Chapter 14

Chemistry of Protein Stabilization by Trehalose

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Carbohydrates are widely used to stabilise dried protein and peptide drugs. Several sugars can prevent damage to proteins during drying, but the products often have a short shelf life at ambient temperatures. There is increasing evidence to suggest that this is due to a progressive chemical reaction between the reducing carbonyl groups of the sugars and the amino groups of proteins, the so-called Maillard reaction. The rational choice of an excipient for pharmaceutical use must thus consider not only the avoidance of desiccation damage but also subsequent degradative chemistries on storage. We present data from comparative studies on the long term stability of a variety of proteins, including enzymes and therapeutic peptides, dried using various carbohydrate excipients, which establish the superiority of the simple disaccharide trehalose as a stabilising excipient. These observations illustrate the chemical stability of trehalose and document the protein degradation observed in samples dried using other carbohydrates.

Stability at room temperature is the ultimate goal for pharmaceutical formulations. The dual role of water, as a nucleophile in hydrolysis reactions and as a plasticiser which increases the molecular mobility of reactive chemical species, makes aqueous protein formulations inherently less stable than their dry counterparts. This increased stability of dry protein formulations has focused attention on techniques of drying and led to the development of freeze-drying as a popular method of water removal (1-4). However, despite its widespread use, many freeze-dried products are still unstable at ambient temperatures (4-6). Detailed theoretical analyses of the physico-chemical events during freeze-drying have led to a substantial literature on the use of cryoprotectants as stabilising excipients (2, 5-7). Various carbohydrates have been advocated as stabilising excipients in freeze-drying, and these are proposed to act via the generation of an amorphous, glassy solid state in the freezing step (2-4, 7). Nevertheless, the freezing step remains a major variable, as evidenced by the equivocal values for the experimentally measured glass transition temperature of the maximally freeze-concentrated unfrozen matrix (T'g) for various carbohydrate excipients (3, 7-9), and is suggested to be the major cause of protein damage during freeze-drying (4, 6, 10). Recent attention has thus focused on the techniques of ambient temperature drying, as

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these not only eliminate the freezing step but are more rapid and energy-efficient in the removal of water during drying (6, 11-17).

In nature, there exist organisms that have evolved the remarkable ability to dry out at ambient temperatures, remain metabolically dormant for long periods under harsh environmental conditions and yet regain full metabolic activity when rehydrated (17, 18). These cryptobiotic or anhydrobiotic organisms are found in many phyla in both the plant and animal kingdoms and the better known examples are the brine shrimp *Artemia salina,* the soil nematode *Ditylenchus dipsaci*, the tardigrade *Adoribiotus coronifer,* the resurrection plant *Selaginella lepidophylla* and baker's yeast *Saccharomyces cerevisiae.* Studies on cryptobiosis in the bakers yeast, *S.Cerevisiae,* have shown that the synthesis of a simple disaccharide, trehalose (α-D glucopyranosyl α-D glucopyranoside), is both necessary and sufficient to account for the protection of all the biomolecules in the organism during drying (20, 21). Recent work has shown that this protection can be replicated *in vitro* by drying biological molecules in the presence of trehalose at ambient temperatures (6, 11-15, 24). Concordant with the fact that the dehydration of cryptobionts occurs at ambient or higher temperatures, this reproducible stabilisation of biomolecules dried at ambient temperatures is in sharp contrast with previously reported work using trehalose as an excipient during freeze-drying (3-7, 11-15, 22-24).

A feature of biomolecules stabilised using trehalose technology is their ability to be stored for extended periods at high temperatures without any apparent damage, as assessed by the full recovery of biological activity on rehydration (11-14). Studies of other sugars, polyhydric alcohols and oligosaccharides under identical conditions showed that this degree of stabilisation is unique to trehalose, even though some of these excipients do protect the biomolecules from damage during the drying process itself and confer more limited tolerance to high temperatures (6, 11, 14, 22-24). Some of these data are presented below, together with the results of studies on the mechanism of action of the disaccharide which suggest that the chemical inertness of trehalose may be important. These data highlight a previously ignored feature of the use of carbohydrate excipients in protein formulations, namely the spontaneous chemical reaction between stabilising agent and protein product. This feature is well recognised in the food industry, under the umbrella of the non-enzymatic browning or Maillard reaction, which is one of several causes of spoilage of many food products during storage (25-27). In these foodstuffs containing high amounts of sugar and protein the Maillard reaction is even observed during refrigerated storage at low temperatures (25-27) and is a particular problem during the processing of these food products by drying (28).

Trehalose stabilisation of biomolecules

Our early work has shown that antibodies, air-dried in the presence of trehalose, are undamaged, and full biological activity is recovered on rehydration, even after several years storage at room temperature or 37°C (11, 13, 14). Similar results were obtained with a variety of enzymes, hormones and blood coagulation factors, suggesting that this process may be generally applicable to biological molecules (11-15). As a stringent test of this technology to preserve labile biological molecules, the enzymes used in molecular biology, which are notoriously fragile and thus usually transported and stored at or below -20°C, were studied in detail (11, 14, 15). We have shown that both restriction endonucleases and DNA modifying enzymes can be dried from trehalose solutions at ambient temperatures without loss of activity (11, 14, 15). Furthermore, these dried enzymes show stability on storage for extended periods even at elevated temperatures (11, 15, Fig.1, Table 1).

Illustrated below (Fig.1) are the results obtained in an accelerated ageing study with the restriction enzyme *Pst*I vacuum-dried with supplemental heating to

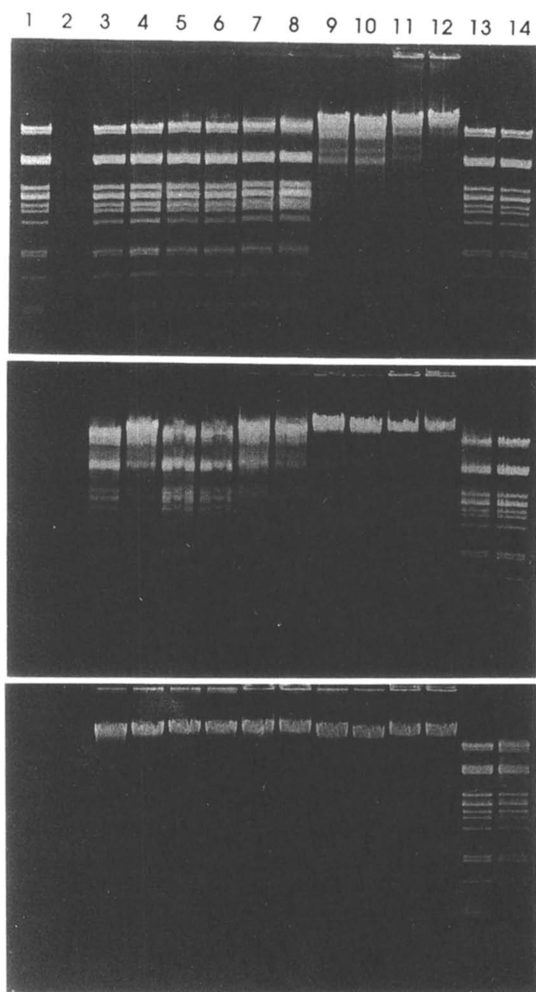

Fig.1. Accelerated ageing study on the restriction enzyme *Pst* I. Five units of fresh enzyme control (track 1) was compared, for the ability to cut bacteriophage λ DNA, with 2.5unit (even numbered tracks) or 5unit (odd numbered tracks) of enzyme dried using various carbohydrate excipients and stored for 35 days at 37°C (top panel), 55°C (middle panel) and 70°C (bottom panel). The carbohydrate excipients used were Glucopyranosyl-mannitol (tracks 3 & 4) or sorbitol (tracks 5 & 6), reduced isomaltose (tracks 7 & 8), sucrose (tracks 9 & 10), maltose (tracks 11 & 12) and trehalose (tracks 13 & 14). As can be seen only trehalose shows any stabilising effects at the two higher temperatures studied.

Table 1. Stability of PstI dried in various carbohydrate excipients

CARBOHYDRATE	CHEMICAL NAME	RED. SUGAR	TEMP °C	TIME days	ACTIVITY
MONOSACCHARIDES AND ALCOHOLS					
Glucose	α-D-glucopyranose	+	37°	1	+
			"	14	-
Sorbitol	sugar alcohol of glucose	-	"	14	+
			"	35	+
			"	70	-
Galactose	α-D-galactopyranose	+	"	1	-
Galactitol	sugar alcohol of galactose	-	"	1	-
Mannose	α-D-mannopyranose	+	"	1	-
Mannitol	sugar alcohol of mannose	-	"	1	-
DISACCHARIDES					
Trehalose	α-D-glucopyranosyl-α-D-glucopyranoside	-	"	98	+++
			55°	70	+++
			70°	35	+++
Maltose	4-O-α-D-gluco-pyranosyl-D-glucose	+	"	14	++
			"	7	-
Maltotriose	O-α-D-glucopyranosyl(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose	+	"	14	-
Lactose	4-O-ß-D-galacto-pyranosyl-D-glucopyranose	+	"	14	-
Lactulose	4-O-ß-D-galactopyranosyl-D-fructose	+	"	14	+
				35	-
Sucrose	ß-D-fructofuranosyl-α-D-glucopyranoside	-	37°	14	++
			"	35	-
POLYMERS					
Inulin	Polymer of 1-O-ß-D-fructofuranosyl-D-fructose	-	"	7	-
Dextran	Polymer of α-(1→6) -D-Glucopyranose (1→3,1→4 branch)	+	"	7	-
Ficoll	Polymer of ß-D-fructo-furanosyl- α-D-glucopyranose	-	"	7	+

<u>Quantitation of activity</u>
- \- no detectable activity
- \+ some activity (10-20% of titre)
- ++ partial activity (25-40% of titre)
- +++ full activity

<u>Reducing properties</u>
- \+ reducing sugar
- \- non-reducing sugar

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a residual moisture content of 2.6-3.6% in a number of carbohydrate excipients and stored for one month at either 37°C, 55°C or 70°C. Although some of the carbohydrate excipients stabilised the enzyme during drying and on storage at 37°C (Fig.1, top panel, Table 1), only the samples stabilised using trehalose retained activity when stored at either 55°C or 70°C (Fig.1, middle and bottom panels, Table 1). These results have now been correlated with real-time data and emphasise the finding that, with respect to long-term stability, trehalose is a better stabilising excipient than the other carbohydrates tested under identical drying and storage conditions (Table 1). All the monosaccharides were ineffective, whether reducing or non-reducing, as were polymers such as inulin, ficoll and dextran (Table 1). Reducing sugars, such as lactose and maltose, failed within a month at the lowest temperature studied, 37°C, as did the non-reducing disaccharide sucrose (Table 1). The chemically more stable non-reducing sugars, the sugar alcohols, showed better stabilities than their reducing counterparts, but still failed within a month at 55°C (Table 1).

With regard to the results presented above and in our other studies, it must be emphasised that the stabilisation effects seen with trehalose in our experiments cannot be ascribed to any transition metal effects (4). Previous work has suggested that transition metal ions may in part be responsible for the enhanced stabilisation observed with trehalose (23, 38). However, this does not apply to the studies we have reported as the trehalose used in our experiments was not purified by methods that use deproteination by zinc and barium salts (29), and no trace heavy metals were detectable by atomic absorption spectroscopy. Furthermore, the confusion over the effects of zinc (4, 23) arises from the studies on the enzyme phosphofructokinase as a model for drying, where the effects of the transition metal are mediated largely in the liquid phase (24) and probably reflect the divalent metal ion requirement of this enzyme.

Mechanism of action of trehalose

There are two main hypotheses that have been postulated with respect to the molecular mechanism by which trehalose stabilises biological molecules (30, 31, 36-38). The water replacement theory states that, being a polyol, trehalose can make multiple external hydrogen bonds which could replace the essential structural water molecules that are hydrogen-bonded to biomolecules and thus maintain their molecular structure (30, 38). The glassy state theory postulates that, as the drying trehalose solutions undergo glass transformation, this results in an amorphous continuous phase in which molecular motion, and thus degradative molecular reactions, are kinetically insignificant (31, 36, 37). Our results (11, 14, 15 and below) are not consistent with either hypothesis being a sufficient sole explanation for the mechanism of action of trehalose, but suggest that the chemical inertness of the sugar may be an equally important feature in its mechanism of action.

The water replacement theory suggests that, as polyols, other sugars should also be effective as stabilising excipients, and, if the specific spatial combinations of hydroxyl groups are the crucial feature, then glucose should be as effective as trehalose. However, glucose was in fact among the least effective of the sugars tested (see Table 1), and none of the other polyols tested was found to be as effective as trehalose (see Fig.1 and Table 1). Furthermore, if molecular mimicry of water was important, as might be expected for water replacement, then scyllo-inositol (with all its hydroxyl groups being axial) should, in theory, be the most effective carbohydrate, but it is among the least effective in practice (S.Sen unpublished). The water replacement theory can thus not be a complete explanation for the mechanism of action of trehalose. Similarly, the glassy state theory alone cannot explain the stability conferred by trehalose. In the high temperature storage stability data reported in Fig.1 above, the glass transition temperatures of the samples dried in trehalose to a water content of 2.6-3.6% were

all below 37°C as measured by differential scanning calorimetry (Y.Newman *et.al.* manuscript in preparation). Thus, their stability persists at well above their glass transition temperatures, and although the glassy state may be important in other systems, it appears not to be a factor in the long-term high temperature stability of biomolecules dried in trehalose.

It appears that the relative chemical stability and non-reducing nature of trehalose (32, 33) may be significant features in its mechanism of action, especially with regard to the long term stability observed at high temperatures. This was first suggested by an interesting feature observed in the accelerated ageing trial described in Fig.1 above. The development of a brown coloration was noted in a number of the sample wells at all three temperatures after just two weeks of storage of the sample (Fig.2). Furthermore, the extent of the coloration appeared to correlate with the reduction in enzymatic activity in these samples. Increasing coloration was observed in the samples stored at higher temperatures (Fig.2), which also showed the greatest loss of activity (Fig.1). This coloration was highly reminiscent of the non-enzymatic browning seen during the processing and storage of food products. This non-enzymatic browning is the result of the spontaneous reaction between the reducing sugars and proteins that are natural constituents of these foodstuffs, and has been widely studied in food chemistry under the umbrella of the so-called Maillard reaction (Fig.3, 25-27).

The Maillard reaction is actually a cascade of chemical reactions initiated by the spontaneous condensation of reactive carbonyl and amino groups such as those commonly found in reducing sugars and proteins, respectively (Fig.3). The activation energy of the initial condensation to form a Schiff's base is only of the order of 10-15 kcal, is reversible in the presence of water, and the equilibrium is largely in favour of the reactants in aqueous environments (Fig.3, 25-27). The subsequent spontaneous Amadori or Heyns rearrangement of the Schiff's base is irreversible and triggers a complex series of reactions that ultimately result in the production of brown melanoidin pigments and both fragmentation and cross-linking of the proteins involved (Fig.3, 25-27). In the food industry, the Maillard reaction has been widely studied, as it is one of several causes of spoilage, especially of dried food products, during storage. It has even been observed during refrigerated storage of foodstuffs with high protein and sugar contents (25-27). The Maillard reaction is a particular problem with dry foodstuffs, as the equilibrium of the reaction is forced towards the formation of the Schiff's base by the loss of water, and many of the subsequent reactions are accelerated at low water activities (27, 28). We have examined whether the Maillard reaction is a major problem during storage of protein formulations containing carbohydrate excipients, and some of the results are presented below.

Evidence for Maillard reactions in dried protein formulations

Our initial studies attempted to correlate changes in biological activity with the development of brown pigments, as observed in the accelerated ageing studies on restriction enzymes (Fig.2). These studies were carried out on samples of the enzyme alkaline phosphatase which were dried, under the conditions described in the experiments above, from solutions containing glucose, fructose, maltose or trehalose and stored for various periods at 55°C, before enzymatic activity was re-assayed. The results showed that with glucose and fructose, all enzymatic activity was lost within 10 days (Fig.4), with maltose and sucrose, a steady decline in activity was observed, with 15% of the enzymic activity lost within 3 weeks, compared to no detectable loss in the samples dried in trehalose (Fig.4). Perhaps surprisingly, the non-reducing sugar alcohol maltitol showed a much greater loss of activity than its reducing counterpart maltose, with 50% loss of activity within 3 weeks (Fig.4). The most rapid loss of activity was, however, seen with the samples

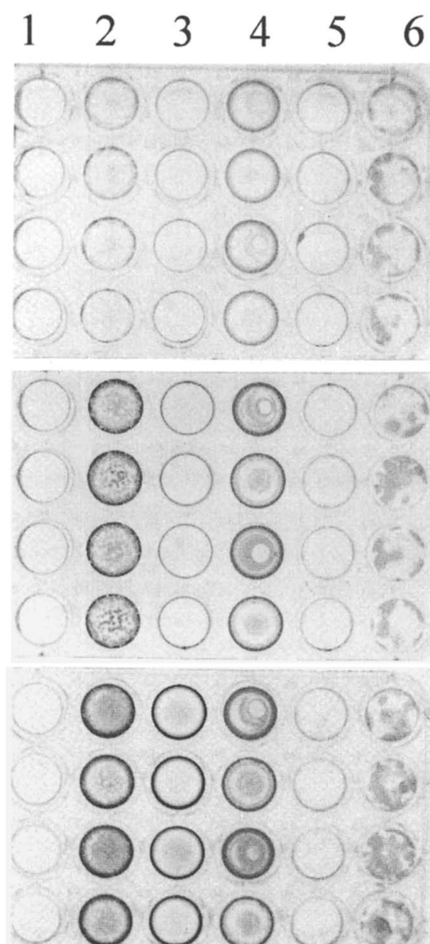

Fig.2. Non-enzymatic browning observed in the samples used in the accelerated ageing study reported in Fig.1 after two weeks storage at 37°C (top panel), 55°C (middle panel) and 70°C (bottom panel). The carbohydrate excipients used were trehalose (row 1), sucrose (row 2), maltose (row 3), reduced isomaltose (row 4), glucopyranosyl-sorbitol (row 5) and glucopyranosyl-mannitol (row 6).

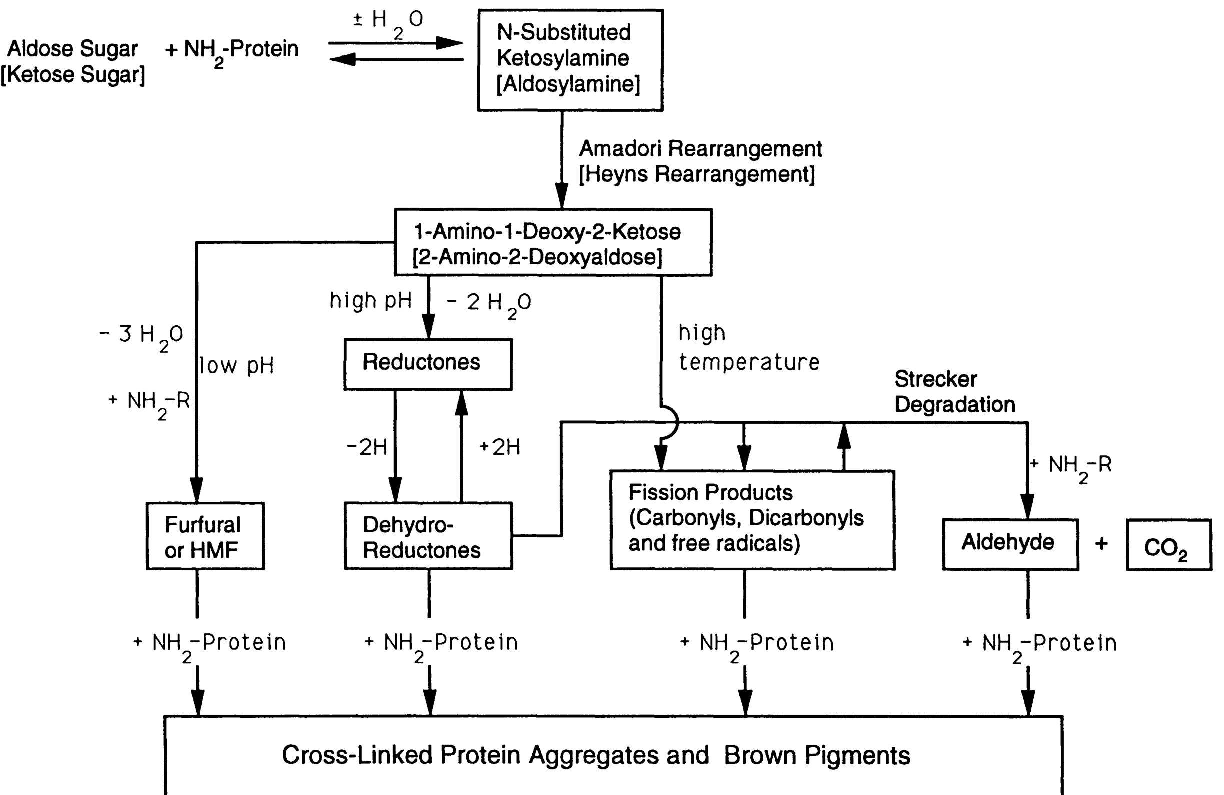

Fig.3. Schematic of the Maillard reaction. The reaction is initiated by the reversible spontaneous amino-carbonyl condensation to form a Schiff's base and followed by a cascade of subsequent reactions resulting in the cross-linking of the proteins involved and the generation of brown melanoid pigments.

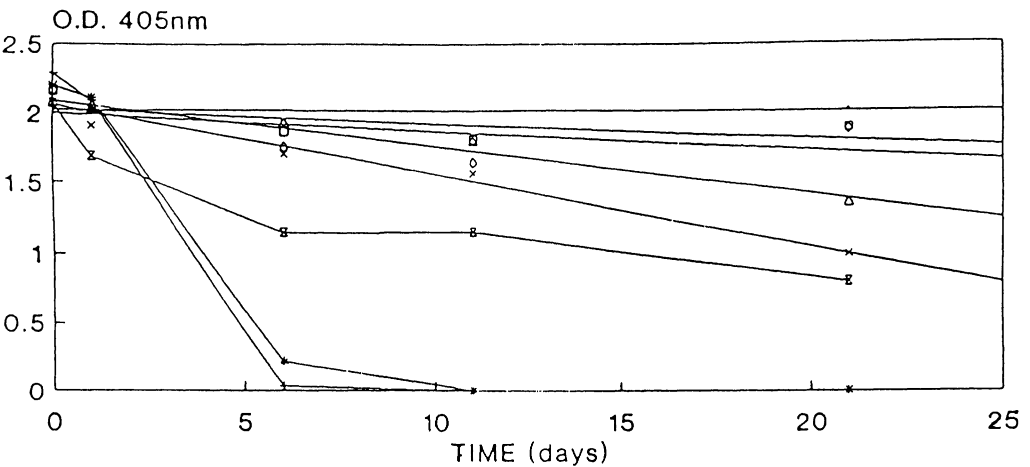

Fig.4. Residual activity in samples of alkaline phosphatase dried in the presence of various carbohydrate excipients and assayed colorimetrically after storage at 55°C. Enzymic activity (O.D. 405 nm); (·) trehalose, (□) maltose, (◊) sucrose, (×) maltitol, (+) glucose, (*) fructose, (Δ) no additive, (X) wet control.

dried in glucose and fructose (Fig.4), which also showed visual changes with the development of a brown coloration in the sample wells. This development of the brown pigments, as assayed by the increase in absorbance between 277 and 290 nm, did not correlate directly with the rate of loss of enzymatic activity. The production of melanoid pigments occurred later than the loss of enzymic activity assayed colorimetrically (Fig.5). This is consistent with the fact that the generation of brown melanoid pigments occurs in the terminal stages of the Maillard reaction and thus cannot be used to predict enzyme inactivation due to the early reactions of the cascade (Fig.3). Similarly, analysis of the samples by SDS-PAGE showed a complex pattern of protein breakdown and cross-linking in all samples, except those dried in trehalose, and the complexity of these patterns precludes the use of this technique in determining the extent of protein modification by the Maillard reaction.

A surprising result was obtained on analysis of the residual sugar contents of the samples in the studies described above (Fig.4). When dried in glucose or fructose, only the individual sugars were detectable in the samples immediately post-drying (Fig.4). However, on loss of enzymatic activity after high temperature storage, these samples were found to contain mixtures of the two sugars (Table 2). A similar isomerisation was observed in the samples found to contain a mixture of glucose and maltose immediately post-drying, presumably due to partial hydrolysis of the maltose. On loss of activity after high temperature storage, these samples were found to contain a mixture of both glucose and fructose, as well as maltose (Table 2). The absence of any mannose production detected in this non-enzymatic isomerisation is indicative of a chemical reaction pathway involving a common addition compound intermediate, similar to the formation of osazones in the Fisher reaction, by the reversible condensation of phenylhydrazine with reducing sugars (34). Such a reaction could theoretically occur, if the amino acid side chains of the proteins being dried substituted for the amino groups normally contributed by phenylhydrazine in the classical Fisher reaction, and this potential mechanism is currently being tested. It is interesting to note that a recent study of the decomposition of Amadori compounds under physiological conditions has reported the reversal of the Amadori rearrangement of the Schiff's base (39). This results in a similar isomerisation of the aldose or ketose sugar moieties initiating the Maillard reaction.

To enable a more detailed analysis of these chemical modifications of proteins by carbohydrate excipients, we studied a more relevant pharmaceutical model system. The protein modifications of a therapeutic peptide, glucagon, dried for 18 hr under a vacumn of 30 milliTorr, with a shelf-temperature rising from 25 to 42°C, were studied. Formulations containing various carbohydrate excipients were analysed by reverse-phase HPLC analysis, and the comparison of glucose and trehalose is presented in Fig.6. In the samples dried in the presence of glucose, an additional peak that might correspond to Schiff's base derivatives of the peptide was detectable, even immediately post-drying (Fig.6, a), and after just 4 days storage at 60°C, this peak already represented the major fraction of the total peptide detected (Fig.6, b). After storage for 2 weeks at 60°C, this peak represented ~80% of the total protein, and a number of additional peaks corresponding to other reaction products were detectable (Fig.6, c). In the samples dried using trehalose, however, no peaks corresponding to either the addition compounds or other reaction products were detectable, even after 3 weeks storage at 60°C (Fig.6, e). Furthermore, the generation of brown pigments was only observed in the samples dried in glucose. These results suggest that the chemical modification of proteins by carbohydrate excipients such as glucose, can adversely affect their stability during subsequent storage. The results also suggest that the difference between glucose and trehalose as carbohydrate stabilising excipients may be due mainly to their respective chemical reactivities.

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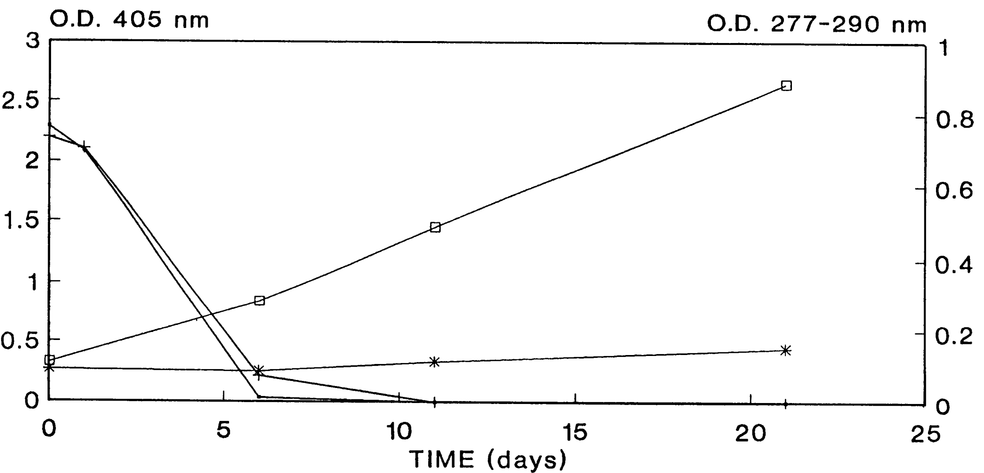
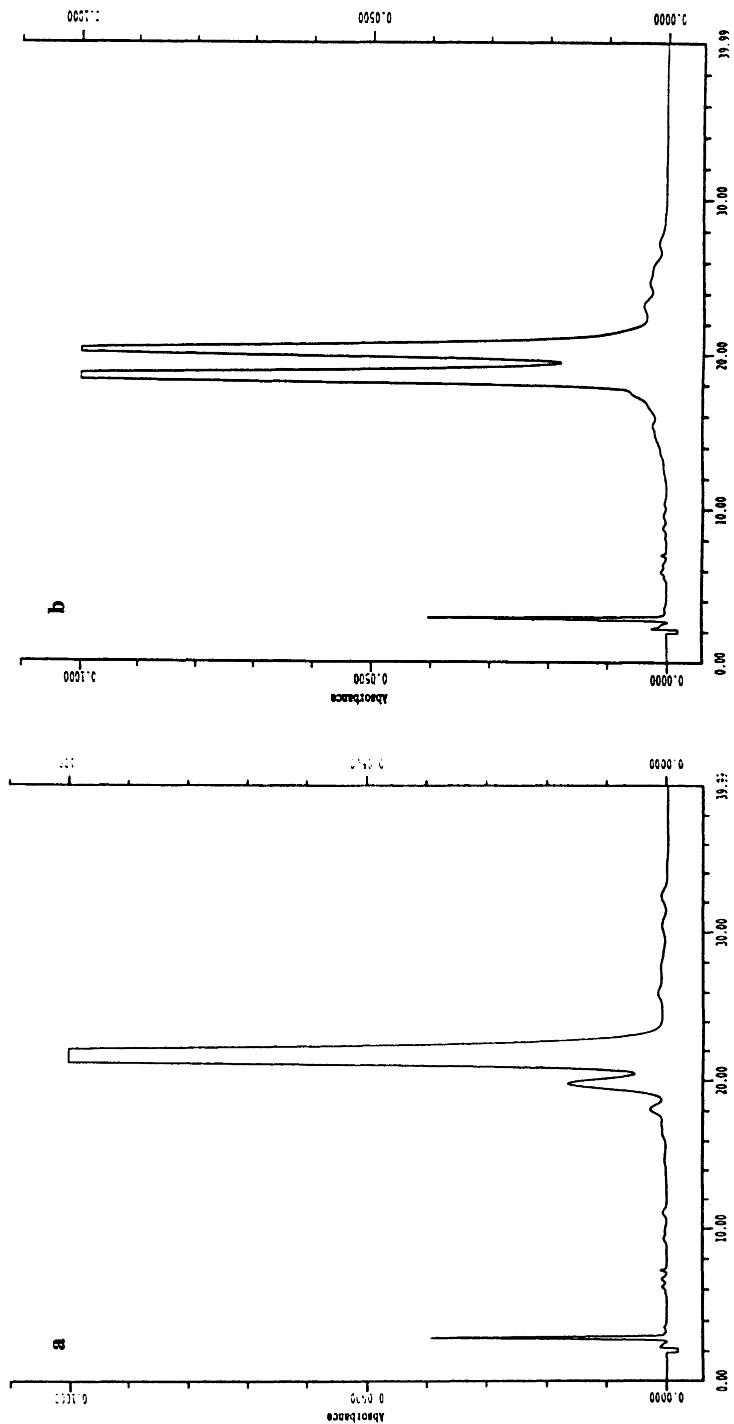


Fig.5. Development of brown coloration assayed by absorbance between 277-290nm compared with loss of enzymic activity in samples of alkaline phosphatase dried in fructose and glucose after storage at 55°C. Color development (O.D. 277-290); (·) glucose, (+) fructose. Enzymic activity (O.D. 405); (*) glucose, (□) fructose.

Table 2. HPLC analysis of carbohydrates in formulations dried in various excipients

Carbohydrate excipient used in two formulations	Post drying activity	Post drying sugar analysis	Activity after 2 weeks	Hplc analysis after 2 weeks storage
1. Glucose Sample A	+	Glucose	-	Glucose + Fructose
2. Glucose Sample B	+	Glucose	-	Glucose + Fructose
3. Sorbitol Sample A	++	Sorbitol	-	Sorbitol
4. Sorbitol Sample B	++	Sorbitol	-	Sorbitol
5. Fructose Sample A	+	Fructose	-	Fructose + Glucose
6. Frucrose Sample B	+	Fructose	-	Fructose + Glucose
7. Maltose Sample A	+++	Maltose + Glucose	-	Maltose + Glucose + Fructose
8. Maltose Sample B	+++	Maltose	+/-	Maltose + Glucose + "Others"
9. Sucrose Sample A	+++++	Sucrose	+++	Sucrose
10. Sucrose Sample B	+++++	Sucrose	++	Sucrose
11. Trehalose Sample A	+++++	Trehalose	+++++	Trehalose
12. Trehalose Sample B	+++++	Trehalose	+++++	Trehalose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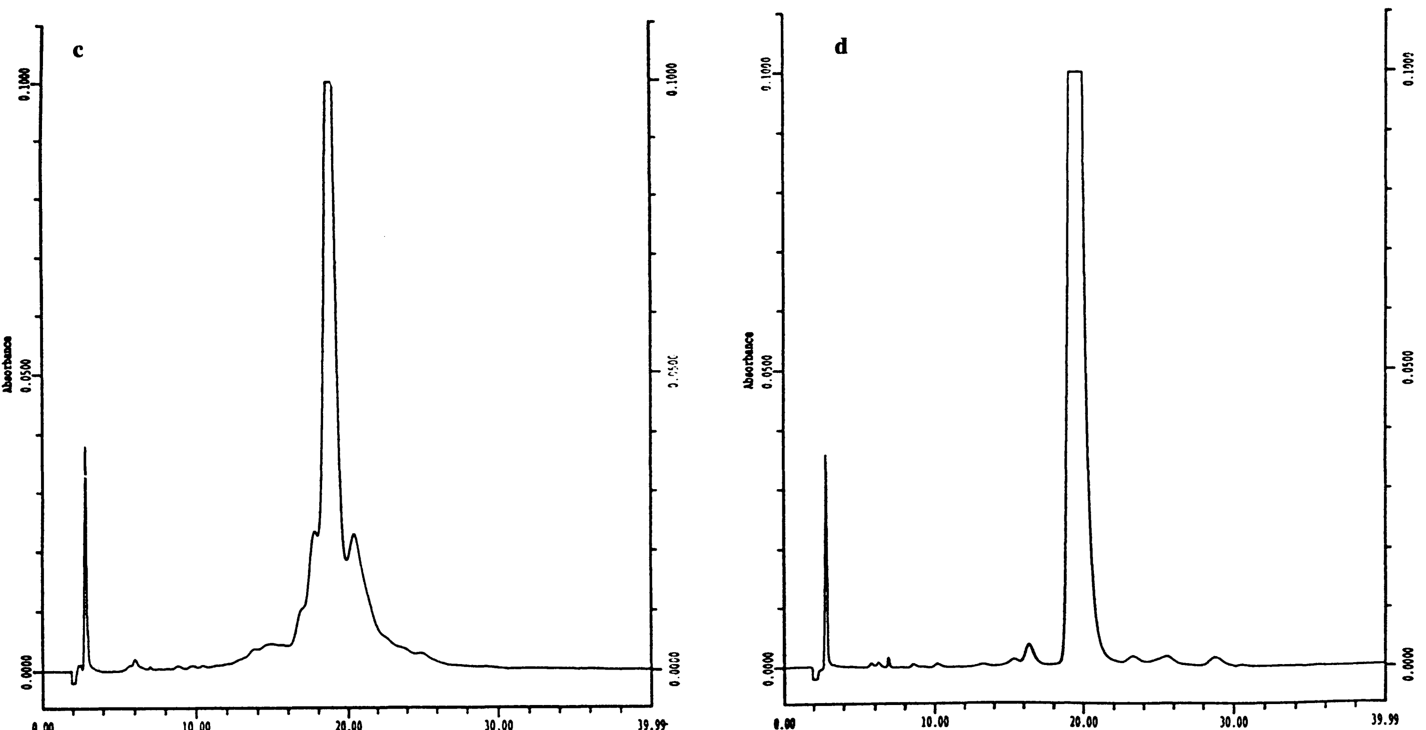

Fig.6. Reverse phase hplc analysis of the therapeutic peptide glucagon dried in the presence of glucose (a,b,c) or trehalose (e,f). The wet control formulation (d) was compared to the glucose formulations immediately post-drying (a) and after storage at 60°C for 4 days (b) and 2 weeks (c), and the trehalose formulation immediately post-drying (e) and after storage at 60°C for 3 weeks (f).

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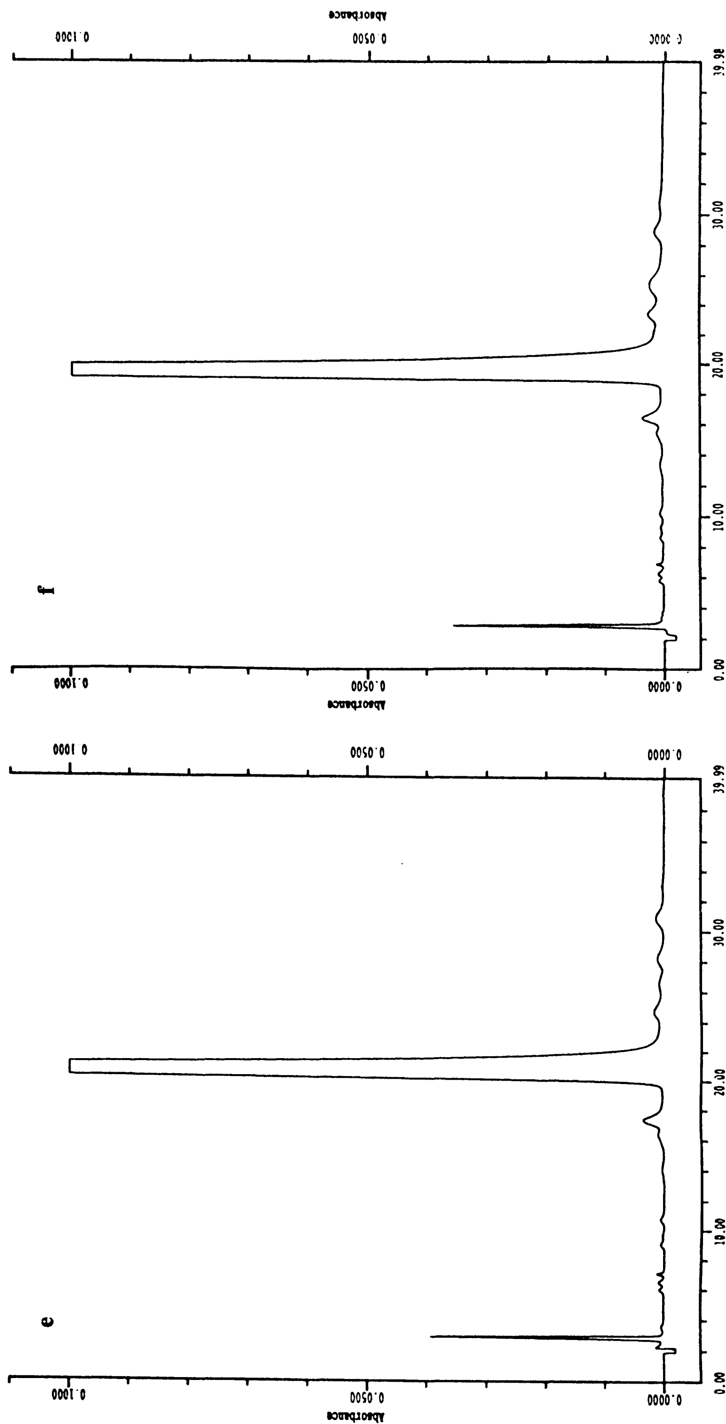

Figure 6. Continued.

The modifications observed in our studies of conventional excipients are consistent with the spontaneous formation of Schiff's bases by amino-carbonyl condensations that initiate the Maillard reaction, as they correlate with published data on the relative chemical reactivity of these sugars in this reaction in food chemistry (25-27), and with the absence of these modifications in samples dried using the non-reducing disaccharide, trehalose (Fig.6, d). Preliminary studies with other non-reducing mono- and disaccharides, mannitol and sucrose, also showed no detectable modifications immediately post-drying, though the appearance of additional peaks was observed in these samples after prolonged storage (results not shown). This might reflect the relative stabilities of the various non-reducing sugars, as compared to trehalose, sucrose is easily hydrolysed (33), and the monosaccharide sugar alcohols are prone to autocatalytic oxidation (35) that yields compounds such as dicarbonyls that are extremely reactive in the Maillard reaction (26, 27). This suggestion is also consistent with the non-enzymatic browning observed in the initial accelerated ageing studies described above, as the more intense browning seen in the sucrose wells, compared to the maltose wells (Fig.2), correlates with the respective reactivities of the hydrolysis products, fructose and glucose (see Fig.5). In the context of the hydrolysis of non-reducing sugars, it is important to also note that some of the reactions in the Maillard cascade may also result in changes in pH, and these changes may in turn accelerate other reactions of the cascade (Fig.3).

Finally, to directly investigate the effect of residual moisture on chemical reactivity, a model system containing lysine with the two non-reducing excipients trehalose and sorbitol, dried and stored at 3 different defined residual water contents, was used. Chemical reactivity was ensured by spiking the drying mixtures with a 5% trace of glucose, and its reaction with the lysine was measured by the quantitation of the glucose remaining after storage at 50°C. In the trehalose samples, chemical reactivity was reduced as the residual water content decreased, and essentially no reactivity was observed at a water content of around 5% (Fig.7a). In contrast, in the sorbitol samples, the chemical reactivity was accentuated under the driest conditions (Fig.7b). These results strongly implicate Maillard-type reactions to yield Schiff bases, as these reactions are driven to completion in systems where water activity is limiting (26, 28).

In conclusion, our results suggest that the Maillard reaction could be an important factor in determining the long term storage stability of dry protein formulations containing carbohydrates as stabilising excipients. Although this preliminary conclusion may be unfamiliar in the context of pharmaceutical formulations, it has long been recognised in the food industry, where the Maillard reaction is known to be a particular problem during the drying of various foodstuffs with high sugar and protein contents (25-28). This is due to both the shift in the equilibrium of the initial amino-carbonyl condensation reaction towards the formation of the Schiff's base by the removal of water during drying and the acceleration of the subsequent reactions at low water activities (27, 28). Most previous work on the stabilisation of dry protein formulations has emphasised the ability of stabilising excipients to replace structural water and/or provide an amorphous or glassy solid matrix in the dry state. This is not entirely incompatible with our findings, as the chemical reactivity of these excipients, documented above, occurs during the storage of the formulations rather than during the drying process itself. It is possibly the unique combination of the properties of water replacement, glass transformation and chemical inertness that might explain the parallel evolutionary selection of trehalose by cryptobionts in many different phyla and the empirically determined superiority of trehalose as a stabilising excipient for dried protein formulations.

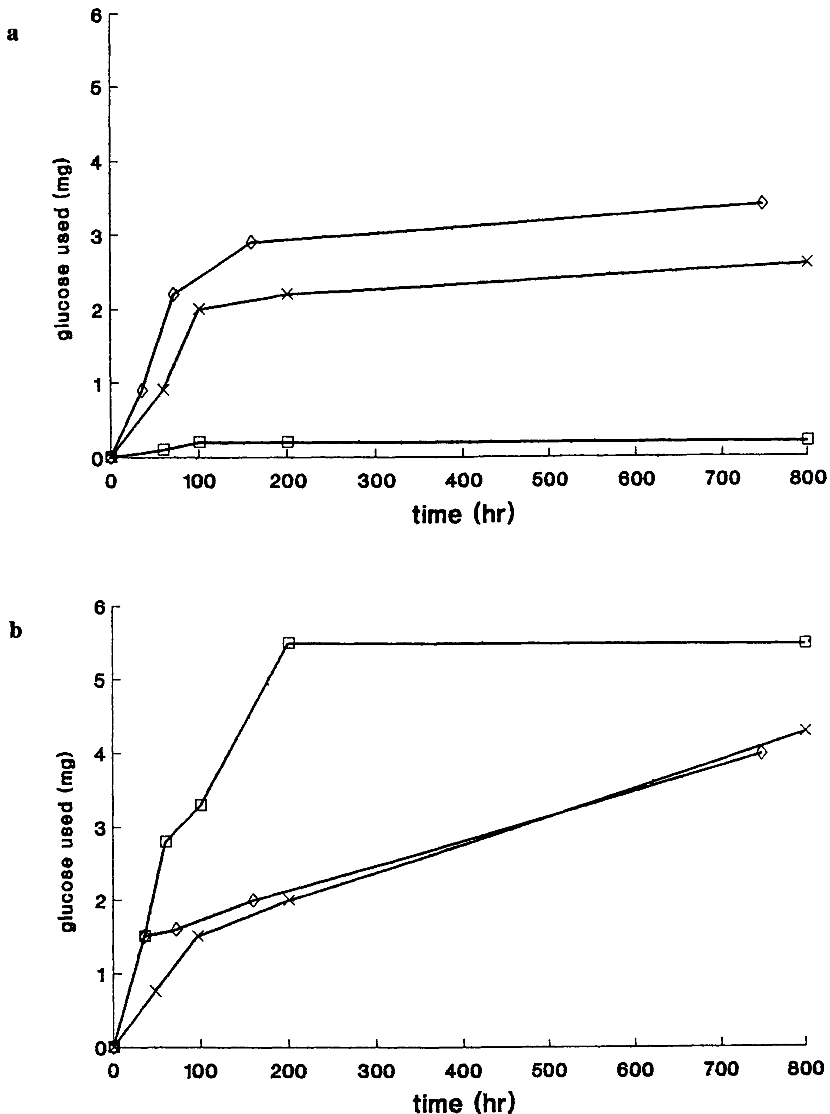

Fig 7. Effect of residual water content on chemical reactions in model excipients. Solutions of 10% w/v lysine and 5% glucose in 85% trehalose (**a**) or 85% sorbitol (**b**) in water were freeze-dried with primary drying at -50°C for 48 hr and secondary drying for a further 24 hr at 20°C. The desired water content was achieved by storage at 20°C over anhydrous P_2O_5 followed by exposure to a saturated water vapour atmosphere for either 0 hr, 8 hr or 25 hr. The actual final water content of the samples was determined by thermogravimetry using a Kahn microbalance.

a:- (□) 4.59% water, (◊) 15.09% water, (x) 22.89% water
b:- (□) 5.42% water, (◊) 13.12% water, (x) 23.62% water

References

1. Pikal M.J. *Biopharm* **1990,** *3,* 18-27.
2. Pikal M.J. *Biopharm* **1990,** *3,* 26-30.
3. Franks F. *Cryoletters* **1990,** *11,* 93-110.
4. Pikal M.J. 1993 this vol.
5. Carpenter J.F. and Crowe J.H. *Cryobiol.* **1988,** *25,* 244-255.
6. Crowe J.H., Carpenter J.F., Crowe L.M. and Anchordoguy T.J. *Cryobiol.* **1990,** *27,* 219-231.
7. Levine H. and Slade L. *Pure Appl. Chem.* **1988,** *60,* 1841- 1864.
8. Ablett S., Izzard M.J. and Lillford P.J. *J.Chem.Soc. Faraday Trans.* **1992,** *88,* 789-794.
9. Roos Y. *Carbohydrate Res.* **1993,** *238,* 39-48.
10. Carpenter J.C. 1993. this vol.
11. Roser B.J. *Biopharm.* **1991,** *4,* 47-53.
12. Roser B. and Colaco C. *New Scientist* **1993,** *138,* 24-28.
13. Blakeley D., Tolliday B., Colaco C.A.L.S. and Roser B. *The Lancet* **1990,** *336,* 854-855.
14. Colaco C.A.L.S. Blakeley D.,Sen S. and Roser B. *Biotech.Intl.* **1990,** p345-350. Century Press. London
15. Colaco C., Sen.S., Thangavelu M., Pinder S. and Roser B. *Biotech.* **1992,** *10,* 1007-1011.
16. Franks F., Hatley R.H.M. and Mathis S.F. *Biopharm.* **1991,** *14,* 38-55.
17. Franks F. and, Hatley R.H.M. in *"Stability and stabilization of enzymes"* Eds. W.J.J.van den Tweel, Harder A. and Buitelaar. Elsevier, Amsterdam. 1993, p 45-54.
18. Crowe J.H. and Clegg J.S. *Anhydrobiosis.* Dowden, Hutchinson and Ross, Stroudsburg, PA. 1973.
19. Leopold A.C. *Membranes, metabolism and dry organisms.* Cornell Univ. Press, Ithaca. 1986.
20. Coutinho C., Bernardes E., Felix D. and Panek A.D. *J.Biotechnol* **1988,** *7,* 23-32.
21. Gadd G.M., Chalmers K. and Reed R.H. *FEMS Microbiol Letters* **1988,** *48,* 249-254.
22. Crowe J., Crowe L., Carpenter J.F. and Aurell Wistrom C. *Biochem J.* **1987,** *242,* 1-10.
23. Carpenter J.F., Martin B., Crowe L. and Crowe J. *Cryobiology* **1987,** *24,* 455-464.
24. Carpenter J.F. and Crowe J.H. *Cryobiol.* **1988,** *25,* 459-470.
25. Reynolds T.M. *Adv.Food Res.* **1965,** *14,* 167-283.
26. Finot P.A., Aeschbacher H.U., Hurrell R.F. & Liardon R. *The Maillard Reaction in food processing, human nutrition and physiology.* Birkhauser, Basel. 1990.
27. Lendl F. and Schleicher E. (1990). *Ang.Chem.* **1990,** *29,* 565-594.
28. Nursten H.E. in *Maillard browning reactions in dried foods in Concentration and drying of foodstuffs;* Ed. D. Macarthy. Elsevier Applied Science. London. 1986; p53-87.
29. Stewart L.C., Richtmyer N.K. and Hudson C.S. *J.Amer.Chem.Soc.* **1950,** *72,* 2059-2061.
30. Clegg J.S. in *Membranes, metabolism and dry organisms*; Ed. A.C. Leopold Cornell Univ. Press, Ithaca. 1985; p 169-187.
31. Burke M.J. in *Membranes, metabolism and dry organisms;* Ed. A.C.Leopold. Cornell Univ. Press, Ithaca. 1985; p 358-363.
32. Lee C.K. in *Developments in food carbohydrates*; Ed. C.K.Lee. Applied Science Publ. Ltd. London. 1980; vol 2, 1-90.

33. Tewari Y.B and Goldberg R.N. *Biophys. Chem.* **1991** *40,* 59-67.
34. Percival E.G.V. *Advances Carbohyd.Chem.* **1948**. *3,* 23-25.
35. Wolff S.P. et.al. *Free Radicals Biol. and Med.* **1991.** *10,* 339-352.
36. Green J.L. and Angell C.A. *J.Phys.Chem.* **1989,** *93,* 2280-2882.
37. Levine H. and Slade L. *Biopharm.* **1992,** *5,* 36-40.
38. Crowe J.H., Crowe L.M. and Carpenter J.F. *Biopharm.* **1993,** *6,* 28-37.
39. Zyzak D.V., Richardson J.M., Thorpe S.R. and Baynes J.W. *J.Biol.Chem.* **1993,** (in press).

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