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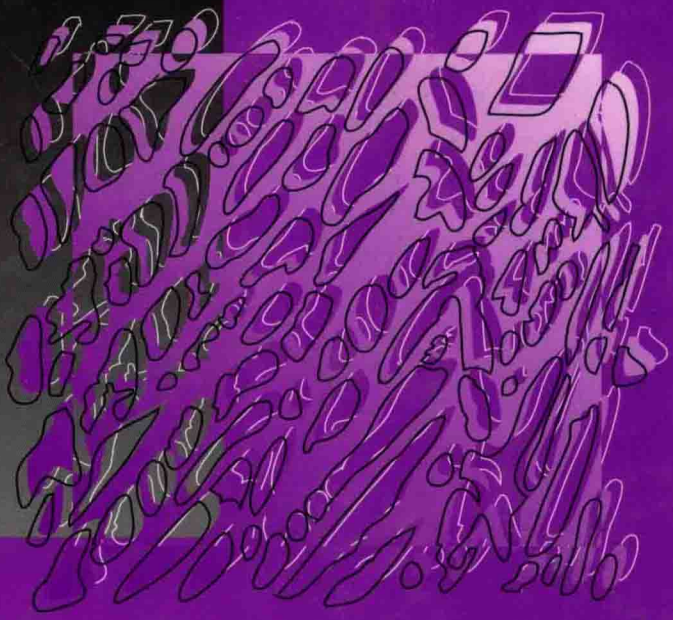

Amorphous Food and Pharmaceutical Systems

edited by HARRY LEVINE

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Preface

The Amorphous Aqueous State – Some Personal Reminiscences

Water and its mysteries have dominated and still continue to dominate my professional life, which has now spanned some 50 years. Curiously, I now find that the first 25 years were devoted to studies of dilute aqueous solutions *in* water, and the second 25 years to studies of very dilute solutions *of* water. The interest in 'residual water' arose during a marketing brainstorm that my then-employer, Unilever Ltd, was in the process of conducting. The aim was to persuade consumers about the wholesomeness of frozen food products. A new word was coined and added to the marketspeak vocabulary: 'frozeness'. This was seen as a desirable attribute, because it was associated with 'freshness'.

At about the same time, the middle 1970s, Unilever developed an interest in plant cell and tissue culture. Because I 'knew all about water', my colleagues and I were charged with the scientific backup for 'frozenness', and also the development of suitable cryopreservation methods for the maintenance of plant embryos, destined for eventual growth into palm trees. We soon found that all this was easier said than done. Thus began the long and tortuous path that has eventually led, by a random path, to the 2001 Cambridge conference, proceedings of which are collected in this book.

None of us had prior hands-on experience of freezing or cryobiology. So, like most physically trained scientists, we began our pilgrimage with a study of the effects produced by freezing model systems, in our case water-soluble polymers. Calorimetry seemed to be a useful method to monitor such effects, and a somewhat dilapidated Perkin Elmer DSC-2 was available. With help of friends in the Engineering Division, it was soon given an overhaul and converted to make it suitable for studies at subzero temperatures. The initial chart recorder traces of cooling and heating runs provided more questions than answers. As is frequently recorded by others in the scientific literature, so we, too, rediscovered the wheel several times over. Thus, we discovered undercooling, nucleation, and

eutectic phase behaviour. What puzzled us was the universal appearance of a discontinuity in the heat output curve in the neighbourhood of $-30\,^{\circ}C$, irrespective of the solution under study. Eventually, we were driven to the conclusion that we were observing glass transitions in the frozen solutions. We called it T_g', but without quite understanding its significance. Others have tried since then to give it different names, but that has only confused the issue. T_g' has fortunately become embedded in the literature, and by now, we all know what we are talking about, or do we?

The universality of T_g' no longer needs emphasising; it now forms the basis of several process technologies, mainly in the food and pharmaceutical industries. In 1974, however, we were intrigued by the amorphous material that remained after water had been removed by freezing. What might be its ultrastructure, and why did it still contain water? This all led to some sophisticated electron microscopic studies by Patrick Echlin and Helen Skaer, both at the University of Cambridge. In 1977, we jointly published our first T_g' paper.[1] At that time, my personal future was still shrouded in the mists of uncertainty, but the collaboration with my Cambridge colleagues continued for many years and led to some firm friendships. Had a fortune teller told me at the time what my future held for me, I would not have believed it.

After my departure from Unilever, I was fortunate to be able to continue the 'glass' studies at the Department of Plant Sciences in Cambridge, without the need for monthly project reports and budget forecasts. By that time, I had become fascinated by the concept of undercooled water as a means of preserving live cells and tissues. It actually worked, but the procedures were too complex to lend themselves to commercial exploitation. It did, however, give me the opportunity of adding Pierre Douzou, of cryobiochemistry fame, to my circle of friends. In the world of Plant Sciences, we must have been unique, because much of our experimental material consisted of mammalian erythrocytes. When lysed and centrifuged, the outcome did, however, resemble the beetroot juice that was spattered around the laboratory walls. During the undercooling work, I was brought face to face with the fact that the effects produced by freezing, on the one hand, and low temperature, on the other, have nothing in common; low temperature preserves and stabilises, but freezing kills! Indeed, I began to realise that on this planet, cold (freezing) is the most widespread threat to life.

That brought us back to glasses and glass transitions, perhaps even *in vivo*. My colleagues and I became interested in the physics and chemistry of natural survival mechanisms, in freeze-tolerance and freeze-resistance phenomena, and by a roundabout route, that brought us to supersaturated solutions and glasses of polyhydroxy compounds. I went for help to the glass experts in the Materials Science Department. They listened politely to my story, but when it came to water-soluble glasses, they looked at me as though I was talking metaphysics. It was only when I reminded them of sugar candy and candy floss that a look of recognition returned to their faces. Since then, they, too, have become obsessed with the materials science of aqueous solid solutions.

This all happened at around the time when I received my first visit from Harry Levine, which eventually led to a wonderful friendship with him and Louise

Slade. They 'bought into' my stories, and jointly we developed the new branch of technology, until it was ready to be presented. They then threw themselves with vigour into measuring hundreds of T_g values, writing papers and preaching the gospel of glass transitions to the food processing industry, with amazing success. I later tried the same approach with the pharmaceutical industry, but was not nearly as successful. Even today, the myths of water activity and bound water are hard to kill!

In the meantime, we came to realise that freeze- and drought-tolerance, as exhibited by many species, probably relies on *in vivo* vitrification mechanisms and is promoted by the biosynthesis of lyoprotectants of different chemical origins, with PHCs predominating. Trehalose received extensive press coverage and was even claimed, mistakenly, by some to be unique as protectant against desiccation. It was only one step to suggest that similar mechanisms might be applied to the *in vitro* stabilisation of labile molecules, of supramolecular structures, and perhaps even of intact cells and tissues. Initial experiments proved to be encouraging, and some pharmaceutical companies began to take an interest. This persuaded us to file for patents, but the University was reluctant to assist in such activities. That is how Pafra Biopreservation came into existence, a startup (or upstart) enterprise, located in the Cambridge Science Park. Forced to exist under stringent financial controls, our small group was yet able to develop the stabilisation technology to the point that we were runners-up (after Marconi) in the Prince of Wales Award for Technology competition, and also received several government awards and grants.

During the twelve years of its existence, our Cambridge laboratory was able to welcome and host twelve scientists, from the Netherlands, USA, Japan and Russia, from graduate student level to professors on sabbatical leave. My colleagues and I had to work hard to keep our heads above water, financially speaking. In the meantime, the visitors were able to advance our collective understanding of *in vivo* ice nucleation, of glassy carbohydrates, and of phenomena relating to nucleation and crystallisation in such glasses, their hydrates, and their ability to stabilise proteins. Those were exciting times for us all.

At one stage, my fellow directors at Pafra Board of Directors put it to me that we were good at spending money, but what might we do to earn some money. It was this suggestion that got us into freeze-drying. It dawned on us that this capital-, labour- and energy-intensive process was universally used in the pharmaceutical industry, but that there appeared to be little understanding of the technology. It was strictly a trial-and-error operation, too often with expensive errors. Even worse, the process did not receive any mention in chemical engineering texts, and there appeared to be no freeze-drying research in any university engineering departments anywhere in the world. With our accumulated knowledge of water, drying, carbohydrates, glasses and stability, we set about to develop a freeze-drying consulting service to industry. Tony Auffret, our Technical Manager, was mainly responsible for creating an enviable reputation for this enterprise. The development had another beneficial spinoff; it put us in touch with Mike Pikal, then in far-away Indianapolis, surely the undisputed King of Freeze-Drying. Thus, another friendship was formed and cemented. By the time

Pafra Biopreservation was sold in 1997, our clientele (or patients?) included 18 of the world's 20 largest pharmaceutical companies, in addition to many smaller ones. To us, it was a case of David and Goliath, and we were constantly astounded by how megaPharma, where millions of dollars are spent annually on R&D, could be so ignorant of a technology right at the heart of their operations. Our archives bear witness to the number of 'hospital cases' we received for treatment. While Harry and Louise were touring the globe, visiting bakeries wherever they went, teaching the polymer/material science approach to food processing, such as baking cookies and crackers, so we did a similar job for the pharmaceutical industry.

At some stage, Harry, Louise and I reached the conclusion that the science and technology so basic to our respective industrial interests needed tidying up. There were too many holes, too many unanswered questions, and there appeared to be few well-directed research approaches. We set about the construction of a highly subjective short list of 'experts' who were familiar with the outstanding problems and actively engaged in relevant research. And so it was that the first Amorph conference was put together in 1995. It was completely sponsored by industry, which enabled us to bring together 35 invited 'experts' at Girton College, Cambridge. The format was novel, because no participant was permitted to speak for longer than 5 minutes; it was to be a true Discussion Conference. Louise kept a record, apparently of every word that was spoken, and the proceedings were written up in the form of an informal report, which is attached (see Appendix I) to this Preface.

This book constitutes the record of the 2001 follow-up (see Appendix II) to the 1995 discussion conference. During the intervening years, the list of 'experts' has grown. More scientists have become fascinated by the puzzles of water-soluble amorphous systems, their properties and their applications. Important contributions by the 'newcomers' feature in this book, alongside those of the old-timers. The reader is left to judge whether all the problems and questions highlighted in 1995 have been resolved. If not, then what else is required?

Although the significance of water-based amorphous states has become more widely recognised, there is plenty of tutorial work left, and I hope still to be able to make a contribution. My most recently acquired friends at Inhale Therapeutic Systems Inc. allow me annually to 'indoctrinate' newly employed scientists, but also to discuss with their experienced colleagues matters relating to drying, stability and amorphisation. There are also still Intellectual Property issues, associated with our former patents, that rumble on in various law courts and require attention.

The BioUpdate Foundation, which I helped to found, in association with yet another friend, Andre Schram, provides post-experience courses on various aspects of biotechnology. The amorphous state forms an important part of the courses on protein stability. Our freeze-drying course is also an evergreen, and continues to attract participants from many European pharma companies, whenever and wherever it is presented.

In summary, I have been fortunate to get to know, and often to befriend, so many scientists in so many countries. It is said that a rolling stone gathers no

moss. This rolling stone has gathered plenty, both in dilute solutions and, more recently, in aqueous glasses.

Reference

1. F. Franks, M.H. Asquith, C.C. Hammond, H.B. Skaer and P. Echlin, *J. Microsc.*, 1977, **110**, 223.

Felix Franks
London, March 5, 2002

Appendix I: Summary Report of the Discussion Symposium on Chemistry and Application Technology of Amorphous Carbohydrates

Girton College, Cambridge, UK, April 4–6 1995. Symposium Organizers: Felix Franks and Harry Levine, Symposium Manager: BioUpdate Foundation. Report compiled by Felix Franks, from notes supplied by Louise Slade.

The Premise

The physical properties of amorphous carbohydrates in the anhydrous state or at a low moisture content play an important role in the processing and product quality of cereal-based and various other foods and the stabilization of pharmaceuticals and biotechnological products (*e.g.* as excipients in freeze-drying). There is an increasing awareness that, in all such applications, thermomechanical properties partly determine the choice of suitable formulations. Despite their increasing importance in food and pharmaceutical process technology, the chemistry of such amorphous sugars is substantially unexplored. Formulations and recipes are usually arrived at on a hit-or-miss basis with little basic understanding of the reasons for success or failure.

The Objective

The Symposium was convened to discuss and define the relevant problems, rank them in some order of importance and suggest effective experimental, theoretical and computational approaches for their study.

The organizers of the Symposium express their gratitude for the generous support by the sponsoring companies.

Participation

Participants included 30 invited scientists with known interest and expertise in

the subject, and an equal number of observers, nominated by the sponsoring companies.

Report

This report is not intended to be a printed version of the full Symposium proceedings. It is compiled in note form as a summary of *significant aspects* of the discussions. In its layout it conforms approximately to the format of the Symposium and should be read in conjunction with the Symposium programme and the list of participants.

The discussions relating to each session have been 'tidied up' and are summarized in precis form, according to subject matter, rather than in the chronological order in which they were introduced during the session. Contributors to each discussion topic are indicated but remarks made during the discussions are not attributed to individuals. Participants are reminded that the contents of the agenda document and of this report are privileged information and must not be quoted or referred to without the explicit permission of the individual contributors, whose identities can be obtained from the BioUpdate Foundation.

Follow-up

The principals of the BioUpdate Foundation are now considering suitable follow-up actions to what was considered to be (by the majority of participants) a most productive and novel exchange of ideas.

Topic 1 – Relationship Between Molecular Structure and Glass Transition

Contributors: Le Meste, Ablett, Randall, Slade, Huang, Angell, Franks, Cesaro, Brady, McInnes, Zografi, Bizot, Levine, Karel, Foster.

Relationships (if any) between molecular structure, interactions and their temperature dependences; differences between entangling and nonentangling systems; *i.e.* are there molecular and network T_g values. Are some experimental methods more sensitive to one or the other? How is the structure of a biopolymer related to its T_g? Currently T_g needs to be measured, cannot be predicted, *e.g.* from structure and/or interactions.

How can structural features of amorphous carbohydrates be measured? A better definition of 'solid' is required in relation to amorphous phases.

Can parameters of importance in glassy carbohydrates be predicted; *e.g.* the 'universal' constants in the WLF equation. Why does ΔC_p of vitrification decrease with molecular weight for a series of oligomers?

The 'heretical' view was expressed that a liquid formed immediately after completion of melting is NOT an equilibrium state. How does its viscosity/temperature relationship differ from a supercooled liquid?

Is it possible to measure isomerization rates in a sugar melt? Different tautomers might possess different T_g values. T_g depends on annealing temperature; find 12 °C differences, particularly high where other thermal events at higher temperatures are possible. Refer to fructose behaviour; similar behaviour might occur with galactose and ribose.

Recent NMR studies suggest that the H-bonded network structure of a β-furanose might be easily disrupted by a 'foreign' stereoisomer and that a β-pyranose melt should be more viscous than the corresponding β-furanose. This implies that a freshly prepared melt will increase in viscosity during relaxation. Furanose–pyranose conversions typically have an activation energy of 10 kJ mol^{-1}, so that the process could be trapped.

Glycerol (three carbons) and sorbitol (six carbons) exist as single conformers, but fructose exists as a mixture of many possible conformers. Presumably conformers should be miscible? Immiscible amorphous phases might coexist. A pure pyranose crystal could give rise to isomer mixtures or discrete phases on fusion, certainly on a 5–10 nm scale. Hence could have time-dependent entropy and viscosity changes. For instance, the NMR spectrum of fructose held at 120 °C reveals the appearance of different isomers with time; this might lead to a depression of T_g. There was agreement that the conformer composition of sugars is temperature and concentration dependent.

Sorbitol and mannitol also have preferred conformations, at least in solution; they are solvent-dependent. In the crystal, the two polyols adopt different conformations (planar zig-zag *vs* 'sickle'). Nothing is known about the fused state. Speculation that α and β sugars may well have diffusion coefficients that differ by 10%.

Many sugar sub-states exist with measurably different energies (boats, chairs). They would affect entropic contributions to glass transitions. How much entropy is trapped in a glass, compared to the entropy of fusion? Compare fructose and sucrose: apparently more 'trapped' entropy in fructose. Relevance to T_m/T_g and 'fragility' concept, because T_m location depends on the entropy of fusion. Also consider the contribution of tautomeric mixtures in this context.

Contrast sucrose with lactose, raffinose and trehalose, all at low moisture contents: Gordon Taylor equation fits, except for sucrose, where the effect of water on T_g is larger than calculated. How is the 'structure' of the dry sugar related to its T_g? Do internal hydrogen bonds play a role? Possibly, but rotations of C–C bonds do occur.

The vitrification potential of salts was mentioned. Mg gluconate is a particularly good glass former, with $T_g = 80$ °C; an amorphous 50:50 mixture of sucrose and Mg gluconate is stable. Other glass-forming salts include Na gluconate and Na citrate.

Iso-maltose is claimed to be more flexible and has lower T_g than maltose. Other evidence to the contrary. The hydrodynamic volume is important.

T_m/T_g is used as an indicator of 'fragility' of fluids. Thus, the ratio is 2.0 for water (strong) and 1.0 for the most fragile liquids. Actually should use T_b/T_g as the true relation, because both temperatures refer to the liquid phase.

The question of 'unique' sugars was raised. As regards fragility, stachyose

$\gg$ maltose; implies high W_g, (as well as T_g and high mol. wt) has a role to play. Trehalose is more fragile than expected.

Are there predictive relationships between structure and fragility? How can fragility be measured by a single method? Perhaps tan δ, measured at a single frequency, say 10 MHz with respect to its value at T_g. Possibly Brillouin scattering?

Topic 2 – Chemical Reactivity of Solid Sugars

Contributors: Karel, Zografi, Hatley, Angell, Huang, Foster, Ablett, Franks.

Reactions do occur below T_g. Often (not always) rate depends on $(T - T_g)$. Freeze-dried materials have porous matrix which collapses above T_g. Loss of volatiles or oxygen uptake can then exhibit reduced rates above T_g. Crystallization events can also affect chemical reactions: when anhydrous sugar crystallises, the matrix is diluted, but when sugar crystallises as a hydrate, the matrix may be diluted or concentrated. The role of crystallization enhanced reactions in food stability was mentioned.

Consider two components + water; one a good glass former (*e.g.* trehalose), the other is an 'additive', but should be considered as a reactant, rather than simply as a plasticizer.

Most reactions require an initial proton transfer step, usually involving water, but sugars can also play that role. Also raised the question of the meaning of pH in a system with 2% moisture.

Should be possible to identify T_g more reliably in complex systems containing proteins, *e.g.* where partly superimposed transitions in tertiary structure (denaturation) can occur. Could have complex behaviour where possibility of tautomerism exists. Fructose shows heat flow discontinuities at 240 and 320 K. Is the upper transition related to an isomerization?

Can WLF equation account for chemical reactions (Maillard)? The rate is said to depend on ΔT and moisture content, but ΔT is itself a function of moisture content. Scepticism expressed about the validity of WLF kinetics in such situations. Chemical reaction rate depends more on diffusion (mobility), perhaps ONLY on translational diffusion. Water acts primarily as plasticizer, but also as reactant (hydrolysis). WLF constants are NOT 'universal' constants. It was suggested that WLF or similar kinetic models are just as universal as the Arrhenius model which is itself a special case.

Long discussion on Maillard reactions; they can occur in glasses, *e.g.* lactose + insulin yield ketoamine products. Most studies refer to prenucleated systems. Crystallization requires both nucleation and growth below T_g. PHB (polyhydroxybutyrate) crystallises *in vivo* ($T_g = 10\,^{\circ}C$) but is found to be amorphous at room temperature after extraction.

Topic 3 – Chemistry and Biochemistry in Supersaturated Carbohydrate Mixtures

Contributors: Hatley, Foster, Karel, DeLuca, Franks, Shalaev.

Discussion of the survival of microorganisms and viruses in glassy matrices, also mention of chemical reactions, *e.g.* enzymatic and acid inversion of sucrose in glasses.

Photochromic material in a glassy matrix requires 2 s at 60 °C to change colour, involves movement of two five-membered rings. Possibly conformational changes can occur in proteins, even in the vitreous state. Reference to Klibanov's work on enzyme–substrate complex in a solvent from which the substrate can be removed by washing, but the protein cannot depress T_g. The protein 'remembers' its conformation when in the presence of the substrate. Suggestion that, within the (limited) resolution of FTIR, protein conformation can be maintained during drying, but slow aggregation can occur during subsequent storage.

In the oxidation of NADH: Arrhenius kinetics apply both above and below T_g, but large decrease in E_A at T_g. Could be due to multistep reaction, each with its own E_A.

Monoclonal antibodies (MCA) lose activity by aggregation after 'conventional' freeze-drying and storage for 60 days at 35 °C. Sucrose and maltose can protect, but in combination, the sugars are more potent than would be predicted from their individual effects. Source of aggregation: one MCA complex dissociates, followed by irreversible misassociation of subunits. If the protection by sugars requires sugar 'bridges', then two types of OH spacings are required to explain the observed effects. Question about the nature of hydrogen bonding patterns between sugar molecules.

The question of intramolecular reactions within glasses was raised, *e.g.* deamidation of aspartate. Not enough data are available. Suggestion that even intramolecular rearrangements or condensations require an intial proton transfer step, usually from solvent. Can a sugar in the amorphous matrix act as proton donor/acceptor in such steps?

The question of protein cold denaturation during freeze-drying was raised which might be responsible for the loss of 'quality'.

Topic 4 – Physical Processes (*e.g.* Crystallization) of, and within Amorphous Carbohydrates; Kinetics; Effects of Residual Moisture
And
Topic 5 – Solid Solutions Involving Carbohydrates

Contributors: Reid, Levine, Zografi, McInnes, Ring, Hemminga, Pikal, Huang, Blanshard, Randall, Roos, Shalaev, Franks, Foster, Angell, Slade, Flink, Mathlouti, Brady.

A general plea was made for better definitions/descriptions of 'amorphous', 'amorphous structure', 'supercooled/supersaturated'. Any difference between quenched liquid and dry milled crystal? What is considered to be the size limit of a 'crystal'? How many unit cells? Freeze-dried amorphous samples left in the freeze-drier at 60 °C for 2 h, 85 °C for 4 h or 120 °C for 1 h (re)crystallize without any indication of melting.

How is (incipient) crystallinity detected. The detection of a crystalline phase requires dimensions of several unit cells, say 10 nm. X-ray diffraction becomes unreliable as a quantitative estimate for degrees of crystallization below 10%. NMR is more reliable in such cases; it detects 'crystallinity' by the existence of specific bond orientations

The technology of creating amorphous materials by milling crystals was discussed; requires particle size 1–6 μm. T_g values of milled and cold quenched materials are identical; reference also made to 'cotton candy' technology.

Crystals subjected to pressure lose their characteristic Raman spectra; when the pressure is released some substances remains amorphous, others revert to crystallinity. The same results have been found with the effects of irradiation on crystals. The results might form the basis of categorising materials as 'good' or 'bad' glass formers.

Crystallization kinetics from amorphous phases need study. According to the literature, no crystallization occurs below T_g, but this is hardly the case. The addition of even small amounts of PVP has a major effect on the crystallization of sugars, although T_g is not markedly affected. A 'magic' inhibition of sucrose crystallization is observed in 7:1 mixtures of sucrose:fructose. Raffinose, trehalose and lactose are also effective crystallization inhibitors. A sorption mechanisms in solution, *i.e.* by poisoning, was acceptable, but how do they act in anhydrous systems?

A case of the drug indomethacin was mentioned: in a formulated product of T_g 45 °C it crystallises in three weeks at room temperature, and (more slowly) even at 20 °C. It can be studied by DSC down to $(T_g - T) = 15$ °C. A fit of the VTF equation and extrapolation suggest a relaxation time of 10 s at T_g. At $(T_g - T) = 50$ °C, crystallization can be prevented for one year. If storage under such conditions is not practical, PVP can be added to raise T_g. For 20% PVP no crystallization occurs in three weeks, even at 4 °C above T_g.

The effect of additives on the crystal forms obtained is of interest; examples were presented of very different effects produced by the addition of glucose and fructose on the crystal habit of sucrose. Quite apart from crystal growth effects, the question was put how crystal nucleation is affected by 'foreign' sugars. Nucleation history also governs how amorphous materials are produced, whether from the melt, from solution by freeze-drying or evaporation, or by spray-dried powders.

The crystallization of mannitol is of particular importance. Homogeneous amorphous mannitol, obtained by spray drying and containing 0.5% water, has a T_g of 36 °C. At room temperature it crystallizes completely within two weeks but without loss of water. Similar results are found with mannitol/glycine mixtures ($T_g = 50$ °C). Monitoring by X-ray diffraction over several weeks shows

gradual polymorphic changes. Crystallization at $-20\,^{\circ}C$ was also reported for mixtures with $T_g = 100\,^{\circ}C$.

Evidence for method of preparation effects on the ability to detect T_g: Maltodextrin/protein mixtures (0.5% moisture) reveal no T_g at room temperature, but T_g becomes visible after storage at $-90\,^{\circ}C$ and subsequent heating.

What can be said about the relative stabilities of systems protein-water-X, where X is sucrose or ficoll? T_g (ficoll) $\gg 132\,^{\circ}C$, T_g (sucrose) $= 70\,^{\circ}C$. Thus, for same T_g, *e.g.* $45\,^{\circ}C$, require more sucrose in formulation. Also free volume of polymer is larger.

Discussion of relative densities of sucrose *vs* sucrose/PVP and sucrose/ficoll. Note also in ternary systems have separation of polymer rich and sucrose rich phases. Mobility of probes in ternary mixtures of water/glucose/NaCl find retardation by NaCl. Two amorphous phases not uncommon in protein/sugar/water mixtures; observe two distinct glass temperatures.

Phase separation also observed in starch/fructose/water systems. Thus, 20% fructose is completely miscible with starch/water, but at higher fructose concentrations observe two separate T_g profiles.

The complex phase behaviour of water/sugar/salt mixtures was highlighted with reference to the solid/liquid state diagram of the water/sucrose/NaCl system. During cooling, depending on the initial composition, especially the sucrose:NaCl ratio, ice crystallization takes place first, but $NaCl.2H_2O$ crystallization can also be induced. Two distinct stages of freeze concentration can now be identified: after the completion of primary ice crystallization and after the subsequent completion of secondary ice and NaCl crystallization. The point of *maximum* freeze concentration after the crystallization of ice and NaCl can be regarded as a quasi-eutectic point in the ternary state diagram. The dependences of glass and softening temperatures on the sucrose:NaCl ratio can be represented in two dimensions and is of some practical importance in freeze-drying operations. If NaCl can be induced to crystallize, it lends mechanical rigidity to the cake during the sublimation of ice. It then becomes possible to perform primary drying above T_g' (or T_s), without danger of product collapse.

Another aspect of ternary mixtures in relation to freeze-drying was mentioned: the use of mass transfer agents. *Tert*-butanol (TBA) accelerates the sublimation of ice from frozen sugar solutions, especially at some specific TBA:sugar ratios. Thus, the freeze-drying of lactose or sucrose is time consuming because of the low collapse temperatures. The addition of TBA permits primary drying above the nominal collapse temperature. Concern was voiced about difficulties experienced with the removal of TBA; some preparations retain 4.5% after drying. TBA appears to be retained (encapsulated) within the sugar.

The question was asked whether solutions, cooled at different rates, but to the same T_g' would exhibit different ice growth rates. The general opinion was in the affirmative.

Question of the existence of sucrose hydrate(s) was raised again: no agreement! 1949 X-ray evidence for hydrate(s) may (not) be reliable/reproducible? Observation of anhydrous crystal at $> 5\,^{\circ}C$, but crystal hydrate of different appearance at $< 5\,^{\circ}C$, with different refractive index. Suggestion to wash out crystals with

ethanol at low temperature. Other suggestions: use temperature gradients (zone refining) to detect and grow crystals.

Discussion of protein denaturation in ternary and quaternary systems with low moisture contents; *e.g.* T(denat) in maltose/water glass is depressed up to 25–35% moisture, but no further depression beyond that. Aggregation immediately follows denaturation. Distinction must be drawn between denaturation caused by unfolding and chemical inactivation which may occur without unfolding.

In water/sugar/protein systems observe preferential hydration of protein. As water becomes limiting, the sugar is dehydrated before the protein. Extensive literature on lysozyme/LDH and the effects of polyols and water on the drying stability.

Glassy films containing 50% ovalbumin in fructose, glucose, sucrose are rehydrated and monitored by infra-red; the effects of the three sugars on the IR spectrum are very different. Is anything known about the hydrogen bonding patterns between sugars/polyols and proteins, as water is removed? Not much. Insulin–lactose adducts prevent insulin aggregation during drying; reason unknown.

The question of the significance and determination of residual moisture content generated much discussion. In a partially crystalline mixture, the 'residual moisture content' *must be referred to the water content per unit mass of amorphous phase* which is not always easy to determine. The question arose if there is a universal relationship between water activity and critical moisture content, or does it depend on the particular composition. A useful definition of critical moisture content is the water content that is able to depress T_g to room temperature.

There exists an extensive literature on hydration and hydration numbers of sugars; what is its relevance? Hydration number is an operational definition, therefore the method of measurement must be defined. The discussion generated much heat. It was suggested that measurements of a_w provide an indication of the deviation from ideal solution behaviour which, in turn, depends on the hydration number. This was contradicted: deviations from ideal behaviour are ascribed to solute–solute interactions which, in the case of sugars, are of a repulsive nature.

Molecular dynamics simulations suggest 2.6 water mol per sugar OH in xylose, but how does this correspond with the reported $n_h = 5$ for sucrose? Presumably hydration might be defined in terms of the distances of nearest neighbour water molecules from sugar OH groups by stipulating acceptable hydrogen bond lengths. It used to be said that 3 mol of water per sugar mol causes collapse of freeze-dried sugars, but that referred to room temperature. The discussion was left unresolved, because (a) there was no agreement about the exact definition of 'hydration' and (b) its temperature dependence.