Bond Cleavage Reactions in Solid Aqueous Carbohydrate Solutions

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Purpose. To investigate chemical reactivity in water soluble glasses.
Methods. Rates of bond cleavage reactions in freeze-dried and freeze-concentrated aqueous carbohydrate solutions were measured above and below the glass transition temperatures (T_g). The kinetics of two reactions have been determined in formulations containing di- and polysaccharides: (1) fission of the Asp-Pro peptide bond in Physalaemin and Hamburger peptide by following the release of proline, using a ninhydrin based reaction and (2) the unimolecular dissociation of 2-(4-nitrophenoxy) tetrahydropyran by following the release of the 4-nitrophenoxy anion.
Results. The results show clearly that reaction occurs below the glass transition temperature, albeit at very reduced rates. No significant enhancement of the temperature dependence of the rate constant was observed near T_g. Different water soluble glasses provide different degrees of stability. The order of stabilisation was sucrose>Ficoll (low mol. weight)>Byco A ≅ Ficoll (high mol. weight)>dextran. The density of the matrix, and therefore the degrees of freedom of mobility of the reactant, is thought to be responsible for these differences.
Conclusions. The storage of therapeutic agents, such as proteins, in glassy matrices below T_g does not confer indefinite stability. When formulating products, notice should be taken of the differing stabilisation properties of excipients.

KEY WORDS: carbohydrate-based glasses; glass transition temperature; hydrolysis reactions; protein stabilisation.

INTRODUCTION

Oligopeptides and proteins are finding increasing uses as therapeutic agents. They are, however, subject to gradual chemical degradation, leading to inactivation, brought about by a variety of different mechanisms. These chemical processes involve the breaking (and possibly the faulty remaking) of covalent bonds, and they are therefore irreversible (1). In addition, proteins are sensitive to conformational destabilisation (denaturation), brought about by environmental factors, such as changes in concentration, temperature, pH, and solvent composition. In principle, these latter effects, which do not involve the breaking of covalent bonds, should be reversible. Indeed, *in vitro* renaturation and reactivation treatments are extensively employed in the downstream processing of recombinant proteins (2). Probably because the conformational stability of proteins can generally be treated by the methods of equilibrium thermodynamics, the denaturation/renaturation phenomenon has received, and is still receiving, a great deal of attention. On the other hand, the irreversible, chemical changes, leading to inactivation (e.g. oxidation, deamidation, proteolysis, disulphide rearrangement) are not so well explored.

Commercial therapeutic products containing peptides and proteins are usually presented in the form of solid products, in recognition of the fact that the rate of deterioration can in this way be substantially reduced, if not prevented altogether. On the other hand, the necessary removal of >99% of water from an initially dilute solution, whether by freezing or evaporation, presents a number of technical problems relating to stability which require recognition and attention. Thus, it has long been recognised that the excessive inactivation of proteins during drying can be prevented in the presence of stabilising additives. Polyhydroxy compounds (PHCs) appear to be particularly effective in this respect. Although these materials have been used for many years, the reasons for their particular efficacy in rendering many labile substances stable in the dried state only began to be explored during the 1980s and have received increasing attention since then (3). The general consensus is that on removal of water (by freezing or by evaporation), from their unsaturated aqueous solutions, most PHCs form supersaturated solutions. On further drying the supersaturated solutions eventually vitrify i.e. undergo a glass transition (4). In the glassy state, compared to dilute solutions, there is evidence that the rates of both physical and chemical processes are reduced by upwards of ten orders of magnitude (5).

More detailed studies of the complex dynamics and reactivities of the water soluble glasses themselves, and of trapped species within the glasses, have revealed that in highly supersaturated aqueous sucrose solutions, approaching the glass transition, rotational and translational diffusive motions become uncoupled. Also, the motions of the two chemical species undergo complex coupling and decoupling processes with increasing sucrose concentration (6). Initial studies suggested that the very process of vitrification would render trapped molecular species in the glass chemically stable (7), but more recent work is demonstrating that proteins can undergo chemical deterioration even in the vitreous state, albeit at very reduced rates (8).

Current reappraisals of several thermophysical aspects of PHC glasses (9) are raising certain questions about relationships, if any, between slow structural, enthalpic and volumetric relaxation processes and chemical stability/reactivity. Most previous studies of slow mechanical relaxations had been confined to "real" materials, e.g. oxide and silicate glasses, where the famous "Kauzmann Glass Paradox", relating to the possibility of a zero configurational entropy, had been explored (10). These investigations led to the specification of an additional characteristic temperature, T_0, the so-called temperature of zero mobility which lies substantially below T_g. Experimental data on the ramifications of such relaxation processes, as they might occur in water-based glasses, are still very limited (11), but the ability to measure the necessary parameters required for tests of the

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new hypotheses should provide further chemical insights into the significance of slow relaxation processes, and how they might affect the stability and performance of commercial products. It is in any case becoming apparent that the established practice of equating the glass transition with absolute chemical stability is due for revision. The major objective of the present study was to test the validity of this hypothesis with the aid of reactions which are characteristic of peptides and the kinetics of which in aqueous solution are reasonably well understood. For the study of materials in their amorphous state, all experimental operations must be carried out with reference to the glass temperature. This is particularly true for the freeze-drying process. In comparisons of different excipients it is difficult to control two dependent variables: T_g and the residual water content. Bearing this in mind, we selected two reactions, the kinetics of which are expected to be independent of the residual water content: the acid hydrolysis of a peptide bond and the hydrolytic decomposition of an acetal.

Although it is common practice to refer generically to "the" peptide bond, the four hundred combinations of the amide link between the protein amino acids all have different stabilities. Some are sufficiently labile to be of concern for the stability of pharmaceuticals, others require boiling in 6M HCl for 96 hours to ensure quantitative cleavage. The reactivity of peptide bonds depends very much on the type of amino acid residue involved, also on the relative position of the peptide bond of interest in the primary chain and on conformational properties. Thus, aspartate residues are cleaved at least a hundred times faster than other peptide bonds in dilute acid. Asp-Pro bonds are particularly labile, more so than other Asp-X or X-Pro bonds, as shown by the half life ($t_{1/2}$) for the hydrolysis (0.015N HCl, 110°C) of Physalaemin's Asp-Pro bond. The half life of 11 minutes compares (12) with 130 minutes for Asp-Phe, 228 minutes for Asp-Lys and more than 24 hours for Gly-Pro or Val-Pro. For Ala-Gly hydrolysis, $t_{1/2}$ is also greater than 24 hours. It has been suggested that the hydrolytic reaction proceeds via intramolecular catalysis by the carboxylate anion, with displacement of the protonated nitrogen of the Asp-Pro peptide bond (release of proline). Rate enhancement is due to the greater basicity of the proline nitrogen compared to other primary amine nitrogen atoms (13). However, the mechanism of degradation of Asp residues in proteins and peptides has not yet been fully elucidated. Whatever might be the correct mechanism in dilute solution, when extrapolated to supersaturated solutions (low water content) and low molecular mobility, a change in kinetics might be expected. Attack of water may become rate-determining and slow down the *overall* reaction, but it will *not* influence the rate of release of proline.

The Asp-Pro bond is not abundant in proteins or naturally occurring oligopeptides. There is thus a low probability of multiple occurrence of this residue pair in a given peptide which makes it an attractive system for studies of chemical degradation by hydrolysis. Mainly for practical reasons, related to attempts to reduce the possibility of interfering side reactions, the following three substrate peptides were chosen for investigation:

1. Asp-Pro dipeptide.
2. Physalaemin, an undecapeptide with the sequence Pyr-Ala-*Asp-Pro*-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2, where Pyr is pyroglutamic acid (a tertiary amine).
3. Hamburger peptide, with the sequence Asp-Ser-*Asp-Pro*-Arg.

In parallel with the studies of the Asp-Pro stability, the hydrolysis of 2-(4-nitrophenoxy) tetrahydropyran (npTHP) was also investigated. At high pH values, the mechanism is a unimolecular dissociation with a large charge separation in the transition state (see Figure 1). The reasons for choosing this reaction were:

1. The kinetics of this reaction, under a variety of conditions, have been intensively studied (14).
2. One of the products, the 4-nitrophenoxy anion, has a strong absorbance at 400 nm at pH 11, which provides a reliable assay.
3. The reaction is easily followed in the glassy state, because samples for analysis only require rehydration.
4. Although the attack of water to form the products might become rate-determining in the vitreous state, due to the limited availability of water, this will not influence the release of the 4-nitrophenoxy anion.

In order to gain significant information and establish any causal relationships between the kinetics and mechanisms of the hydrolytic reactions and the thermomechanical properties of the amorphous reaction matrix (solid solvent), several experimental conditions had to be met:

1. Adequate assay methods for the reactants and/or reaction products and for residual water.
2. A reliable, "best practice" lyophilisation technique (15).
3. A physical method for the determination of phase and glass transition temperatures and for monitoring any physical changes that might take place, in real time, within the dried matrix.

Details of assays and the lyophilisation technique are described below. For studies of the thermomechanical and phase behaviour, differential scanning calorimetry (DSC) was chosen, because the carbohydrate-based experimental systems used in this study are characterised by large changes in their heat capacities in the glass transition temperature range (16). Note that this is not the case for native proteins which also undergo a glass transition but without an easily measurable specific heat change (17).

MATERIALS AND METHODS

Reactants: Physalaemin was purchased from Novabiochem and the Hamburger peptide from Sigma. npTHP (m.pt. 59-61°C) was synthesised by literature procedures (18).

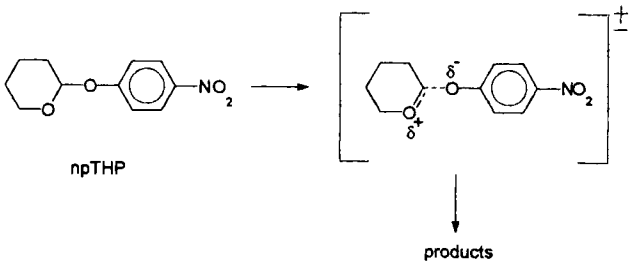


Fig. 1. The hydrolysis of 2-(4-nitrophenoxy) tetrahydropyran (npTHP) at high pH values.

Excipients: Dextran (MW 74,200) and Ficoll (a cross-linked sucrose ethylene chlorohydrin copolymer) (MW 70,000 and MW 400,000) were obtained from Sigma. Sucrose was purchased from Rhone-Poulenc (Prolabo) and Byco type A (a non-gelling gelatine hydrolysate, MW 2,500–4,000) from Croda Colloids.

Other materials were from reputable suppliers and of analytical reagent grade.

Glass temperatures were established by Differential Scanning calorimetry (DSC). All samples were sealed in stainless steel calorimeter pans and loaded into a Perkin-Elmer model DSC-2 equilibrated at 27°C. For measurements of the glass transition temperatures in the freeze-concentrated solutions (T_g'), samples were typically cooled at 5°C/min to −65°C and reheated immediately (again at 5°C/min) to 30°C. Dried samples were cooled at 10°C/min to −25°C/−30°C before heating at 10°C/min. All glass transition temperatures were measured as the mid-point of the transition. Residual water analysis was by coulometric Karl Fischer titration (using a Mitsubishi CA-05 Moisture Meter) with Hydranal AG anolyte and Hydranal CG catholyte solutions. Samples for DSC and residual water analysis were transferred and sealed under dry nitrogen. Spectrophotometric measurements were made with a Perkin Elmer model 557 instrument fitted with a temperature controlled cell compartment.

Freeze-drying was performed in a SB Cold Trap 1000 model drier. Primary drying was performed below T_g' and secondary drying controlled, to ensure that all products were maintained in a glassy state throughout (19). Excipients (10% w/w) were dissolved in distilled water and the pH adjusted either to pH 11 with 1M NaOH (for acetal samples) or to pH 2.5-3.0 with citric acid (1% final w/w) for peptides. All samples (100 μl, 5 mm fill depth) were freeze-dried in disposable 1.5 ml polystyrene spectrophotometer cuvettes. Peptide samples were calibrated to yield either 75 nmol of proline (Physalaemin) or 100 nmol proline (Hamburger peptide) after hydrolysis. npTHP was prepared as a stock solution (ca. 10^{-4} M) in acetone and 60-120μl injected into 2 ml excipient solution. All freeze-dried samples were sealed under dry nitrogen before storage.

Peptide samples were rehydrated with 50 ml water and reacted with ninhydrin according to the method of Troll and Cannan (20), following a slightly changed procedure. In a calibration, reproducible linear relationships between optical density and concentration were found for both Asp and Pro at both 570 and 440 nm. The extent of peptide cleavage was obtained by determining the ratio of

$$\frac{OD_{440,t} - OD_{440,i}}{OD_{440,max} - OD_{440,i}} \times 100\%$$

Here, $OD_{440,max}$ was obtained by taking the calibration value at 100% conversion. $OD_{440,i}$ was the initial optical density obtained from the calibration value at the concentration of peptide which was freeze-dried. $OD_{440,t}$ is the value for the rehydrated sample after a storage time t.

Acetal samples were rehydrated with 1 ml distilled water and the optical density at 400 nm recorded. The reaction was allowed to go to completion and OD_{400} again measured. These values of $OD_{400,max}$ showed only small variations, ascribed to small changes in pH in the rehydrated solutions. Generally, rate constants were reproducible to within 5%.

RESULTS

Asp-Pro Peptide Cleavage

The peptides were freeze-dried in solutions containing the cross-linked sucrose polymer Ficoll. Owing to its hydrophilicity, Ficoll is completely miscible with water. It generally offers good stabilisation to the lyophilised products and it does not crystallise on drying from aqueous solution. In addition, this polymer is available in different molecular weights, allowing investigation of the effects of polymer size.

In addition to the study of Physalaemin and Hamburger peptide, an attempt was made to investigate the kinetics of peptide bond cleavage of the dipeptide Asp-Pro, which was synthesised in the laboratory. Though we have observed reactivity in the vitreous state, we believe that uncertainty about the purity of Asp-Pro and, therefore, the reliability of the data obtained does not allow us to report reliable results.

The Asp-Pro bond in Physalaemin was found to be more labile than the corresponding peptide bond in the Hamburger peptide. This is demonstrated both by the extent of reaction occurring during freeze-drying, presumably during freeze concentration, and the time course of hydrolysis at various temperatures in the freeze-dried glass (see Figure 2). As the lyophilised Physalaemin sample contains 2.2% residual water compared with 4.0% residual water for the Hamburger peptide glass, water concentration is not the cause of the enhanced lability.

Although the hydrolysis data for the Hamburger peptide can be fitted to a first order rate process, in the case of Physalaemin a simple first-order kinetic model does not appear to apply (see Figure 3).

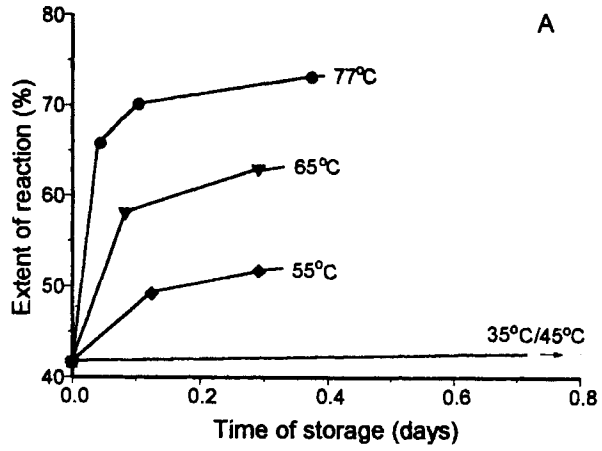
From the observed pseudo first-order kinetics, rate constants were determined and used to construct Arrhenius plots (see Figure 4A). The data have also been plotted versus $T\text{-}T_g$ (so-called WLF-plot, not shown). The WLF-equation is frequently applied to processes which show accelerated kinetics in the vicinity of the glass transition temperature (21). However, no anomalous temperature dependence of the hydrolysis rate was observed.

The Arrhenius plots suggest activation energies of 116 (±19) and 86 (±12) kJ/mol for Physalaemin and Hamburger peptide, respectively.

Results for npTHP Aqueous Solutions

The spontaneous hydrolysis of npTHP in dilute solution has a rate constant of $3.2 \times 10^{-4} s^{-1}$ at pH 11 and 40°C and is retarded in 10% w/w sugar solutions by a factor of approximately 2.5 (14). We investigated the reaction both in freeze-concentrated and freeze-dried glasses for a variety of 10% w/w excipient solutions.

The temperature dependence of the C-O bond cleavage rate was measured in two Ficoll glasses of high molecular weight (400,000), (T_g = 30°C/residual water = 4.3% w/w and T_g = 45°C/residual water = 3.7% w/w), stored at temperatures ranging from 6 to 75°C, for periods up to 80 days. Figure 5 shows the results after storage of the higher T_g glass, as well as combined data for the temperature variation of the first order rate constant. Rate constants are summarised in Table I, together with those determined from storage of a low molecular weight (70,000) Ficoll glass (T_g = 30°C, residual water 8.7% w/w). Both high molecular weight Ficoll glasses show a similar rate



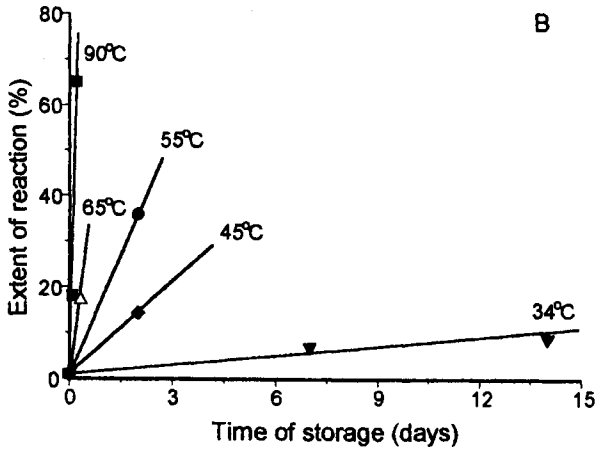
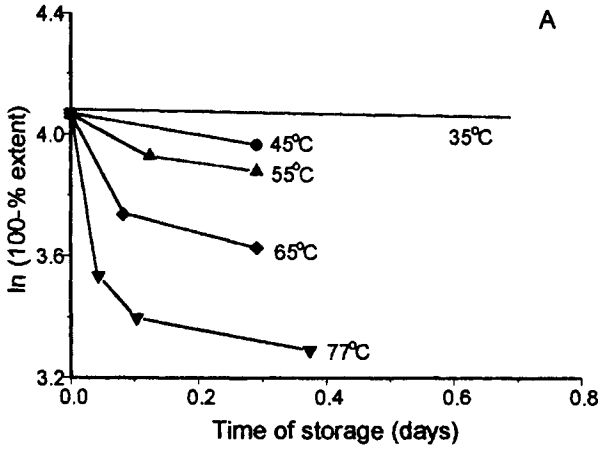


Fig. 2. Extent of release of Pro as a function of storage time for (A) Physalaemin (Ficoll, 400K, T_g = 49°C, water content 2.2%) and (B) Hamburger peptide (Ficoll, 400K, T_g = 46°C, water content 4.0%).



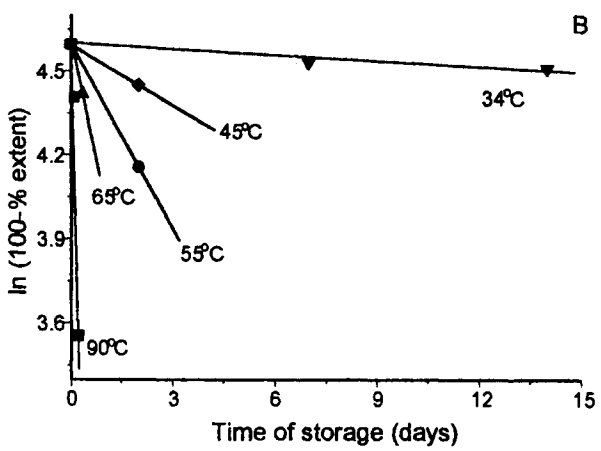


Fig. 3. Kinetic treatment of data from Figure 2, assuming first-order kinetics for (A) Physalaemin and (B) Hamburger peptide.

constant at 6°C i.e. at least 24 degrees below T_g. The ten-fold rate increase on softening of the glass with a lower T_g can be seen by comparing the 34°C data points. Both formulations show a similar rate constant, $1.6 \times 10^{-3}h^{-1}$ (ln k = −6.44), at (T_g+10)°C.

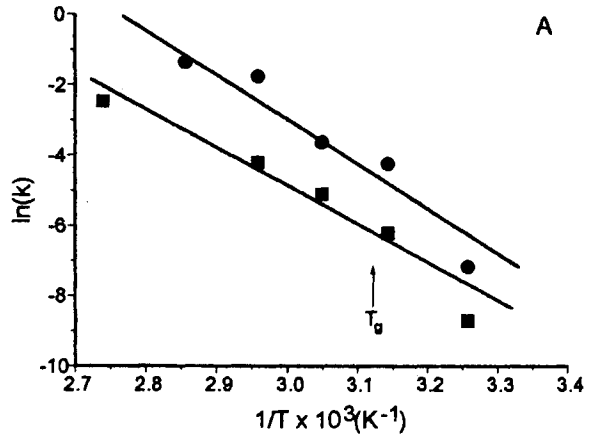
Comparing the 34°C data for the high and low molecular weight Ficoll formulations, the reaction appears to proceed approximately eight times faster in the high molecular weight glass. This may reflect the anticipated higher porosity of this stabiliser, which may facilitate diffusion of the products and thus prevent the reverse reaction from occurring. If the water content was a significant rate determining factor, then the observed rate in the low molecular weight Ficoll glass, (T_g = 30°C, residual water content 8.7% w/w) would be expected to be higher than in the high molecular weight Ficoll equivalent (T_g = 30°C, residual water content 4.3% w/w). This was not found.

We further investigated differences in stabilisation for a series of glasses containing npTHP in sucrose, Byco and dextran matrices. The data are summarised in Table II. The low molecular weight Ficoll is the same as that shown in Table I. Figure 6 shows the temperature dependence of the data with T_g as the reference temperature.

Although there are exceptions (e.g. compare Byco A with Ficoll HMW or LMW), there is a tendency towards lower molecular weight excipients exhibiting greater inhibition of reaction. Dextran, which has a high T_g, is particularly poor, as can be seen for the rate constant at 6°C i.e. (T_g-79)°C, which is about 2 orders of magnitude higher than for sucrose at the same temperature (but only T_g-10°C). Despite the variation in rate between differing excipients at a given temperature, there is an overall similar temperature dependence of the rate constant for all excipients tested.

It is extremely difficult to produce glasses with control over both the glass temperature and the residual water content. Although we have chosen a reaction in which the appearance of products should be independent of the residual water content, the validity of the assumption has not been verified. If the reaction products remain in close contact, then the reverse reaction may be expected to occur. Thus, the overall rate of formation of the 4-nitrophenoxy anion may be controlled either by the diffusion of products away from each other or by the diffusion of water, quenching the oxonium cation.

Although it is difficult to estimate the water content of freeze-concentrated glasses (22), and estimates vary widely, an



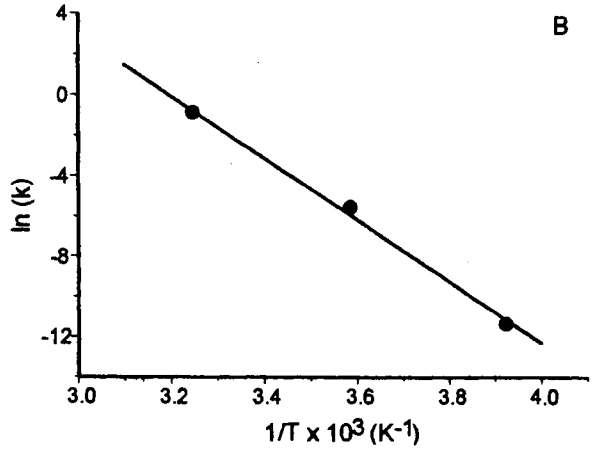
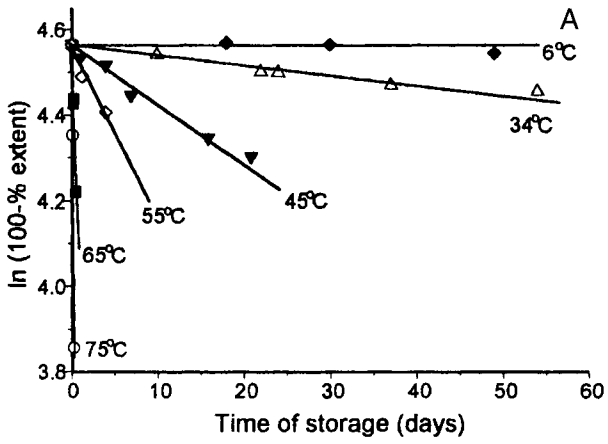


Fig. 4. Arrhenius plots for (A) the hydrolysis of Asp-Pro in Physalaemin (●) and Hamburger (■) peptide and for (B) the hydrolysis of npTHP in 10% (w/w) aqueous solutions of sucrose and Ficoll HMW (data points, taken from Table III, overlap within the experimental error).



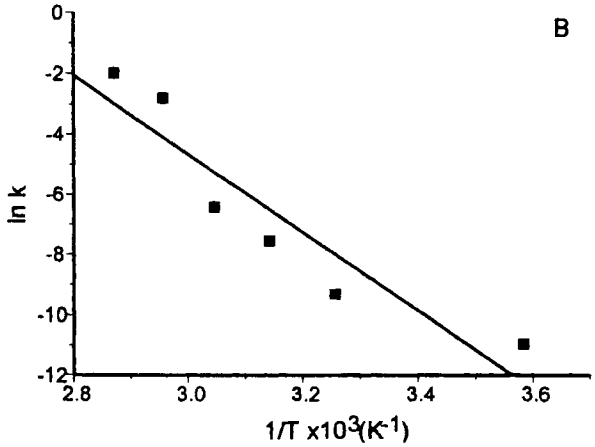


Fig. 5. (A) Kinetic treatment of spectrophotometric data for the hydrolysis of npTHP in Ficoll (MW 400,000), T_g = 45°C, water content 4.3% and (B) the Arrhenius plot.

aqueous solution being freeze-concentrated will lead to a glass containing a significantly higher water content (typically 20–50% w/w) than any other dehydration process. We have studied the decomposition of npTHP in Ficoll and sucrose solutions and in the corresponding freeze-concentrates.

A comparison of the data in Table III for the dilute aqueous Ficoll solution with those for the freeze-dried Ficoll solution shows that, in a glass with T_g = 45°C, the decomposition is slowed by only two orders of magnitude at 6°C and 35°C, compared to a dilute solution. The glass temperatures of the freeze concentrates (T_g') were found to be −21°C (Ficoll) and −34°C (sucrose). In dilute aqueous solution the activation energy at ambient temperature is 98 kJ/mol (14). In 10% Ficoll (400,000 MW) and 10% sucrose our estimates are 127 (±9) kJ/mol for both solutions (see Figure 4B), which is only slightly higher than the literature value for the activation energy in dilute aqueous solution (14). Although some reaction occurred during freeze-concentration, no further reaction was detected at −42°C in either solution stored over 116 days. At −42°C, $(T-T_g)$ = −21°C (sucrose) and −8°C (Ficoll).

Table I. Ln(k) Values for the Hydrolysis of npTHP in Ficoll Glass Formulations Stored at Different Temperatures; k Measured in h^{-1}

Storage temp. (°C)	High MW Ficoll T_g 30°C	High MW Ficoll T_g 45°C	Low MW Ficoll T_g 30°C
6	−11.51	−10.98	−10.56
25	−10.48		−9.90
34	−6.98	−9.33	−9.12
40	−6.44		
45	−6.07	−7.56	
55		−6.44	
65		−2.81	
75		−1.97	

Note: For another high molecular weight Ficoll glass with Tg = 46°C, which is comparable to the glass shown in the second column, but with substantially higher water content, ln(k) = −9.64 at 25°C. This is very similar to the value in the table at that temperature. This shows that the water content of the glass does not govern the reaction kinetics.

Table II. Ln(k) for the Hydrolysis of npTHP in Formulations of Excipients of Different Structure and Molecular Weight; k Measured in h^{-1}

	Sucrose	Byco A	Ficoll	Dextran	Ficoll
MW	342	2.5–4K	70K	74K	400K
T_g(°C)	16.5	34	30	85	45
water content (% w/w)	—	6.8	8.7	8.7	4.3
$T_{storage}$(°C)					
6	−13.92	−9.76	−10.56	−8.95	−10.98
25	−11.18	−9.12	−9.90	—	—
34	−10.98	−8.57	−9.12	−8.18	−9.33
45	—	—	—	—	−7.56
55	—	—	—	−5.99	−6.44
65	—	—	—	−3.65	−2.81
75	—	—	—	−2.65	−1.97
90	—	—	—	−2.41	—
95	—	—	—	−1.90	—

Note: Entries marked by "—" were not determined.

Table III. Ln(k) Values for the Hydrolysis of npTHP in Dilute Aqueous Carbohydrate Solutions; k Measured in h^{-1}

Temperature (°C)	10% w/w Ficoll	10% w/w sucrose
−18	−11.33	−11.33
+6	−5.52	−5.55
+35	−1.01	−0.86

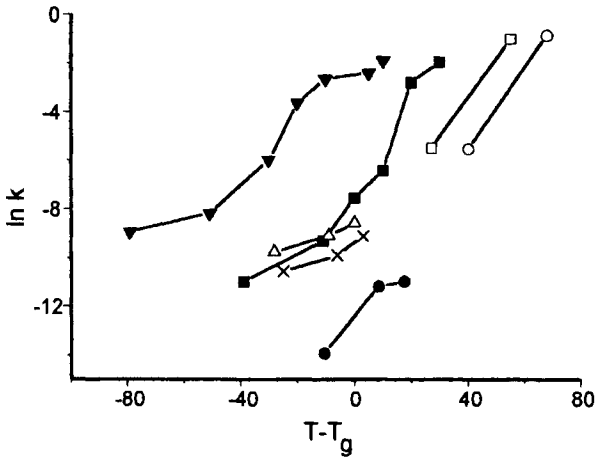


Fig. 6. Temperature dependence of the hydrolysis rate of npTHP with T_g as the reference temperature in freeze-dried 10% (w/w) aqueous formulations of Dextran (▼), Ficoll, HMW, 400,000 MW (■), Ficoll, LMW, 70,000 MW (×), Byco A (△) and sucrose (●) and in freeze-concentrated formulations of Ficoll, HMW (□) and sucrose (○) (with T_g' as reference temperature).

DISCUSSION

The results provide one of the first examples of chemical reactivity studies in water soluble glasses. We studied two different types of reactions in which a covalent bond (C-N peptide bond and C-O acetal bond) is cleaved. Although the reactions are actually hydrolysis processes, our assays measured the release of secondary amine in the first, and *p*-nitrophenoxy anion in the latter case. The effect of the limited availability of water in the highly concentrated aqueous solutions on the overall hydrolysis reaction rate can, therefore, not be established with the experimental methods used.

The two short peptides studied do not have any preferred secondary structures, so that interference of effects due to conformational (in)stability need not be considered. The results obtained for the peptides show that the Asp-Pro peptide bond cleavage is severely retarded in freeze-dried Ficoll glasses. The kinetics suggest that the cleavage does not follow first-order kinetics. Since the bond cleavage is acid catalysed and requires protonation of the carbonyl oxygen prior to intramolecular involvement of the aspartyl side chain carboxyl group, it is likely that the reaction rate depends on the availability of protons in the matrix. The protons can be provided either by water, but more likely, because of their presence in excess, by carbohydrate polymer hydroxyl groups. Alternatively, the rate may be limited by the reduced neighbouring group participation of the aspartyl side chain carboxylate group, due to diminished flexibility of this group.

It is impossible to deduce subtle changes in the mechanism, but peptide bond cleavage does take place at a rate which is significantly lower than in dilute aqueous solution. The glassy Ficoll matrix seems to offer higher stability to the smaller peptide. On the basis of the available kinetic data, the temperature dependence of the cleavage rates follow the Arrhenius equation, with activation energies which are approximately five times higher than in dilute aqueous solution (here we made a comparison to the activation energy of Asp-Gly in a hexapeptide (23)). Since the activation energies of the two peptides are the same within experimental error, it is likely that the difference in reactivity finds an explanation in terms of differences in translational and rotational degrees of freedom (entropy) of the reactant. In view of the results of the acetal cleavage, discussed below, we believe that this can be explained in terms of the fit of the reactant in the vitreous polymer matrix. Possibly, the observed rates are in line with slow relaxation enthalpy processes in the glass, as has recently been suggested for the chemical stability of human growth hormone in sugar glasses, after storage below T_g (8).

That water is not of major importance in the rate equation of the hydrolysis reaction was shown clearly by two experiments in which npTHP was hydrolysed. Results on two glasses, with differing residual water contents but similar T_g values, show that the glass with the higher residual water content actually provides better stability. The cleavage follows first-order kinetics in the vitreous state and is, as in the peptide cleavage, retarded considerably compared to the rate observed with dilute aqueous solutions. The various excipients, in which the acetal was dried, provide different stabilities towards cleavage. When rates are plotted versus $(T-T_g)$ (i.e. WLF plot, Figure 6), dextran provides low stability compared to the other excipients. Sucrose still provides the best stabilising matrix. If, however, ln k values are plotted versus 1/T (Arrhenius plot), it appears that rates are comparable in all glasses except for sucrose, which is still the best stabiliser. Activation energies are all similar for the different excipients and comparable with the activation energy in dilute aqueous solution.

The matrix density is probably an important factor in determining the stability of the embedded product, together

with the size of the excipient molecule in comparison to the size of the reactant. Dextran is a fairly rigid, linear polymer; packing in a matrix is not very efficient, resulting in void space, which provides the reactant with a relatively high rotational freedom. Ficoll is a cross-linked polymer and Byco is a shorter polymer, both are factors for more efficient packing in concentrated solutions. Sucrose is a disaccharide with approximately the same size as the acetal. It is not difficult to imagine a very efficient packing leaving the acetal with little mobility.

Figure 6 also suggests that for storage far below T_g (in the case of dextran glasses), the reaction rate levels off. It would support the supposition that the temperature at which no further reduction in rate is observed equals the temperature of zero configurational entropy, T_0, *i.e.* the point where chemical reactivity really is arrested for all practical purposes.

CONCLUSIONS

Though rates of bond cleavage reactions occur at very reduced rates in carbohydrate-based glasses below their T_g values, it is simplistic to assume that reactivity is completely arrested. It is conceivable that at T_0 (the "zero configurational entropy-point"), which usually lies substantially below T_g, reactant molecules lose all degrees of freedom of mobility necessary for participation in any type of chemical reaction.

Reaction rates vary non-linearly with the viscosities of the carbohydrate solutions, which rise by many orders of magnitude upon transition to a glassy state much more so than the reaction rates of the incorporated compounds decrease, suggesting that the motions of the matrix molecules and the reactants are not coupled.

Not all types of carbohydrate-based glasses provide similar degrees of stabilisation to the reactants. It appears that stability depends on the 'fit' of the reactant in the polymer matrix; low-density glasses provide high mobility and therefore low stability to small reactants, whereas denser glasses maintain small reactants in a lower free volume, substantially reducing the molecular mobility, and hence the rates of chemical reactions.

The mechanisms of chemical reactions are prone to changes in the carbohydrate-based glasses, especially reactions which are catalysed by acids or bases, since there is a reduced availability of water and therefore of protons. It is not unlikely that carbohydrate hydroxyl groups are involved in proton transfer processes. This interesting aspect remains to be investigated in detail.

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