



UNIVERSITÀ DEGLI STUDI DI TRIESTE

Sede Amministrativa del Dottorato di Ricerca

**XX Ciclo del
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***Molecular mobility of trehalose in relation to its
bioprotective action.***

(Settore scientifico-disciplinare CHIM/04)

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The birth of a star

A long time ago, in a desert far, far away, sweet *manna* rained down from heaven in the wilderness to feed the Hebrews during the Exodus. Since some manna have been shown to contain large amounts of the sugar trehalose, this is probably the first episode in the history of mankind that can be traced back to this sugar, raising trehalose's popularity and relevance.

Later on, the discovery that some seeds from *Nelumbo Nucifera* found in the desert could keep vital and germinate after periods as long as 1000 years, seemed as miraculous as the *manna* rain. Breathless scientists found no difficulty in relating this phenomenon to what was already observed in "certain animalcules found in the sediments in gutters of the roof of houses". On the early 700's Leeuwenhoek wrote about his experience with these small animals, describing how they changed shape when dehydrated, but recovered their original shape and "came back to life" when rain water was poured on them. After repeating his experiments and obtaining the same results, he arrived to the conclusion that there was something protecting the animalcules to an extent that they could survive even after months of dehydration. What he probably didn't know is that this "something" was as simple sugar molecule and that processes like the one he described are spread in nature. Life of tardigrades can be suspended for even 100 years. "Large" animals are not capable of withstanding complete desiccation as some micro-organisms, but earthworms and leeches can loose a very high proportion of their water (up to 93%), corresponding to about the 70% of their total body weight (Keilin 1959) Suspended animation was called in many different ways such as viable lifelessness, latent life, etc; but lately the more diffuse terms are the Greek descendents anabiosis and anhydrobiosis. Anhydrobiosis means clearly "life without water" while Anabiosis "return to life". The suspended life taking place at temperatures below the freezing point of water is called cryobiosis. Here again, cold damage to cells is widespread known, yet some organisms manage to survive under extreme temperatures.

Today we know there is a link between all these events, which is the disaccharide trehalose.

The myth

Many organisms presenting suspended animation are known to due their ability to survive from dehydration to some sugars, mostly sucrose and trehalose. Under harsh environmental conditions, these organisms accumulate large amounts of sugars, specially disaccharides. Sucrose is most commonly found in plants while trehalose seems to be

common among animals and microorganisms. In the past it was believed that these sugars, including trehalose, had a neat energetic reserve function, and that their function was to supply simple carbohydrates immediately after rehydration to help cells resume vital functions. Only recently it has been universally accepted that they may play a more active role than what imagined before. Evidence suggests that trehalose production is a response to stress in many organisms. Since the discovery of the exceptional role in nature of this sugar, many theories and experiments have crowded the scientific, and some times not that much, literature.

1.1 - When facts meet the myth

Many papers have been published in the last years regarding the protective effects of this sugar through its interaction with cells, lipids and proteins. Trehalose (α -D-glucopyranosyl-(1 \rightarrow 1)- α -D-glucopyranoside, fig.1.1) is a non reducing disaccharide as sucrose, fact which makes them attractive for uses where oxidation is a threat. It is also known as mycose, since it was first found in fungi. In nonenzymatic Browning reactions the amino (R-NH₂) groups from aminoacids react with reducing carbonyl groups of sugars. They are the main cause of protein functionality loss during storage, and may induce unacceptable changes to nutritional as well as organoleptic properties of stored materials like food or drugs. Experiments comparing the performance of sucrose and trehalose in freeze dried systems at low pH show that rate constants for brown colour formation 200 to 2000 fold greater for sucrose than for trehalose, probably due to its higher stability to hydrolysis² Trehalose dissociates only under extreme conditions as very low pH or the presence of the specific enzyme trehalase.³

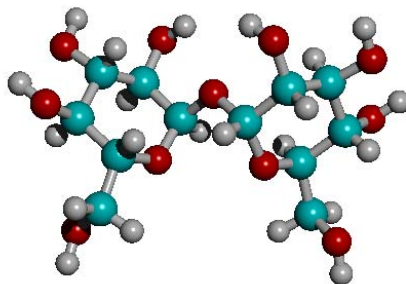


Fig 1.1 - Stick and ball picture of Trehalose molecule; the symmetry of the two glucose arms centered on the glycosidic oxygen is emphasised .

Cell membranes are irreversibly damaged on dehydration, with phases change like crystallisation, fusion and leakage of the membranes taking place. But when, for instance, DPPC (Dipalmitoyl phosphatidylcoline) liposomes are dried in the presence of trehalose at 20% of the dry weight (as in anhydrobiotic organisms) no phase change is observed and upon rehydration they appear morphologically and functionally similar to freshly prepared ones. [Science, 223, 701-703 1984 a]. Results show that when trehalose is added, the lipids behave like hydrated, so sugar is taking the place of water. This gives rise to the first theory trying to explain its particular effects, that is the *Water replacement hypothesis*, proposed by Crowe in 1973⁴. Indeed, aqueous solutions of polar and non polar compounds have been deeply studied in the past as

model systems for interpreting and predicting the behaviour of functional biological macromolecules. The interplay of solute-solute and solute-solvent interactions has been disclosed and sometimes arbitrarily catalogued (see, for example, papers and references therein in the volume of Biophysical Chemistry 2003 dedicated to Walter Kauzmann), nonetheless the stabilization of membranes by several solutes is complex and not assignable to a specific contribution alone.

Yeasts accumulate trehalose in response to stresses. During fermentation, when ethanol concentration rises, it starts poisoning cells triggering a natural signal for them to stop its production. Growth is inhibited, and cell metabolism decreases, so does nutrient intake and ethanol production. Trehalose is likely to improve ethanol tolerance by protecting the plasma membrane from leakage.⁵ Temperature is another stress to consider on fermentation processes. For example when temperature changes from 25°C to 40°C, trehalose is synthesized by cells along with heat shock proteins to enhance their thermotolerance. Proteins would play a key role in the recovery from the heat-stressed state whereas trehalose should be involved mainly in the protection of proteins and enzymes from denaturation as discussed below.³

Some proteins do not get damaged at all when freeze dried, but more labile ones suffer degradation and denature, losing the possibility to come back to their original conformation and as a consequence, losing functionality. Trehalose has proven to protect this kind of proteins, and some significant results attribute an efficiency of almost 90%. Apparently, the extreme flexibility around the glycoside bond, conferred by the lack of intramolecular hydrogen bonding, allows it to adapt to the irregular form of proteins and bond to them. It can form up to four hydrogen bonds per molecule of trehalose. In the stable dihydrate molecule of trehalose, these bonds are formed with water. Nevertheless, IR and Raman experiments studying the interaction between trehalose and the protein lysozyme suggest that rather than binding to it, trehalose keeps the remaining water near the protein avoiding complete dehydration.⁶ Moreover, there was also the belief that as trehalose would have a considerably large hydrated radius, it would be excluded from the hydration shell of proteins. According to Sola-Penna and coworkers, hydrated trehalose occupies 2.5 fold larger volume than sucrose, maltose, glucose and fructose.⁷ As a consequence, this *preferential exclusion* mechanism would be responsible of the preservation.⁸ However, an analysis of the actual experimental viscosity data reported in the paper show that some unclear mistakes occurred in their elaboration, since their results do not agree with most of what reported in literature, nor with the experimental data repeated in our laboratory and reported in another Ph.D. Thesis (F. Sussich, Trieste 2004). In our opinion, both the wrong conclusions of Sola Penna and the reporting of a visual perception (sic!) of

the larger hydration volume induced other researchers to consider this phenomenon highly relevant. Indeed, even if the high viscosity attributed to trehalose is not such, as other measurements show, perhaps this mistake made years ago induced also Magazù⁹ to emphasize that trehalose has a bigger hydration ratio than its isomers sucrose and maltose. Although their calculations are based on a series of approximations, the difference between sugar volumes is not as drastic as that reported by Sola Penna. Although the hydrogen bonding protection mechanism can partially explain the efficiency of trehalose, other mono or disaccharides should act as well as it, if this was the only cause. Nevertheless, glucose is not that effective, mostly when considering freeze-drying. It was shown that besides the hydrogen bonding capability, a good cryoprotectant should be able to conserve the native structure of the protein in the dry state. Protein conformation is known to be severely conditioned by the matrix that surrounds it. At this point, another property of trehalose comes to light, its high glass transition temperature. Comparing to the other disaccharides, trehalose has an exceptionally high glass transition temperature, implying that at drying conditions it is already a glass. Proteins are immobilised in this viscous glassy matrix and therefore they cannot denature. Green and Angell mentioned this feature which constitutes another theory trying to explain the mechanisms behind trehalose bioprotective action, the glass hypothesis. In their paper¹⁰, the comparison of the glass transition temperatures of trehalose and glucose was made and a $T_g = 79\text{ °C}$ was reported for trehalose, that is higher than sucrose and other disaccharides (Note that the true T_g of trehalose is as high as 120 °C , nonetheless this work is continuously quoted for the very high T_g of trehalose; the concept is correct, but the number is wrong). Anyway, they highlight this elevated glass transition temperature (T_g), which became to be known as the trehalose anomaly. Experiments were conducted onto elucidating this issue but even when many features of the glass and solution states are known there are no satisfactory explanations to it. Part of this PhD thesis deals with it and the results obtained will be exposed and analysed in the frame of these and other hypothesis.

All the properties mentioned above make trehalose very attractive for a wide range of applications, going from the biomedical to the food or cosmetic science (see references ¹¹⁻¹³ and references therein). It is used in different areas as moisturizer, preserving agent, sweetener and for the stabilization of liposomes in cosmetics. ¹⁴ It could have an invaluable contribution in stabilising vaccines, immunoconjugates, antisera ecc. during storage at room temperature or even higher, overcoming problems arising from a break in the cold chain and decreasing dramatically the cost of distribution, making them accessible for everyone. After several successful

experiences in freeze-drying of human platelets, mammalian cells, etc the most ambitious are already talking about stabilisation of organs for transplantations and Langherans islet transplants¹⁵⁻²²

So it comes out straightforward that understanding the mechanisms underlying bioprotection would have remarkable implications in the progress of science and technology.

1.2 - Bioprotection: how does it work?

The *water replacement*, *preferential exclusion* and *glass* hypothesis were already mentioned as they were initially found in literature as the answer to the question of bioprotection. In the last years, other hypothesis arose as the formers where not satisfying. The *destructuring effect* mechanism states that trehalose imposes a determined spatial location to the water molecules surrounding it, an effect that was verified with molecular dynamic simulations. According to these simulations^{23,24} it conditions the structure of water up to the third hydration shell. Doing so, tetrahedral coordination is banned and water is unable to nucleate and crystallise, so the damages produced by the formation of ice do not take place. Many authors believe that this destructuring effect is possible because trehalose-solvent/solute interactions are favoured above solvent-solvent ones.⁹ Water would bind stronger to trehalose than to other water molecules in what they call “preferential interaction”. It is interesting to reflect about the implications of these preferential interactions on proteins; several papers show these interactions occur, contradicting the preferential exclusion mechanism.

An appealing hypothesis involves the possibility that some crystalline forms (dihydrate and anhydrous crystals) could be also implied in bioprotection. It was observed that when dried membranes where rehydrated with water vapour, the dihydrate crystal was formed.¹³ The crystal “traps” two water molecules per sugar, so that part of glassy trehalose converts into its dihydrate minimizing the free water content. In this way, the rest of the matrix remains as a glass, as there’s no water plasticizing it.²⁵ This hypothesis was supported by some additional experimental evidences and it is becoming more and more clear since the conversion between the dihydrate crystalline and the anhydrous crystalline phase, obtained under certain particular conditions, is fully reversible.²⁶ In this view, a synergic effect toward the immobilisation of a biomolecule should be ascribed to the formation of the anhydrous crystalline/amorphous mixture, starting from the removal of water from the aqueous solution of concentrated trehalose (see the phase diagram in Figure 1.2). In addition to

1. Trehalose properties

the phase diagram of the water-trehalose system, all the possible interconversions are shown in Figure 1.3.

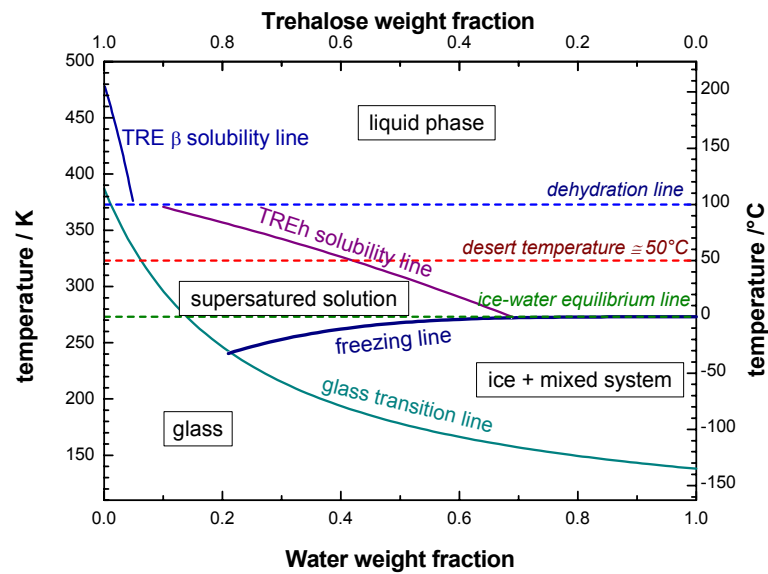


Fig. 1.2 - Phase diagram for trehalose

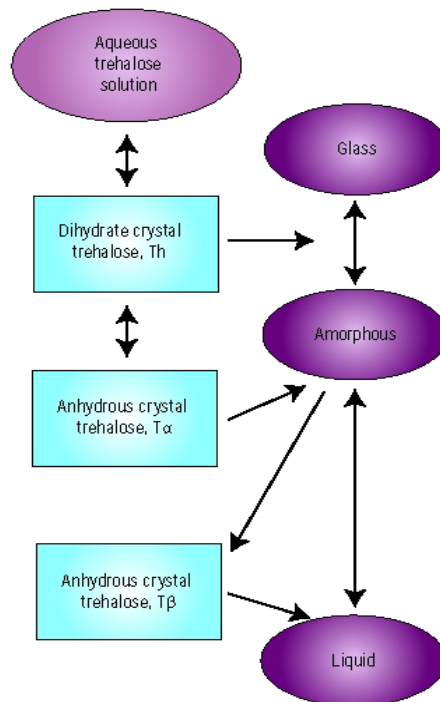


Fig 1.3 - Possible interconversions between trehalose polymorphs

It is worth remembering that trehalose presents polymorphism, a characteristic that makes its phases path-dependent. The thermal history is determinant on the structure of the phase obtained and thus on the polymorph. In an organism, by the moment trehalose starts to be synthesized there is still some water remaining, so the

first species formed is the solution. The latter evolves into the dihydrate crystal as water decreases and the concentration approaches two water molecules per sugar. The three-dimensional sketch of the dihydrate crystal is represented in figure 1.4. The crystal structure^{27,28} is made up by four sugar units $C_{12}H_{22}O_{11} \cdot 2H_2O$ in an orthorhombic cell ($P2_12_12_1$). Water and trehalose molecules are held together in the crystal by a complex system of twelve hydrogen bonds where every hydroxyl group of the trehalose

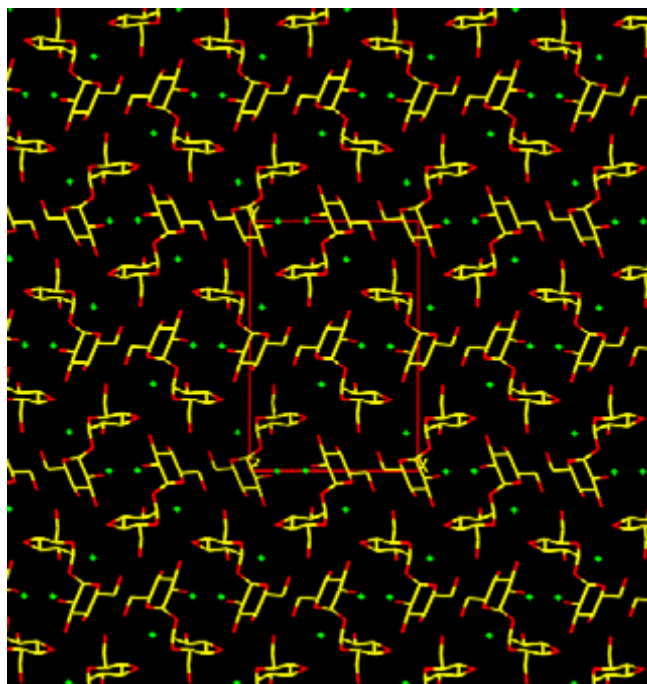


Fig. 1.4 Three-dimensional structure of the di-hydate crystal of trehalose

molecule is both donor and acceptor in the hydrogen bond net. However, in the crystal there are no intramolecular hydrogen bonds such as in sucrose or cellobiose.²⁹

As dehydration proceeds, the dihydrate starts to lose water until when, under mild conditions, becomes anhydrous. The anhydrous phase formed in this way, called *alpha*-trehalose (TRE α) is not amorphous at all in our opinion, but formed by a series of unstable, tiny crystals, as shown by its powder diffraction pattern.³⁰ These crystals can be in the order of a few nanometers and this is the reason why its structure hasn't been determined yet. Another temperature path, which implies the fast dehydration of the dihydrate yields the *beta*-trehalose polymorph (TRE β), a very stable, fully characterised crystal³¹ with a high melting point, around 215 °C. TRE β cannot be involved in the processes occurring in nature since its formation implies the presence of environmental conditions and elevated temperatures which are not verified. Yet it is surely involved in many industrial processes, then it deserves attention for understanding trehalose transformations implies optimising results.

Many papers deal with the formation of the dihydrate crystal from the glassy trehalose matrix as water vapour is added. As mentioned before, part of the bioprotective action of the trehalose is attributed to this form, but there is concern about the reproducibility this process. The amount of dihydrate formed varies from case to case, even when the same rehydration protocol is used³². From this observation it is presumable that the difference comes from the original amorphous phase formed. Some don't accept at all the possibility of polymorphism denying the existence of two different amorphous phases. It is evident from the thermogram that these two polymorphs exist and they have different thermodynamic properties as melting point. Some of our results seem to show that also their dynamical properties differ to some extent, as will be discussed later.

In conclusion, all the theories regarding bioprotection formulated to the date can explain in part the phenomenology involved in it but none is exhaustive. Lately there is a general tendency to accept that a single mechanism cannot justify all the effects. In other words, a full description of the processes taking place can only be drawn if many of the hypotheses coexist to some extent depending on the nature of the stress (freezing, dehydration or thermal shock, etc).

1.3 - State of the art

What do we already know about trehalose?

A considerable number of papers deal with the interaction of trehalose with other cellular components such as membranes or proteins, mainly due to the great interest of the biomedic and food science fields on its potential applications. When it comes to trehalose alone and its solutions (in water), results become fuzzy and contradictory or incomplete. Most investigations dealing with trehalose in water solutions focus on the dilute and semi dilute regime and hardly go beyond the 50% w concentration. The list of papers is very long and mainly refers to several thermodynamic data on the trehalose dilute solutions while some of the solution studies have dealt with physical properties related to conformational^{26,30,33-41}, structural and dynamical aspects.^{23,39,42-49} Thermophysical properties of aqueous trehalose solutions have been reported by Miller et al.^{50,51}, mainly with the intention of providing data for their supplemented phase diagram. Solubility data from several laboratories are collected in the review paper of Chen et al.⁵², together with the freezing curve of trehalose–water solutions. More

recently, a particular attention has been given to trehalose in confined state, where experimentals and MD simulations would be of interest also for the in vivo situation.

On the other hand, many studies have dealt with the thermodynamic, structural and dynamical properties of the anhydrous undercooled liquid of trehalose, mainly to characterise the glass transition region. Here, additional quotation is made to the molecular mobility of undercooled liquid trehalose studied by temperature-modulated differential scanning calorimetry and by dielectric analysis⁵³ and to the work carried out by Lefort et al^{54,55}. The greater molecular mobility of trehalose glass with respect to other sugars, such as lactose and sucrose, has been inferred in this latter recent study by Lefort et al. on the basis of solid state NMR and computational investigation on sugar glasses.

The larger internal mobility of trehalose is however accompanied by the ability of trehalose to form larger clusters than sucrose.⁵⁶ Subsequently, on the basis of the enthalpy relaxation studies of the three sugars, lactose, sucrose and trehalose, during isothermal aging, it has been shown that the size of the cooperative regions in the temperature range between 298 and 365 K is much larger for trehalose than for sucrose and lactose⁵⁷. They all provide some hints in the actual mobility of trehalose molecules in the amorphous/glassy state.

Quasi elastic and inelastic neutron scattering, neutron spin echo Affouard et al.⁵⁸ studied the effect of sugar on water by means of QENS and INS, comparing trehalose and sucrose at 50% w concentration. Their findings suggest that the addition of trehalose and sucrose to water slow down considerably the dynamics of water. NSE experiments evidence the kosmotropic character of these sugars. This kind of molecules impose their own order to water, preventing the formation of the tetrahedral hydrogen bonded network of water, thus hindering ice crystallisation. In agreement with NSE, INS results demonstrate how water translational diffusion is slowed down in the presence of disaccharides and how this effect is more pronounced for trehalose. By their estimation, the dynamics of a 49%w solution of sugar at T=320 K resembles that of pure water at temperatures around 268K in the case of trehalose and 277 K for sucrose solutions. According to this, adding 50% w trehalose to water would have on its dynamics the effect of cooling down to -5°C (freezing). A comparison among trehalose, sucrose and maltose shows that the former has a higher “crystalline-like” behaviour in solution, a higher degree of local order, forming a single entity with water, what would justify its rigidity comparing to other disaccharide solutions.

Therefore, some effects of the sugar trehalose on the water properties seem to be distinctive with respect to other sugars. How these effects converge to the

bioprotectant action of trehalose when water molecules are absent or very rare is not clear yet.

As a further comment, from the literature analysis a gap of information appears on concentrated solutions. Dilute solutions can give useful information about the dynamics of water in the presence of sugar. It is obviously the starting point, and some differences between isomers can already be evident in these range of concentrations. From the commercial point of view, applications dealing with freeze drying and the preservation of solutions, mainly at low temperatures, have certainly taken advantage of these findings. Nevertheless, glass formation in environmental conditions involves concentrated solutions. Considering that T_g of pure (anhydrous) trehalose is around 121°C , and the dependence of T_g with concentration [Fig 1.5], at 30°C a solution should have less than 12% w water to be in the glassy state.

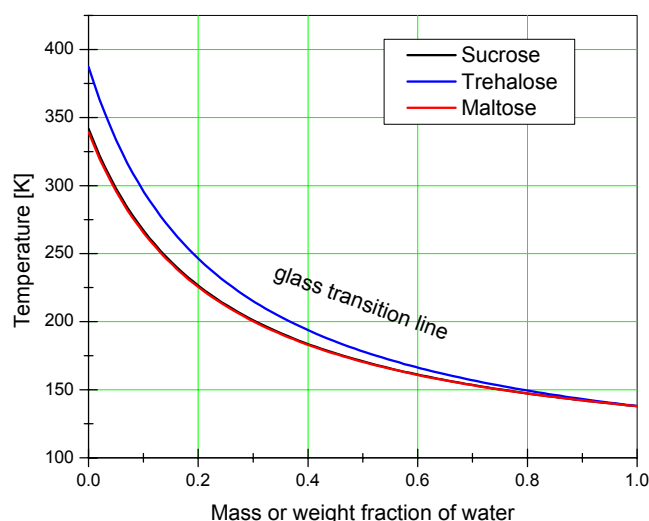


Fig 1.5 – Dependence of the T_g of sucrose, maltose and trehalose with concentration

The aim of the present theses work was to give our contribution in this important range of concentrations. The whole work can be divided in two main parts, one regarding the behaviour of pure sugar and the question of the existence of the anhydrous polymorphs alpha and beta, studied by means of DSC experiments. The other part of our investigations was focused on solutions starting from the dilute regime to follow the evolution of the dynamical properties of trehalose as concentration increases. These studies were performed by means of dynamical light scattering techniques.

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2.1 - What is a glass?

So far the glassy state has been mentioned several times as responsible of many of the exceptional properties of trehalose, but what is a glass?.

Angell¹ gave the following definition: “A glass is a condensed state of matter which has become non-ergodic by virtue of the continuous slow down for one or more of its degrees of freedom”. On this approach matter is seen having a degree of freedom that fluctuates at a rate strongly dependent on temperature and pressure and become so slow for low T or high P that fluctuations are frozen. A phenomenological description of this state follows.

A straightforward way to obtain a glass is heating a solid (for example, a crystalline material) above its melting point, then taking energy away from it fast enough to reach what is called the undercooled liquid state. When going through the crystallisation temperature, this liquid will either crystallise or face the path leading into the glassy state, depending on its intrinsic chemical structure and on operative parameters such as the cooling rate. From the theoretical point of view any liquid, cooled with a proper rate under its crystallisation point, should become a glass, although experimentally cooling rates are finite so there is a limit in the possibility to prove if this affirmation is true for every system. Substances capable of forming glasses with the experimentally available cooling rates are generally referred as “glass formers”, in particular “good glass formers” if the cooling rate is slow.

In the glassy state, the material has many solid-like properties but its X-ray diffraction pattern resembles qualitatively the one for a liquid. In the liquid, molecules rotate, diffuse, etc. As energy taken away from the system (it is cooled), some motions decay as their energy barrier becomes too high to overcome. If this process occurs slowly molecules are able to explore the conformational space and minimise their free energy. As a consequence, without the presence of other conditions (for example steric hindrance) ordering takes place and crystallisation arises. The phenomenon involves a large group of molecules in long range interactions, and is characterised by the periodicity of distribution, homogeneity reflected in a diffraction pattern as a series of Bragg peaks. Conversely, when cooling fast, motion is restricted and molecules are quenched in a configuration away from equilibrium, most commonly in a local energy minimum, preventing ordering. Thus, long range order is not possible and the material formed in this way is not homogeneous. This amorphous solid phase is known as glass.

2.2 - Stability of the glassy phase

The first conclusion arriving intuitively is the liquid to glass transition is not an equilibrium process. The system quenched in a local energy minimum could, in principle, evolve to a global minimum energy state. As there is a finite probability for this process to occur, the system is considered *metastable*. According to the level of probability, the amorphous phase can be classified as *relatively stable or unstable*.

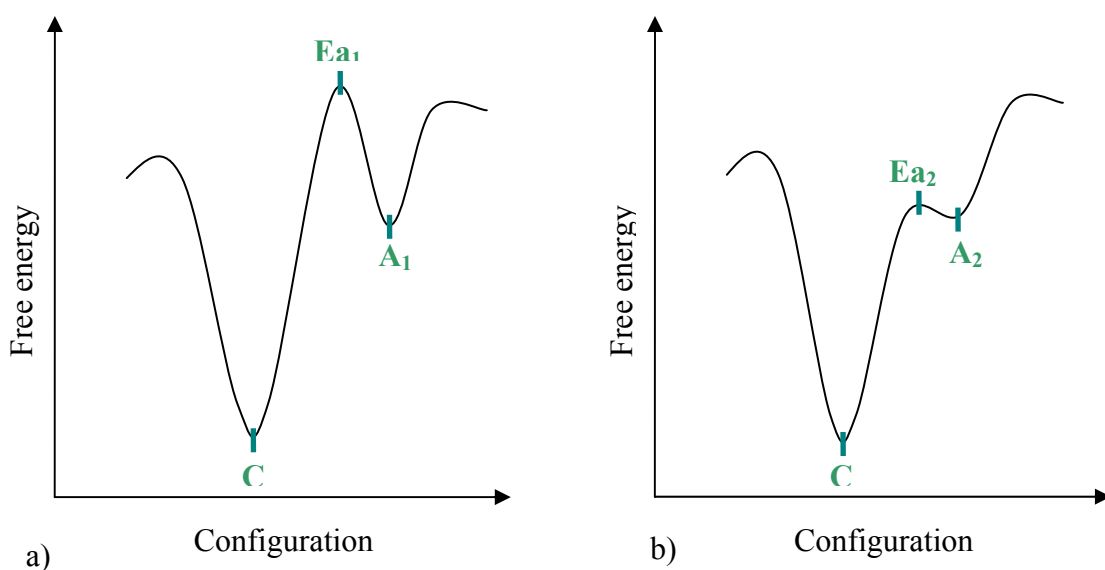


Fig 2.1. Free energy representation of two kinds of amorphous system: a) Relatively stable and b) Relatively unstable. E_a represents the energetic barrier between the two states, C (crystalline) and A_1, A_2 , (amorphous)

Figure 2.1 shows a representation of the free energy diagram for both amorphous phases. When the potential barrier between the configurations of the crystal and the glass is high, the amorphous is trapped in a local minima remaining in its original state and becoming *relatively stable*. In the case of amorphous in b) the activation energy is low so the probability of a transition into the crystalline state is much higher. If a proper amount of energy is added to the system it can easily overcome the barrier and crystallise, what determines its *relatively unstable* classification².

From this viewpoint, it is interesting to note that the beta polymorph would give rise to a relatively stable amorphous phase which tends to remain as such, whereas the amorphous obtained undercooling the fused alpha polymorph crystallises readily, then it would constitute a relatively unstable amorphous phase. From here on, we will

call the amorphous phases obtained from the fusion of alpha and beta crystalline forms as trehalose A and B, respectively. Figure 2.2 shows the different behaviour of A-trehalose and B-trehalose phases on the same thermal cycle. The difference between the amorphous phases could be relevant in the role of trehalose as a bioprotectant. We propose that if this difference exists, it should be reflected on some property of the glass and, chosen the right observable, experiments could demonstrate that the two glasses obtained from alpha and beta behave in a different way under the same environmental conditions. The search of this difference is one of the main motivations of the calorimetric measurements performed.

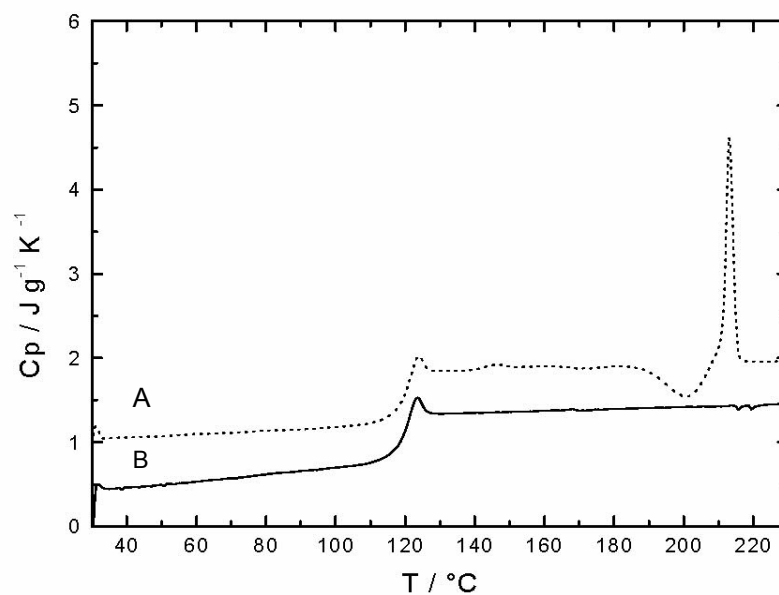


Figure 2.2 – Thermal behaviour of the amorphous phases A trehalose and B trehalose.

It was previously said that the glass can be obtained cooling a liquid under its melting point and that the state achieved is not in equilibrium, so a question emerges: how to know when the phase transition takes place?

2.3 - The glass transition

2.3.1 – Thermodynamic considerations.

During a phase transition there is an evident change in the properties of matter. If a determined property is monitored (e.g. enthalpy, entropy, specific heat, etc.) it is

2. The glassy state

possible to establish when the transition occurs. The glass transition is an exception in this sense. Many properties show anomalies on their dependence on temperature when approaching the T_g resembling a II order transition, although it is not the case. (For a detailed treatment of this argument see ref [1] and references therein). Probably the reason for this anomalies can be found in the departure from equilibrium, as it was mentioned before, during the glass transition the thermic equilibrium of the liquid is lost. This loss is sometimes called “ergodicity breaking” to emphasize the fact that glasses are non-ergodic. On this scenario, a conventional definition of the glass transition is needed.

One of the transport properties changing abruptly during the glass transition is the viscosity. It depends strongly on temperature and can vary up to 14 orders of magnitude when going through T_g . Experimentally it was observed that the viscosity of many materials within this transformation range of temperatures was in the order of 10^{12} Pa.s, so it was adopted universally to define T_g as the temperature at which the system reaches this viscosity value.

Another definition, regarding the thermodynamic aspects of the transition describes it as the phenomenon where the derivatives of the amorphous phase thermodynamic properties change more or less abruptly going from the values for the liquid state to that of the crystal. The temperature at which a discontinuity is observed is called the glass transition temperature T_g . However, some authors¹ recalling the kinetic character of this phenomenon prefer to talk about a glass transformation range rather than temperature and underline the word transformation for what they hesitate to call a transition.

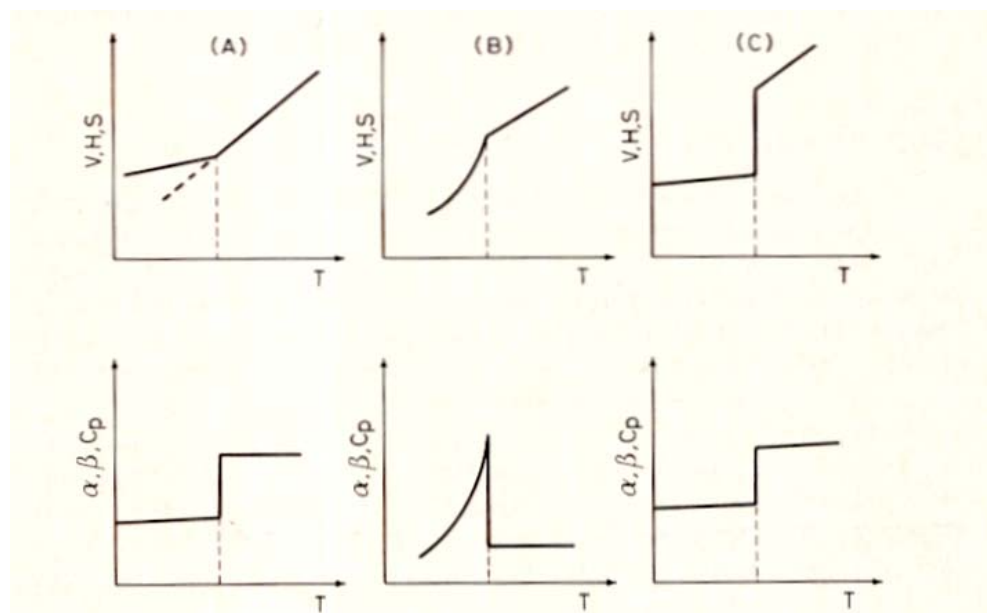


Fig. 2.3 – Comparison between three different types of transition. a) Glass transition; b) second order transition; c) first order transition.

Figure 2.3 shows the temperature behaviour profile for enthalpy, entropy and specific volume as an example for three different types of transition. A first order transition (Fig. 6-c) presents a jump in the observed variables and also on the derivatives, while a continuous change in variables (Fig6-a) characterises the glass transition. This feature makes it similar to a II order transition (Fig.6-b).

The fact that a property change gradually makes it difficult to establish a unique T_g . The most widely used technique to determine T_g is calorimetry. In a calorimetric experiment, energy exchanged between the sample and the instrument is measured against a reference as a function of time. The representation of that power versus temperature is called a thermogram. If the system is in equilibrium at all times, the magnitude represented corresponds to the heat capacity C_p . A typical thermogram including the glass transition region is shown in 2.4.

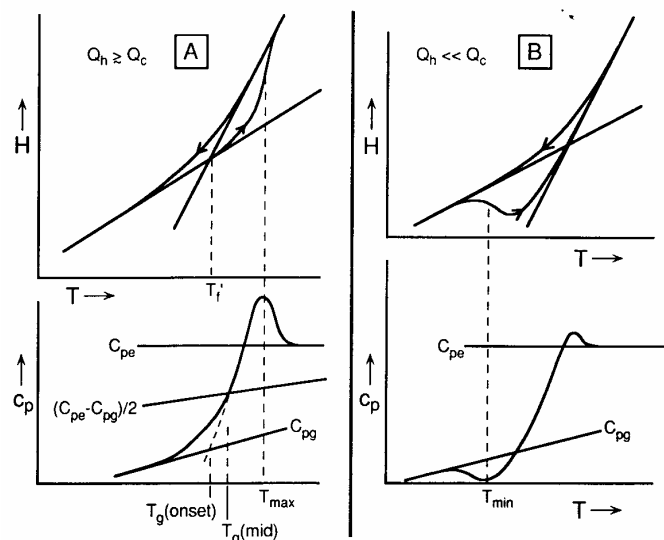


Fig. 2.4 – C_p and enthalpy curves for a glass on heating (a) and cooling (b). Definition of T_g onset, T_g midpoint.

There are several ways of determining T_g from a thermogram. The procedure consists always in finding the cross point between the extrapolation of the properties of the glass and the liquid. The most common one is T_g onset, which is the temperature corresponding to the cross point between the extrapolations of the glass C_p and the tangent at the inflection point of the sigmoidal baseline representing the glass

transition. Another used parameter is the Tg midpoint, which represents the point where half of the mass has trespassed the glass transition. Tg Half-Width represents the point on the curve that is halfway between the onset and end points. Still the Tg inflection point is the point between the limits at which the slope of the curve changes from increasing to decreasing or vice versa. The existence of different criteria for assigning Tg explains in part the extensive range of temperatures attributed to the Tg of many compounds, in our case trehalose.^{3,4} reported a Tg of 79°C; many authors agree that Tg onset of trehalose is somewhere around 121°C. But there are other more relevant factors conditioning the determination of Tg as will be discussed next.

2.3.2 – Kinetic considerations

It is clear at this point that the glass transition is a kinetically controlled phenomenon. The characteristics of the glass are strongly conditioned by the methods used to reach that state. Tg measured depends not only on the state of the glass (thermal history, ageing etc) but also, and dramatically, on the scan rate. Generally speaking, from this viewpoint the liquid has no “memory” and Tg determined during cooling is more stable than Tg determined by heating. It was proposed that rather than an arbitrary Tg with no physical meaning, a more representative parameter as Plazek’s volume crossover or the Hodge cooling fictive temperature could be adopted.¹ However, due to intrinsic instrumental problems, determining Tg on cooling may not be the better choice in terms of precision, Another fact to consider is the entity of the transition. For some materials, the Cp difference between the liquid and the glass may be small, so the transition is difficult to detect.

Considering a Tg determined on heating, the higher the scan rate, the more will the Tg be moving to higher temperatures, and viceversa for the cooling rate. It was mentioned before that when the cooling rate is high, molecules have little time to reorganise before the freezing of motion. It follows that a faster cooling will produce a glass further from equilibrium than one obtained by slow cooling. Then, if the cooling rate is very slow, the glass transition should be approximately similar to a real phase transition. On this base, Anderson defined the *ideal glass transition* as the one occurring over infinite times (rates near to 0), having the characteristics of a phase transition.²

2.3.3 – The Kauzmann’s paradox

Kauzmann used the difference in entropy of the liquid and the crystal at the melting point, ΔS_f to rescale the data for 6 different substances, as seen in fig. 2.5. The extrapolation to zero excess entropy led to provocative results for the most “fragile” glass former he studied, lactic acid. Its excess entropy decreases so quickly that it would vanish at a temperature around $2/3$ the fusion temperature. This thermodynamic inversion is prevented because of the occurrence of a kinetic phenomenon, the glass transition. Wouldn’t it, the crystal would have a higher entropy than the glass, which is not possible.

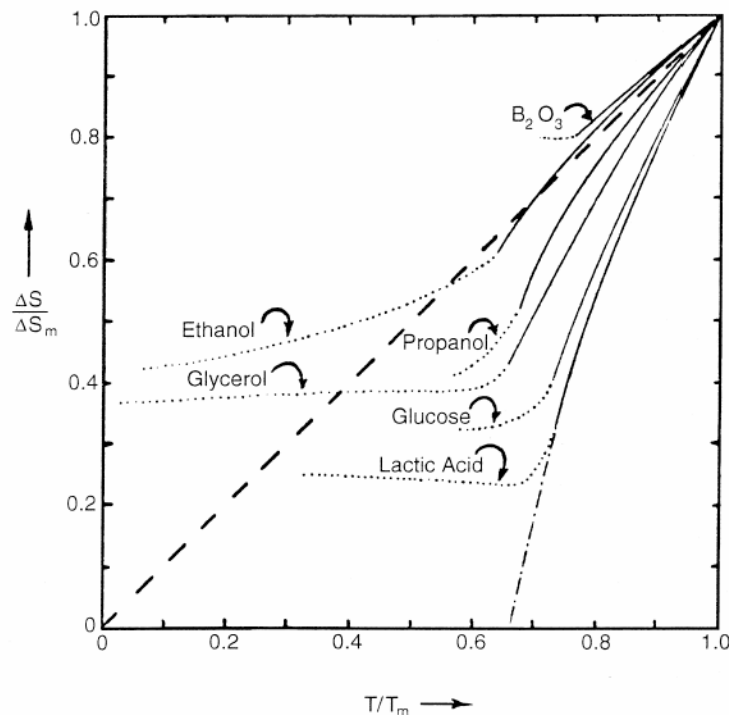


Fig 2.5 - The Kauzmann paradox. From ref. [1]

The glass transition “traps” the system in a conformational energy minimum, a ground state, at the temperature where the entropy would vanish, called the Kauzmann’s temperature T' . For this reason it is assumed that if there would be any thermodynamic phase transition, it would occur at this temperature T' . At the moment, this statement remains a philosophical exercise, since the extrapolation $\Delta S \rightarrow 0$ implies for certain materials infinite times, not accessible experimentally.

2.3.4 Structural relaxation

At temperatures below but not so far from T_g , some diffusive motions are still active in the glass. If a glass is let to “age” at this temperature for some time, molecules reorganise in a tentative to minimise their energy and approach equilibrium. This rearrangements are called structural relaxations and they are at the origin of what is known as physical aging or annealing.

Goldstein and Gibbs⁵ established that the arrangement of molecules when a liquid is cooled below T_g must be near the bottom of a potential energy minimum, otherwise the glass would flow, which is known not to happen at temperatures below half of the T_g . On the other hand, the existence of the ageing phenomenon on temperatures near T_g implies that there are many minima which differ slightly in energy one from another, also called “configurons”, that the particles explore until they arrive to a sort of ground state. This state has an energy still well above that of the crystal and is into it that the system tends to settle as excess entropy tends to vanish.

It is well known that ageing is strongly dependent on temperature. It is very fast near the T_g and becomes slower as T decreases, mainly for two reasons. The first regards the energy barrier between two minima, which gets harder to overcome as the system loses energy. The second considers the entropic barriers raised as minima become more distant. As a consequence, the rearranging group or the number of successive rearrangements must be larger as temperature decreases.

The dependence of the viscosity with temperature in a simple liquid follows an exponential behaviour, well represented by an Arrhenius like equation:

$$\ln \eta = A + \frac{Ea}{RT} \quad 2.1$$

This formula is also suitable for describing the viscosity dependence at $T \leq T_g$ for η intervals of 5 to 6 decades. Experimental data show a bending from this law at a certain temperature, as if the activation energy would increase with decreasing temperature. This was accounted for in the Vogel-Fulcher law:

$$\eta_{VF} = \eta_{VF}^0 \exp(T_{VF} / (T - T_0)) \quad 2.2$$

The VF law fits viscosity data for intervals of η changing 10 to 12 orders of magnitude and anticipates a singularity at $T=T_0$ for T_0 located somewhere below T_g . A fit of viscosity data with this two models is shown in figure 2.6 for comparison.

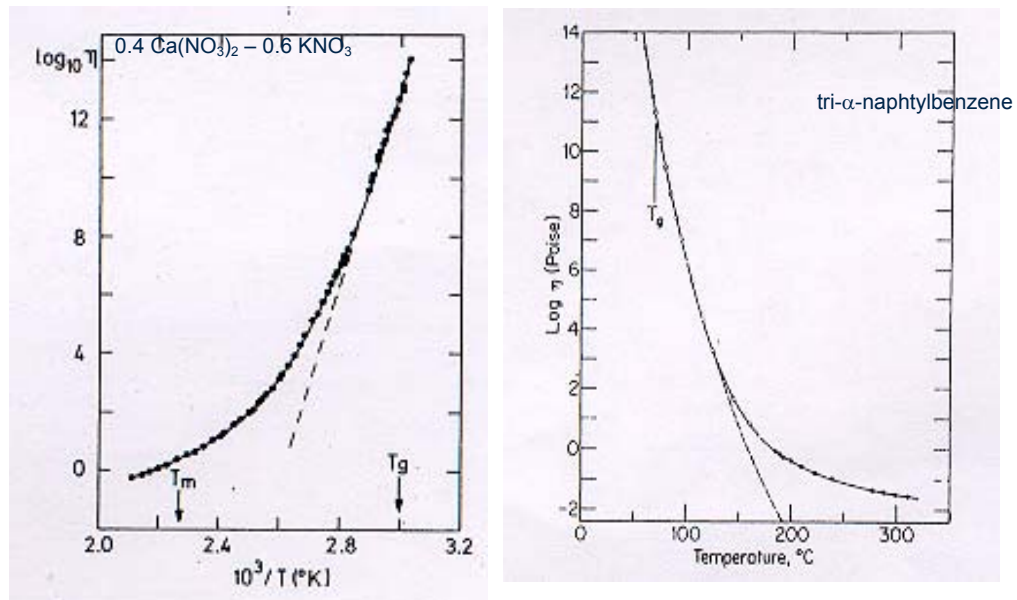


Fig. 2.6 - Viscosity of CKN, T_g 333K, T_m 438K. fitted with an Arrhenius law: $\text{Log}_{10}\eta = -90 + 3430/T$ and TNB, T_g 69°C, T_m 199 °C fitted with a VF law, $\text{Log}_{10}h = -17.46 + 4100/(T-200)$. Taken from ref. [6]

From this figure it is also comes out that there is a certain critical temperature where the curve $1/\eta$ vs T changes trend, which implies also a change in the behaviour of the structural relaxation. This temperature T_c , although not evident for some kind of glass formers, has an important physical meaning, as the crossover temperature between the dynamics of the liquid and the glass.

The most widely used expression to describe the evolution of the viscosity when the Arrhenius behaviour fails is the Williams-Landel-Ferry equation, valid above T_g . Applied to the viscosity it yields

$$\frac{\log \eta}{\log \eta_{T_g}} = \frac{-C_1(T - T_g)}{C_2 + (T - T_g)} \quad 2.3$$

where η_{T_g} is the viscosity at the T_g ; C_1 and C_2 are fitting parameters, with values around 17.4 and 51.6 respectively, changing slightly depending on the material. The WLF semiempirical equation relates the viscosity of a liquid to a temperature scaled by T_g therefore behaving as a universal law. However, Ferry and others expressed their

concerns about the interpretation of eq. 2.3 pointing out that it is somehow model dependent as the value of the parameters C_1 and C_2 could change according to the choice of T_g .

The characteristic time of molecular rearrangements τ is known to be in several systems, according to a simple viscoelasticity model⁶ proportional to the viscosity by the relation:

$$\tau = \eta / G_\infty \quad 2.4$$

Where G_∞ is the high frequency limit of the shear modulus. By this relation it is possible to observe that if the viscosity changes in 14 orders of magnitude through the glass transition, the structural relaxation and as a consequence the characteristic structural relaxation time τ also does. τ can be in the order of 10^{-12} s in the liquid state to 10^0 - 10^2 s near the calorimetric glass transition when the dynamic of the system slows down. In the approximation of weakly interacting spherical molecules, the decrease in the translational diffusion can be described quantitatively, as it is also proportional to the viscosity, by the Stokes-Einstein relation:

$$D = \frac{K_B T}{6\pi\eta a} \quad 2.5$$

Where D is the translational diffusion coefficient and a the effective radius of the object diffusing, in this case the molecular diameter.

Analogously, the rotational diffusion is inversely proportional to the viscosity by the Stokes-Einstein-Debye relation

$$D_r \equiv \frac{1}{6\langle\tau_c\rangle} = \frac{K_B T}{8\pi\eta a_r^3} \quad 2.6$$

where D_r is the rotational diffusion coefficient, $\langle\tau_c\rangle$ the mean rotational correlation time and a_r the spherical radius,

It is worth noting that although eq. 2.5 holds practically for all temperatures above T_g , for many substances eq. 2.4 holds only for temperatures $T > 1.2 T_g$ ⁷. The latter was verified for example, in a solution of sucrose in water, with fluorescein as tracer (similar in size to sucrose⁸), WLF and SE relations were combined with the same results, supporting the supposition that around T_g diffusion would no longer be controlled by the viscosity. Apparently, for temperatures below $1.2 T_g$ there is a pronounced

enhancement of translational self-diffusion, (measured by indirect methods). The diffusion coefficient would assume the form

$$D_t \propto T / \eta^\xi \quad 2.7$$

where $\xi \leq 1$. This type of behaviour could be an evidence of dynamical heterogeneities in the glass which some authors correlate with spatial heterogeneity. In the field of polymers, Schönhals and Schlosser⁹ attributed the structural relaxation to cooperative molecular motions, extending over some nanometres in length. The *principle of scaling*, which relates the characteristic time of molecular motions to their spatial extension, supports this hypothesis. According to this model, short characteristic times correspond to fast local motions and long times are due to cooperative effects. Nevertheless experiments are controversial regarding these theories, giving rise to another speculation on the difference of the way observables weight the many body interactions¹⁰.

The relaxation phenomena present in a glass originates resonances which show up as peaks in many susceptibility spectra. Structural relaxation extends over a wide spectral range, generally around two decades in frequency, and may assume different shapes according to the nature of the glass forming material. One of the most widely used models for the relaxation function is the Kohlrausch-Williams-Watts stretched exponential function (KWW), expressed in the time domain:

$$\Phi_{\text{KWW}}(t) = \exp\left[-(t/\tau_k)^{\beta_k}\right] \quad 2.8$$

where τ_k is the characteristic relaxation time and β_k is the stretching parameter accounting for the shape of the distribution; $0 \leq \beta \leq 1$.

In the condition $\beta = 1$, the relaxation can be described by a simple exponential function. The equation for $\beta = 1$ is called Debye relaxation function, and although it can describe a limited number of systems, it is very useful as a first approximation for a compound when β is unknown or approximate to 1, and in the description of the relaxation as a sum of many independent Debye contributions. Gotze et al¹¹ describe it as “the paradigm for a dynamics without memory” since it is derived assuming random forces.

Unfortunately, an analytic expression of the Laplace-Fourier transform of the KWW function does not exist, but other functions are capable of describing as well as

the KWW the relaxation in the frequency domain. The Havriliak-Negami (HN) function assumes the form:

$$\Phi_{HN}^* = \frac{1}{[1 + (i\omega \tau_{HN})^{\alpha_{HN}}]^{\gamma_{HN}}} \quad 2.9$$

where τ_{HN} is the characteristic relaxation time, α_{HN} and γ_{HN} are form parameters with values between 0 and 1.

Alvarez et al¹². combined the two expression to find a relation between them. Each coefficient of the HN model is obtained as a polynomial function of the KWW coefficients. Through this procedure it is possible to compare the results obtained in frequency vs. time domain.

A widely used empirical function in the frequency domain is the Cole-Davidson equation. It is characterised by a nearly single relaxation time (Debye) low frequency response, and an extended high frequency tail in the loss¹³. Cole Davidson and Cole Cole functions are particular cases of HN for $\alpha=1$, $0 \leq \gamma \leq 1$ and $0 \leq \alpha \leq 1$, $\gamma=1$ respectively. The equations take the form:

Cole-Davidson:	2.10	Cole-Cole:	2.11
$\Phi_{CD}^* = \frac{1}{[1 + i\omega \tau_{CD}]^{\gamma_{CD}}}$		$\Phi_{CC}^* = \frac{1}{1 + (i\omega \tau_{CC})^{\alpha_{CC}}}$	

Lyndsay and Patterson¹⁴ gave a detailed comparison of the Cole-Davidson function with the KWW finding similarities despite their different distribution function, and found the relation between parameters of the two models. The comparison of the equations in the time and frequency domain allows the rescaling of the results obtained by means of different techniques, thus enhancing the characterisation of the dynamics of a system in a wide spectral range.

An interesting feature of these models describing the shape of the relaxation function is that it is possible to attribute the high frequency part of the distribution to short range motions sensitive to intramolecular interactions, and the low frequency part to long range cooperative motions involving intermolecular interactions. Considering

these dynamical aspects, Schonhals and Schlosser⁹ predict two different limit behaviours for the relaxation function:

$$\Psi(t) \propto t^{-m} \quad t \gg \tau_0$$

$$\Psi(t) \propto t^n \quad t \ll \tau_0$$

which in the frequency domain yields:

$$\chi''(\omega) \propto \omega^{-n} \quad \omega \gg 1/\tau_0$$

$$\chi''(\omega) \propto \omega^m \quad \omega \ll 1/\tau_0$$

where χ'' is the susceptibility function, accounting for dissipative processes. It corresponds generally to the imaginary part of the response function of the system, as C_p^* , the calorimetric imaginary heat capacity in modulated DSC experiments, ε'' , the imaginary part of the dielectric constant in dielectric spectroscopy, and is proportional to $I(\omega) \cdot \omega$ in Brillouin spectroscopy, being $I(\omega)$ the intensity of the spectrum. The parameter $n = (1+\lambda)/2$ is inversely proportional to the *obstacle* in local diffusion, while m depends on the molecular structure of the system, ranging from 1 for perfectly ordered crystals and 0 for totally disordered systems⁹. The parameters m and n determine the shape (or the length scale) of the susceptibility function and assume values ranging from 0 and 1 depending on temperature. It is worth mentioning that HN function can be inferred from these trends, with $m = \alpha_{HN}$ and $n = \alpha_{HN} \gamma_{HN}$.

The characterisation of the degree of cooperativity in a system like trehalose could give a reasonable explanation for the high T_g of this disaccharide compared to its isomers. Trehalose has a T_g as high as that of some tetrasaccharides. This would mean, from the molecular point of view, that cooperative motions are still “blocked” at the temperature other disaccharides are approaching the glass to liquid transition and something about intermolecular interactions would resemble that of oligosaccharides. The temptation to attribute to trehalose a higher intermolecular bonding energy, as one of its exceptional properties is high, nevertheless most of the experimental evidence, as well as theoretical simulations, seem to find for trehalose an overall similar intermolecular interaction energy. An interesting feature many studies pointed out is the high flexibility of the glycoside bond between the two monomers. Rotations around the glycoside oxygen are so enhanced that molecular simulations face repeatedly the need of artificial temperature increases to allow the comparison of configurational information

with experimental results^{15,16}. Would this high flexibility (or any other property) give some “polymeric” character to trehalose, it should be reflected in cooperative effects that would show up in a proper description of the structural relaxation dynamics around the glass transition.

The study of the mobility in the glass brought to light that even when structural relaxation arrests, other kind of motions are present, below as well as above T_g . These fast relaxations involve however only local motions, as vibrations of atoms or bonds, reorientation of small groups of atoms, etc. They are named with a greek letter according to their relative position to the main α relaxation, as $\beta, \gamma \dots$ etc. The most widely studied of this group are the β or secondary relaxations, perhaps because they are accessible for many materials with dielectric and other spectroscopic techniques. Due to their non cooperative character, the activation energy of these processes is relatively low. Although extensively studied, the nature of their origin is not completely elucidated and their assignment is controversial. In polysaccharides they have been attributed to the rotation of lateral groups (at low temperatures) or to local conformational changes of the main chain⁸ for $T_\beta > T_\gamma$. De Gussemé et al.¹⁷ found for pure trehalose two different secondary relaxations, which they called β_1 and β_2 . In Figure 2.7, extracted from this paper, it is possible to see three different relaxations, one is the structural (α) and the two β . β_1 , the one at lower temperatures, shows a non Debye behaviour with α , the stretching exponential ranging from 0.29 at 193K to 0.55 at 273 K. Conversely, α for β_2 remains almost constant in the temperature range 277-325 K at the value 0.46. The origin of these relaxations is still not clear, but a comparison with homologous disaccharides, glucose and oligosaccharides led to suppose that it must be related to some particular feature of oligosaccharides, as the secondary relaxations present in glucose are completely different, with a nearly Debye behaviour¹⁸ ($\alpha=0.94$). The activation energy of β_1 relaxation is similar to that attributed to local motions in chain segments of polysaccharides (e.g cellulose), suggesting that whatever the origin of this relaxations is, it brings to light the “polymeric-like” character of trehalose dynamics. β_2 cannot be related to any physical process but again this kind of relaxations have been observed in complex polysaccharides.

β relaxations originate from thermally activated processes, being frequently well represented by an Arrhenius law:

$$\tau = \tau_0 \exp\left(-\frac{E_a}{K_B T}\right) \quad 2.12$$

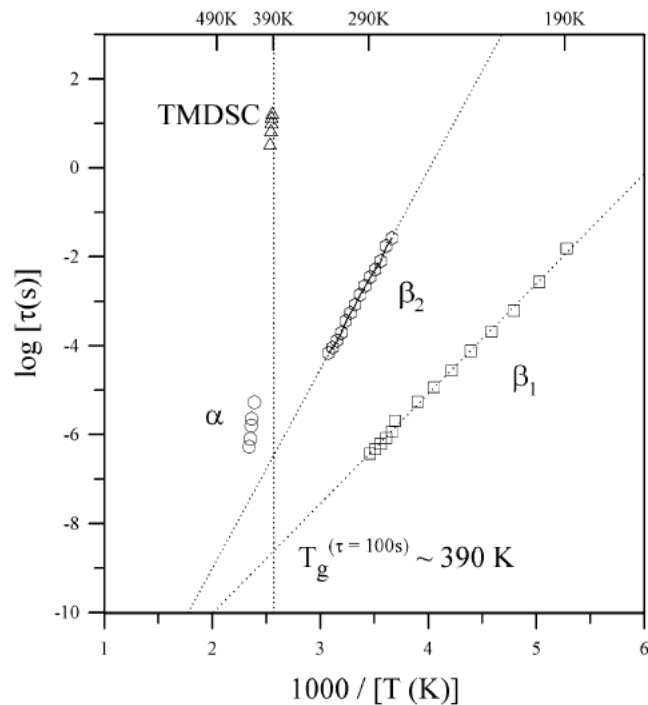


Fig. 2.7- Arrhenius plot of the different relaxations for pure trehalose investigated by dielectric spectroscopy and temperature-modulated DSC. $T=390$ K is the calorimetric T_g of trehalose. The secondary relaxations β_1 and β_2 show an Arrhenius behaviour for their characteristic times τ .

The behaviour of this relaxations is generally analogous to the one shown in figure 9 for trehalose: at low temperatures, the characteristic time of secondary relaxations becomes more and more divergent, and different relaxations can be identified and characterised independently. The fact they are referred to as sub- T_g relaxations reflects they are generally evident at temperatures below T_g away from primary relaxations. As temperature raises and approaches T_g , relaxations merge and most of the times are masked by the more intense α structural relaxation.

2.3.5 – Physical ageing

When a liquid is cooled and goes through T_g it forms a glass. If the system is not cooled enough, structural relaxation generates an evolution of the glass to a more stable thermodynamic state. As a consequence, the properties of the material change, i.e a reduction the free volume is seen. This process takes place because of the presence of molecular motion at temperatures around and below T_g , to at least T_g-50 K

in an observable time scale, and was called physical ageing by Struik¹⁹ to distinguish it from the changes induced by chemical reactions, degradations or changes in crystallinity, as the effects of this phenomenon should be totally reversible on thermal cycling. It was already pointed out that this phenomenon is strongly dependent on control parameters as temperature and pressure, and also on the intrinsic chemical structure of the material. Therefore a glass near T_g and one 30K below will age at totally different rates. The technological relevance of ageing comes out readily, as during this process and due to the structural rearrangements the physico-mechanical properties (i.e. viscoelasticity, creep, stress-strain, etc) also change. Many advanced technical materials applications involve the glassy state at a temperature below T_g . In the field of food and drugs, the stability and properties of the glassy state have serious implications on the processing and storage. Shelf-life can be determined by the ageing rate of a material, and some researchers have already tried to find a connection between this phenomenon and the chemical reactivity of a glass for applications in drugs²⁰.

Physical ageing had been already noted many years ago, and efforts for its quantitative treatment can be dated to the early 60's. Kovacs' pioneer work²¹ on volume relaxations dealt with this phenomenon. Later, physical ageing has been studied also by means of mechanical techniques (stress-strain, stress-relaxation, dynamic mechanical and thermo mechanical analysis) and calorimetry.

2.3.6 Enthalpy recovery

The changes in properties introduced by the rearrangements during physical aging can be quantitatively measured through the evolution of a state variable. The heat capacity is one of the properties affected by aging. The energy minimisation reached during aging has to be compensated before the system undergoes through the transformation into a liquid. As a consequence, if energy is added to the material there will be a more or less regular increase in temperature until the point, in the proximity of T_g , when part of this energy will be used for breaking the interactions formed during ageing, what yields an enhancement of the mobility thereafter. In a calorimetric measurement, this "sink" of energy is represented by an endothermic peak over the glass transition region, as shown in figure 2.8. As this phenomenon arises as a consequence of relaxation, it has been also called enthalpic relaxation, or T_g overshoot. The magnitude of the overshoot peak is determined by the extent of aging, and it can be studied to obtain precious information about the structural relaxation and

its characteristic time. The study of enthalpic relaxation is of great interest both for the kinetic information it provides as for the characterisation of a determined aged state. The quantitative treatment of this phenomenon will be discussed later in the experimental section.

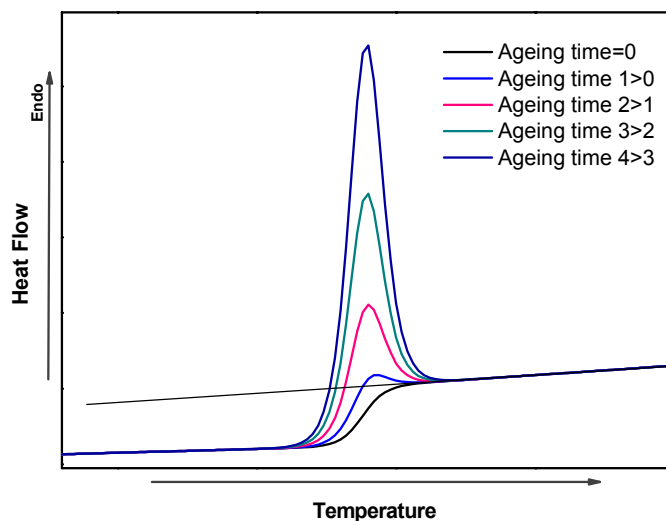


Fig. 2.8 – Enthalpy recovery for progressive ageing times.

Recalling the hypothesis of polyamorphism for trehalose, one of our objectives was to study differences in the dynamical behaviour of these. The motivation of this research was to find out if these dynamical differences would manifest through the observable C_p as an indicator of the properties of the glasses obtained from the polymorphs α -tre and β -tre. The proposal then is to assert if it is possible, beyond the polymorphism, to support the hypothesis of a polyamorphism. Should the amorphs (glasses) of both crystalline forms behave different, it would be possible to distinguish them, giving place to polyamorphism. The first evidence of the presence of this phenomenon arises from the different crystallisation behaviour. It was mentioned in chapter one that the A-glass crystallises into the β -form, while the B-glass remains stable, under the same environmental conditions.

We focused on calorimetric techniques since our laboratory has a somewhat long tradition and a well developed know how in this field.

