS pH 6.0 (△). The data shown are the means of two to four measurements. The standard deviation was less than 1°C in all cases.

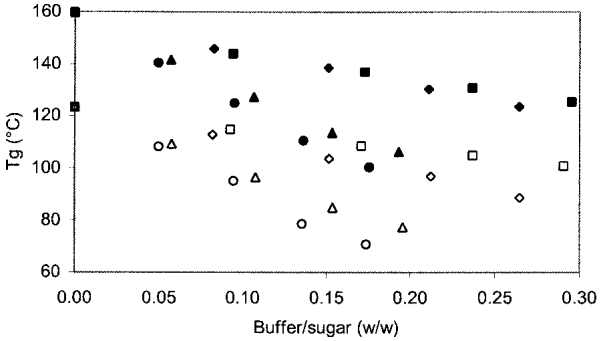

Fig. 5. The Tg of amorphous trehalose and inulin with different buffers, pH 9.8. Inulin/CHES (■), inulin/Tricine (♦), inulin/TRIS (▲), inulin/ammediol (●), trehalose/CHES (□), trehalose/Tricine (◇), trehalose/TRIS (△), trehalose/ammediol (○). The data shown are the mean of two to four measurements. The standard deviation was less than 2°C in all cases.

an effect of an increasing water content in the freeze-concentrated solution. Because water is a plasticizer the Tg′ will decrease with an increasing amount of water present in the freeze-concentrated solution.

The results of the borax-containing samples (Fig. 3), on the other hand, were completely different. Instead of showing Tg′ values similar at both pH 6.0 and pH 9.8, the Tg′ actually changed in opposite directions with increasing concentration of borax at the two pH values. We suggest that this is caused by a different kind of interaction between the sugars and borax at the two pH-values tested. It has indeed been found by others that, in contrast to high pH, there is no complex formation between sugars and borax below pH 7.5 (17,25). This means that at pH 6.0 sugar and borax form a homogeneous mixture in the freeze-concentrated fraction as for the other sugar/buffer combinations investigated in the study presented here.

Effect of Buffers on the Tg

It is generally accepted that a sugar glass with a protein incorporated should be stored below the Tg. This is to diminish the risk of degradation of the protein, which otherwise can occur when the glass structure turns into its rubbery state. The rubbery state allows a higher mobility and thus a higher reactivity of the molecules (6,27). In addition, in the rubbery state crystallization can occur, which leads to phase separation and thus to a complete loss of protection of the protein (6,7,28,29). It has even been shown that the molecular mobility in sugar glasses increases when they are stored at temperatures 50°C below their Tg (30), suggesting that degradation can occur even in samples stored far below their Tg. As can be seen in Fig. 5, all buffers, except for borax (see Fig. 6), suppress the Tg of trehalose and inulin. Similar to the Tg′, the Tg of these trehalose samples is in all cases lower than the Tg of the corresponding inulin samples. Also similar to the Tg′, ammediol and TRIS have the largest effect on the Tg followed by Tricine and CHES. The rapid decrease of Tg with increasing amounts of buffer present in the sample indicates that high amounts of these buffers should be avoided. For homogeneous mixtures the Gordon-Taylor equation applies (31). The values from the measurements of the Tg of the freeze-dried material were fitted in the Gordon-Taylor equation:

$$T_g = \frac{w_1 \cdot Tg_1 + k \cdot w_2 \cdot Tg_2}{w_1 + k \cdot w_2}$$

In this formula Tg is the glass transition temperature of the mixture, Tg_1 is the glass transition temperature of the sugar, w_1 is the weight fraction of the sugar, Tg_2 is the glass transition temperature of the buffer, and w_2 is the weight fraction of the buffer. The parameter k is used here as a fitting parameter. Excellent fits were obtained for all mixtures (see Table II). The good correlation coefficients indicate that the sugars and buffers were homogeneously distributed on a molecular level. The Tg values of the trehalose and inulin

Table I. The Average Number of Ionized Groups of the Buffers at Different pH

		Average number of ionized groups per buffer species	
Buffer	pK_a	pH 9.8	pH 6.0
Ammediol	8.8	0.091	1.0
TRIS	8.1	0.020	1.0
Tricine	8.1	1.0	1.9
CHES	9.3	1.2	2.0

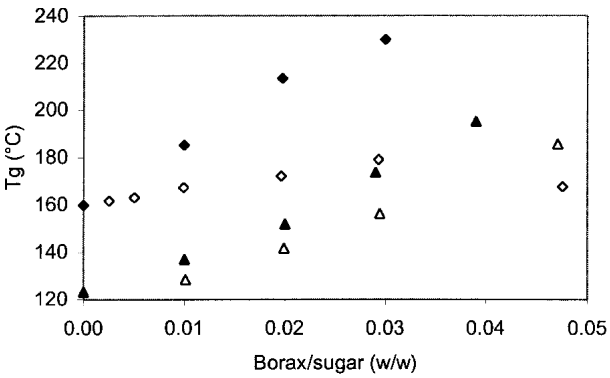

Fig. 6. Tg of amorphous trehalose and inulin in borax, pH 6.0 and pH 9.8. Inulin/borax pH 9.8 (♦), inulin/borax pH 6.0 (◇), trehalose/borax pH 9.8 (▲), trehalose/borax pH 6.0 (△). The data shown are the mean of two to four measurements. The standard deviation was less than 2°C in all cases.

Table II. The Results from the Gordon-Taylor Fit of the *Tg*-Measurements from Samples Freeze-Dried from Solutions at pH 9.8

	Trehalose		Inulin	
Buffer	Correlation coefficient	*k*	Correlation coefficient	*k*
Ammediol	0.993	0.86	0.999	0.43
TRIS	0.998	0.63	0.998	0.44
Tricine	0.997	1.12	0.994	0.56
CHES	0.998	0.47	0.996	0.31

samples that were freeze dried with ammediol, TRIS, Tricine, or CHES at pH 6.0 showed no significant differences from those of the corresponding samples that were freeze dried with the respective buffers at pH 9.8 (data not shown). These results show that in the dry amorphous state the pH, and therefore also the charge, of the buffer in the original solution has no significant influence on the Tg of the final dry product. The results for borax are shown in Fig. 6. The Tg of the samples freeze dried from solutions at pH 9.8 increased with increasing borax content. This is probably explained by complex formation between the sugars and the borax. The Tg of trehalose freeze dried with borax pH 6.0 was slightly lower than the Tg measured for the corresponding samples freeze dried with borax at pH 9.8. Also, for inulin that was freeze dried with borax at pH 6.0, the Tg values were substantially lower than the Tg at pH 9.8. As indicated by the Tg′ measurements, borax does not form complexes with sugars in solution at pH 6.0 (Fig. 3). However, the increase of the Tg with increasing borax content suggests that, in the dry state, complex formation is possible to a certain extent at this pH. For borax, the Gordon-Taylor equation does not apply because it does not take the complex formation between the borax and the sugars into account. Therefore, no *k* values were calculated.

Effect of Freezing on the pH of Different Buffers

In Table III the results of the investigation of the pH change of six different buffers during freezing are given. All buffers except the phosphate buffer changed by 1 pH unit or less. As previously reported, the pH of the phosphate buffer decreased by 3 pH units (11–13). In a study by Croyle *et al.* (15) it was found that when phosphate buffer pH 7.4 was frozen with no sugar present, the pH dropped about 2.4 units. In the study by Croyle and co-workers (15) it was also found that when 0.5 or 1 M trehalose was present, the pH dropped by only approximately 1 unit, and we found no significant difference for phosphate buffer whether sugar was present or not. However, in the study by Croyle and co-workers (15), the concentration of the phosphate buffer was 10 mM, whereas in the present study the concentration was 50 mM. Our results for TRIS and Tricine are contradictory to the results found by Orii and Morita (12), who found an increase in pH after freezing. However, they used another starting pH (pH 8.1 for TRIS and pH 7.0 for Tricine), which may explain the different results. When ammediol, TRIS, and Tricine were frozen in the presence of sugar, the pH did not change, but the pH of solutions containing CHES or borax dropped by 1 unit. When sugar was added to the borax solution, the pH dropped by one unit before freezing, probably because of complex formation. On freezing the pH dropped by another unit. It seems that trehalose and inulin both act as pH stabilizers of ammediol, TRIS, and Tricine during freezing but not for the other buffers investigated here.

Table III. The Influence of Freezing on the pH Shift of Various Buffers as Visually Observed from Color Changes[a]

Buffer	No sugar	Trehalose	Inulin
Ammediol	–1 to 0	0	0
TRIS	–1 to 0	0	0
Tricine	–1 to 0	0	0
CHES	–1	–1	–1–0
Borax	–1 to 0	–1	–1
Phosphate	–3	–3	–3

[a] The pH of the buffers before freezing was 9.8 except for borax and phosphate, where the pH was about 9 and 7.3, respectively.

Effect of Buffers on the Enzymatic Activity of Alkaline Phosphatase from Bovine Intestine

After freeze drying and storage at 20°C for 6 days, the enzymatic activity of BIAP was fully maintained for all samples (data not shown) except for some of the borax samples (see below for further comments). The enzymatic activity of BIAP freeze dried with trehalose or inulin in the presence of different buffers was also evaluated after 6 days of storage at 60°C (Table IV). The enzymatic activity was fully preserved in all cases, except for the freeze dried 2.5% w/v-solutions of trehalose in ammediol or TRIS (and borax, see below). In the products freeze dried from the 2.5% w/v solutions of ammediol or TRIS, the buffer content was 17% or higher. Because trehalose has a lower Tg than inulin, and because ammediol and TRIS induce the largest decrease of the Tg, these samples will have the lowest Tg of all samples under investigation. Therefore, the poor enzymatic recovery is most likely caused by the low Tg. This is confirmed by the results of the corresponding inulin samples. These samples will have a higher Tg. Consequently, the enzymatic activity was fully maintained.

The samples freeze dried from 2.5% w/v solutions with borax demonstrated a loss of enzymatic activity after freeze drying and storage at 20°C. The remaining enzymatic activity was 90% and 65% for trehalose and inulin, respectively. For the samples that were stored for 6 days at 60°C, the remaining enzymatic activity was also substantially lower (see Table IV). Similar to the samples stored at 20°C, the enzymatic activity was higher for the trehalose samples than for the corresponding inulin samples. As already mentioned, at pH 9.8 borax forms complexes with the hydroxyl groups of sugars. Apparently, the chemical modification of the sugars by complexing with borax results in a partial loss of the stabilizing properties of the sugars during freeze drying and storage. Both samples freeze dried with trehalose and with inulin lost a substantial amount of activity, but the trehalose samples showed higher activities than the corresponding inulin samples. Because inulin is an oligosaccharide, the mole ratio for inulin/borax is lower than for trehalose/borax. Possibly, this can explain the different stabilities obtained. The loss of enzymatic activity could not have been caused by a pH drop during the freeze-drying process because no dramatic pH shifts were observed

Table IV. The Remaining Activity (%) of BIAP after Freeze Drying with Sugars and Various Buffers after 6 Days of Storage at 60°C[a]

Concentration sugar+BIAP in solution before freeze drying (% w/v)	Sugar/BIAP (w/w)	Ammediol		TRIS		Tricine		CHES		Borax	
		Trehalose	Inulin	Trehalose	Inulin	Trehalose	Inulin	Trehalose	Inulin	Trehalose	Inulin
10	9/1	110.3	106.9	120.5	109.6	116.6	111.8	116.4	102.2	94.8	105.3
10	4/1	111.6	108.9	118.5	100.9	111.4	112.1	110.7	94.4	97.1	91.4
2.5	9/1	*1.1*	100.9	105.2	104.0	109.3	103.1	112.8	108.3	*66.2*	*37.9*
2.5	4/1	*16.7*	99.8	*69.4*	94.1	101.4	120.6	107.4	95.1	*71.1*	*36.3*

[a] The mean is reported, n = 4. The standard deviation was less than 4.5% in all cases.

in the freezing experiments described above. In a previous study it was found that above pH 5 no loss of enzymatic activity of occurs (32). In the present study the pH was always higher than that.

CONCLUSIONS

Stabilization of pharmaceutically active proteins by freeze drying is often performed from solutions containing a sugar and some buffer. However, applications of buffers in these formulations may have a detrimental effect on the enclosed protein. As found in other studies, employment of phosphate buffers may lead to a strong pH drop during freezing, leading to degradation of the protein. In this study, it was found that buffers can also have a large impact on the Tg′ and the Tg of sugar samples, potentially leading to problems with the stability of the product. As a result of the suppression of the Tg′ and Tg by the buffers, it is recommended to choose a sugar with as high Tg′ and Tg as possible, e.g., inulin instead of trehalose, in order to ensure stabilization of the protein during and after freeze drying. Furthermore, buffers that form complexes with sugar should be avoided because they may severely affect the stabilizing capacity of the sugar. Also bear in mind the fact that these buffers might also form complexes with the sugar moieties of glycoproteins, which might harm the protein in question. In addition to these considerations, the buffer giving the best stability must also be compliant with the regulations of the FDA regarding toxicity if the product is to be used as a pharmaceutical agent intended for human use.

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