AN INVESTIGATION OF THE EFFECT OF CRYOPROTECTIVE AGENTS ON INTERMOLECULAR DISULFIDE FORMATION

by

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The undersigned, appointed by the Dean of the Graduate Faculty, have examined a thesis entitled An Investigation of the Effect of Cryoprotective Agents on Intermolecular Disulfide Formation

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CHAPTER I

INTRODUCTION

A cryoprotective compound is one which prevents or decreases injury caused by freezing living material. Several of these agents are known. It is not known why or how cryoprotective agents protect. It is the purpose of this study to investigate one possible explanation, on the basis of the sulfhydryl hypothesis. The major question to be answered by this study is: Do cryoprotective compounds which prevent damage in a living system due to freezing also protect proteins against intermolecular disulfide bond formation?

The model system Thiogel was used because of ease of measurement of intermolecular SS[1] formation and lack of complicating factors found in living systems. Sulfhydryl groups on the protein molecules of Thiogel are oxidized slowly in air to disulfide bonds. Freezing increases the rate of this oxidation. When the Thiogel is frozen slowly, the ice forms as a shell on the gel surface. It is the removal of water from the Thiogel to this ice shell which causes the increase in the rate of formation of disulfide bonds. As the water is removed the protein molecules come

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[1] The following abbreviations will be used throughout this manuscript: disulfide--SS, sulfhydryl--SH, dimethyl sulfoxide--DMSO.

closer together, catalyzing the oxidation reaction.

Disulfide bond formation is detected by an increase in the melting point of the gel (13). According to the sulfhydryl hypothesis any substance which would decrease the rate of disulfide bond formation as shown by prevention of a rise in the melting point of the Thiogel would presumably also protect proteins in living cells.

CHAPTER II

REVIEW OF THE LITERATURE

A. Cryoprotective agents. Substances which protect organisms against freezing injury have been known since the early 1900's (10). Bartetzko, in 1909, reported protection of *Aspergillus niger* with dextrose (10). Maximov, in 1912, obtained protection when freezing higher plant cells by simply immersing the cells in a solution of glucose (10). Maximov also investigated the protective properties of glycerol, ethanol, $CaCl_2$, KCl, NaCl, and methanol. He thought the protective action of these agents to be related to their concentrations and not to any special chemical nature of the agents (9).

Keith (8), in 1913, noted bacteria could live in frozen milk, eggs, and other foods. Since bacteria could not survive freezing in pure water he suspected these foods might be protecting bacteria. Investigations by Keith showed milk, glycerin, cane sugar, and glucose to protect bacteria against freeze killing. Keith concluded these substances to be protecting by providing spaces in the ice where the bacteria would avoid being crushed by the ice and hence killed. Since water (which freezes solid) would have no spaces or channels in the ice, the bacteria would not survive in pure water.

Between 1913 and 1949 several other investigators used sugars, glycerols and related compounds for protective agents (4). Until 1949, all investigators attributed the protective action of these agents to some non-specific effect, such as passageways between the ice crystals. Then in 1949, Polge, Smith, and Parker, while working with spermatozoa and glycerol, recognized that these protective agents gave some direct protection against injury. This discovery started the revolution leading to renewed interest in cryobiology (4).

Another important discovery in the field of cryoprotective agents was made by Lovelock and Bishop in 1959 (14). Lovelock and Bishop stated that the following properties were necessary for a substance to be a good protective agent: (1) nontoxicity, (2) low molecular weight, (3) high solubility in aqueous solutions, and (4) ability to permeate living cells. On these grounds they then predicted dimethyl sulfoxide (DMSO) would be a good protective agent. Today DMSO is one of the most satisfactory protective agents in use.

At present the study of cryoprotective agents is well under way. For although cryoprotection works in practice, it is not known how it works. Many agents are known to give moderate to strong protection; and equally as important, many substances have been investigated which show no protection. A study of the properties of agents (those which do and those which do not protect) may eventually determine the basis for cryoprotection (4).

The present state of investigation is mainly concerned with the mechanism of action of cryoprotective agents. Several theories have emerged concerning this mechanism of action. One of the most prominent is Lovelock's theory. His theory of freeze damage states that injury is caused by an increased electrolyte concentration in the cells as water is converted to ice. He believes that cryoprotective agents protect by buffering this salt concentration and therefore prevent damage to the cell (14).

Another concept of cryoprotection deals with hydrogen bonding. Doebbler states that there is a correlation between the hydrogen bonding ability of an agent and cryoprotection. According to his theory, those compounds which protect can create an extended region of hydrogen-bound water around their molecules. During rapid freezing this bound water cannot be completely transferred to the growing ice crystals. Providing ice crystals are the agents of freeze injury, these compounds protect by preventing complete freezing (5).

Vinograd-Finkel et al. believe protective agents protect by allowing for the vitreous phase in freezing (6). Ho et al. postulate that cryoprotective agents bind at certain sensitive cellular sites. They protect these sites and prevent freeze damage (17).

It is emphasized that a single protective agent does not protect all organisms. Even those that are protected do not react to the same degree under the same conditions. For example, Sakai showed sucrose to protect cabbage cells, but Terumoto found it gave no protection to a green alga *Aegagropilla Sauteri* (9). Although protection by sucrose is mainly associated with plants, there are a few cases where it has given some protection to animal cells. Vos and Kaalen found that sucrose protected tissue cultures of human kidney, but the protection was not as good as that given by DMSO, glycerol, and glycols (18). Bender found sucrose gave good protection to bone marrow cells (18). Mazur has found several sugars which protect microorganisms against freeze damage (15). Fry has also obtained fair protective results with the sugars sucrose and glucose when freeze drying some microorganisms (7). Doebbler, Rowe, and Rinfert obtained protection for red blood cells with glucose (6).

A survey of the literature on cryoprotection points up these quantitative and qualitative differences in protective agents. A few further facts should be noted here concerning these agents. The agents can be divided into two groups, those which penetrate the cell and those which do not penetrate the cell. Sugars fall into the latter group. Meryman states that penetrating agents appear to be the only ones which protect nucleated animal cells during slow freezing (16). As shown above, some exceptions to this statement are found in the literature. Meryman also states that the non-penetrating agents give protection to erythrocytes or red blood cells (16).

Another factor which affects the protection by these agents is the freezing rate. In general, a rapid freezing rate (several degrees per second) causes more injury than a slow freezing rate (one degree per minute) (16). Glycerol added to erythrocytes, which are frozen slowly, gives protection, but if the freezing rate is rapid more damage is done than if nothing at all were added (16). The non-penetrating polymers give protection with fast freezing rates. The exception to this slow freezing rule in animals appears to be only erythrocytes and in some cases spermatozoa (16). Rapid freezing rates cause the formation of intracellular ice which is lethal (16). If red blood cells are frozen rapidly enough to cause intracellular ice then they too are injured. Meryman believes the reduced size of the red blood cells and their high permeability to water allows for their survival with rapid freezing (16).

Glycerol and DMSO are two agents which penetrate the cell. These agents primarily protect animal cells, but a few exceptions will be noted here. Maximov found 2N glycerol protected cabbage epidermal sections to some extent, but he obtained better protection with 2N glucose (9). Krull got good results when using DMSO on cabbage sections (9).

B. Sulfhydryl-disulfide hypothesis. In 1962, a hypothesis on frost injury was published by Levitt (12). Since this hypothesis is the basis for the problem investigated in this manuscript, it will be stated briefly here for the convenience of the reader.

The hypothesis is known as the sulfhydryl-disulfide hypothesis. It states that freezing injury is due to denaturation of structural proteins in the cell. This denaturation is caused by intermolecular SS bond formation. Intermolecular SS bonds are formed when water is removed to ice loci outside the cell during freezing. This removal causes molecules of protein to come within close proximity of each other, allowing for SH-SS interchange or SH-SH oxidation to SS bonds. Upon thawing, water reenters the cell causing stress on the protein molecules. This stress is relieved by the breaking of the intramolecular bonds (hydrogen bonds and or hydrophobic bonds) and the subsequent unfolding and denaturing of the protein molecules (12).

At present this hypothesis explains all of the known facts related to freezing injury and frost resistance (12). Although direct evidence is still lacking for this hypothesis, several predictions based on the postulates of the hypothesis have been vindicated (11).

C. Thiogel. In 1959, Benesch and Benesch (1) discovered a relatively simple method for thiolating proteins.

Application of this technique was used by Schwarz Bioresearch Inc. to produce three different thiolated gelatins called Thiogel. The various Thiogels (designated A, B, and C) differ from one another in molecular weight and equivalent SH groups per gram.

Benesch and Benesch (1) found their thiolated gelatin to have the following properties: (1) In dilute solutions of gelatin-SH (.2 per cent solution), intramolecular SS bonds form upon addition of the oxidizing agent ferricyanide. (2) In a concentrated solution of gelatin-SH (5 per cent protein concentration), intermolecular SS bonds form upon the addition of ferricyanide. The intramolecular disulfide gelatins have a greatly reduced tendency to form gels. Benesch and Benesch believe this is caused by the SS bonds interfering with the normal hydrogen bonding which is responsible for gelation of gels. The intermolecular disulfide gelatins form a rigid gel which will not melt at 100°C. It is believed that the SS bonds largely replace the hydrogen bonds, which usually cause the gelation, resulting in an extremely high melting point.

It is for property two, above, that Thiogel was chosen for the study reported in this manuscript, namely the ability to form detectable intermolecular SS bonds.

It is noted that thiolated gelatin is a relatively recent discovery and has been available commercially only a few years. Consequently, literature available on the subject and its various uses is limited.

CHAPTER III

MATERIALS AND METHODS

Thiogel C, a thiolated gelatin, was used in this study. Thiogel is a protein which is in the denatured state. Its molecular weight is approximately 100,000 with an average of 6 SH groups per molecule. One gram lots of Thiogel were placed in large test tubes to which 10 ml of a buffered solution were added (see below). The contents were mixed with a glass rod. The test tubes were then placed in a hot water bath (between 50° and 60°C) so that the Thiogel would dissolve. Temperatures above 60°C were avoided because they increased the rate of disulfide formation to the extent that the results obtained were not accurate. Two drops of a 2% neutral red solution were added as a pH indicator and for better visibility of the otherwise nearly colorless gel.

When the Thiogel was completely dissolved, it was poured into a plexiglass plate specially constructed for flatness in order to obtain uniform thickness of the gel. These plates were 9 cm square with a depth of 5 mm. The plates were placed in a cold room with an average temperature of 2°C. Care was taken to place the plates on a level surface since varying thickness of the gel could affect the results. After 15 to 20 minutes the gel was solid enough to cut into 1 cm squares. This was done in the cold room with a number two glass coverslip. Metal cutting instruments were avoided to prevent catalysis of sulfhydryl oxidation. Squares from a single plate were used for both the frozen and cold room treatments, since the gel may vary with storage and treatment. The squares of Thiogel were placed on slightly larger pieces of filter paper and put in covered petri dishes. Half of the squares were left in the cold room and the other half were placed in a deep freeze with a temperature of -2° or -3°C. While the Thiogel in the deep freeze was coming to equilibrium, a melting point was determined for those samples in the cold room. After reaching equilibrium the Thiogel samples in the deep freeze were inoculated with ice crystals. This allowed for extragel freezing rather than intragel freezing. In a plant frozen in nature extracellular freezing normally occurs. This is to say, ice crystals form on the outside of the cell rather than inside the cell. Because of this, extragel freezing was desired in this study.

Since the temperature at which the frozen samples are stored affects the rate of disulfide bond formation (13), a standard temperature of -8°C was chosen. After inoculation, the temperature was lowered to -8°C and maintained there for the remainder of the experiment. It took approximately 3 hours for the deep freeze to reach this temperature. The deep freeze was equipped with a thermostatically controlled element and a fan in order to maintain the temperature constant at -8°C.

For melting point determinations a 250 ml beaker fitted with a zinc-galvanized wire screen and a thermometer were used. Deionized water was heated in the beaker to a predetermined temperature. Squares of Thiogel were placed on the screen with a pair of forceps. The standard procedure adopted was to give one-half to one minute for the gel to begin melting. Melting was completed when all the gel had poured through the screen. If the gel did not melt completely in this time limit the temperature of the water was raised until a fresh square of gel did melt in the time alotted. Since melting points of 50°C and above are exaggerated (13) the experiment was not continued after the control gel reached a melting point of 50°.

The pH of the solution can affect disulfide formation (13). A neutral pH is desirable. Since Thiogel is acidic, it was dissolved in a basic solution of K_2HPO_4 to yield a pH of approximately 7. The control gel, *i.e.*, the one without a protective agent, was dissolved in 10 ml of .1M K_2HPO_4. Solutions containing a protective agent had 1 ml of 1M K_2HPO_4 and 9 ml of the agent. The agents sucrose, glycerol, DMSO and urea were used in 1M concentrations. Sodium chloride was used in .5M and .1M concentrations.

Sucrose was chosen because it has long been associated with protection from frost injury in plants. Sucrose is not effective on animal tissue, however. Glycerol and DMSO were chosen because they do protect animal tissue. Urea and sodium chloride were used because they are injurious rather than cryoprotective agents.

It is emphasized that deionized water should be used to make all solutions and for the melting point determinations. When deionized water was not used, results were inaccurate. It is suspected that the copper in the water catalyzed the oxidation of the sulfhydryl groups, causing the rate of rise of the melting point of the gel to increase.

Although only 2 or 3 sets of results are reported here, several months of preliminary experiments were performed. Several methods were tried and the one chosen for the final experiments was the one which gave the most consistent results. It is this method which is described above.

CHAPTER IV

RESULTS

Immediately after solidifying, Thiogel samples containing no protective agents (Thiogel control) melted at temperatures of approximately 30°C. Upon freezing, there was a sharp increase in the rate of rise of the melting point. The Thiogel control frozen at -8°C for 9 3/4 hours did not melt at 50°C (Fig. 1). Under the same conditions samples of Thiogel containing 1M sucrose and 1M glycerol melted at 36°C. This shows a definite protective action by these compounds, since the rise of the melting point was decreased approximately 15°. The same results were obtained with 1M DMSO and 1M urea (Fig. 3)---*i.e.*, these compounds showed protection to approximately the same extent as sucrose and glycerol.

No protection occurred in the unfrozen state, since Thiogel samples both with and without protective agents kept at 2°C for 11 hours showed a total rise of only 5 or 6 degrees (Fig. 2 and 4). Samples at 2°C had a rate of rise of the melting point approximately the same as those samples with protective agents which were frozen.

Sodium chloride also showed protection in the frozen samples (Fig. 5). Two concentrations of this substance were used. The .5M NaCl gave protection to the same degree as the other agents. The samples containing .1M concentrations

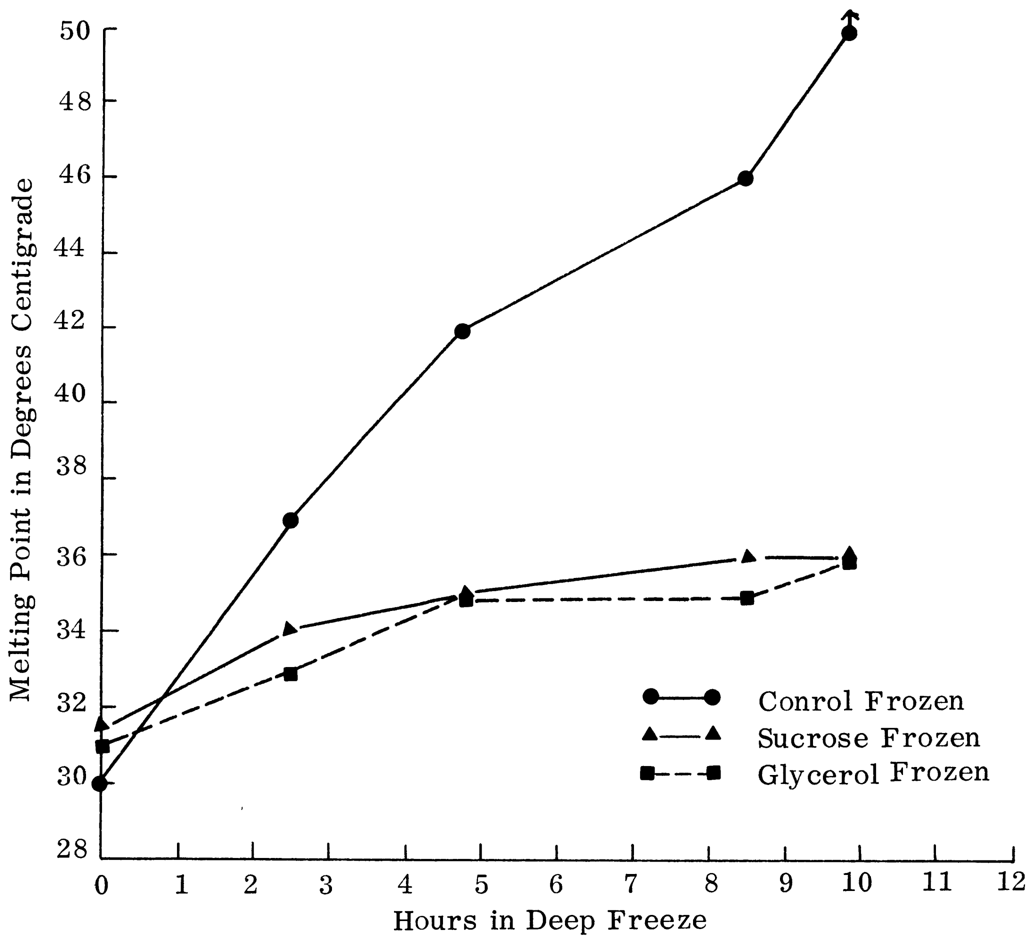


Figure 1: Rate of rise of melting point ot Thiogel control, Thiogel with 1M sucrose, and Thiogel with 1M glycerol when frozen -8° C.

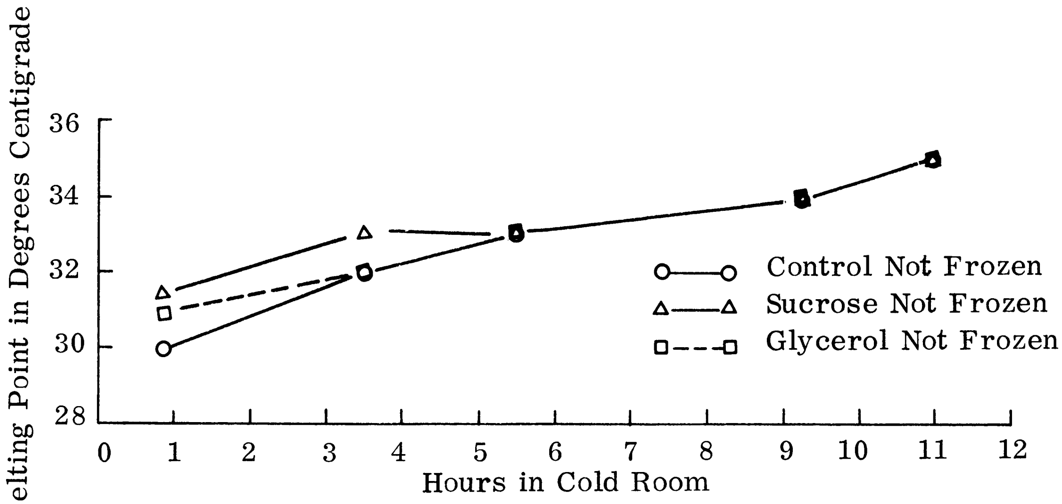


Figure 2: Rate of rise of melting point of Thiogel control, Thiogel with 1M sucrose, and Thiogel with 1M glycerol when unfrozen at 2° C.

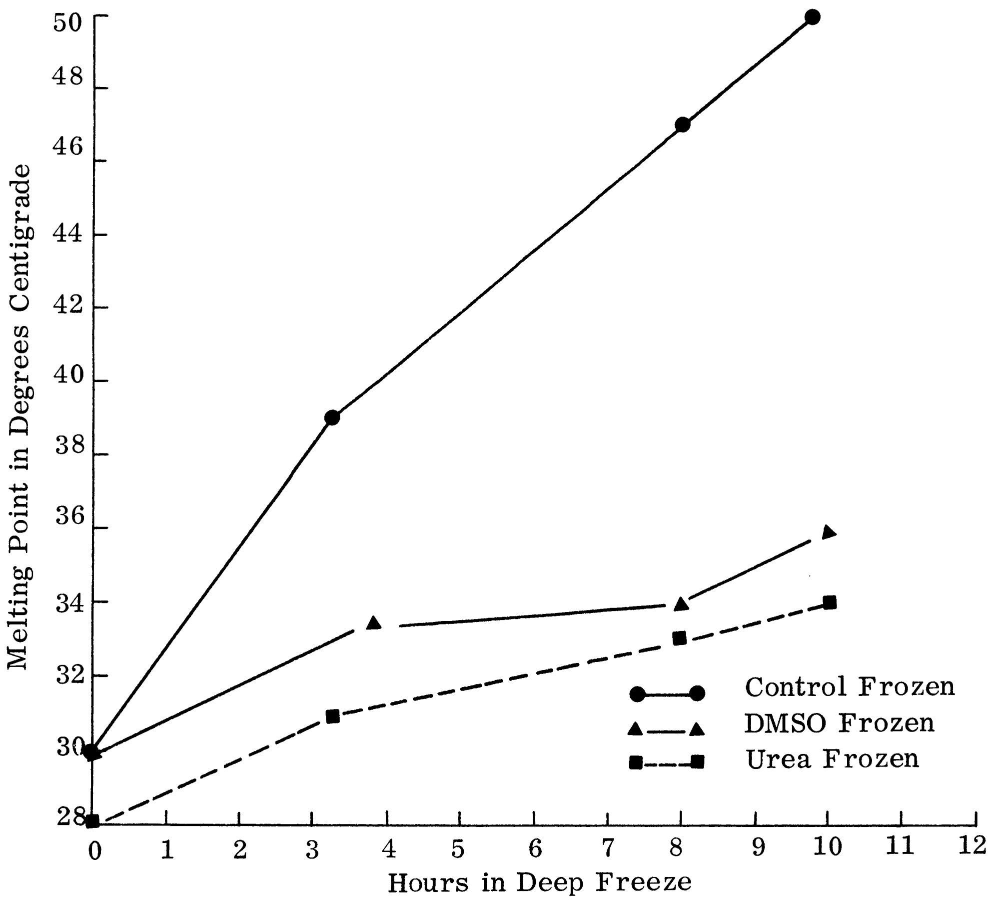


Figure 3: Rate of rise of melting point of Thiogel control, Thiogel with 1M DMSO, and Thiogel with 1M urea when frozen -8° C.

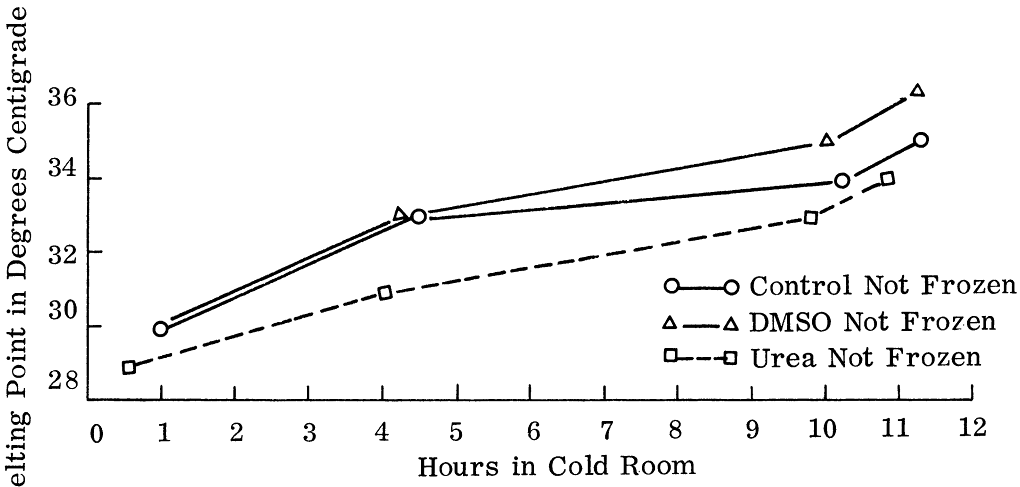


Figure 4: Rate of rise of melting point of Thiogel control, Thiogel with 1M DMSO, and Thiogel with 1M urea when unfrozen at 2°C.

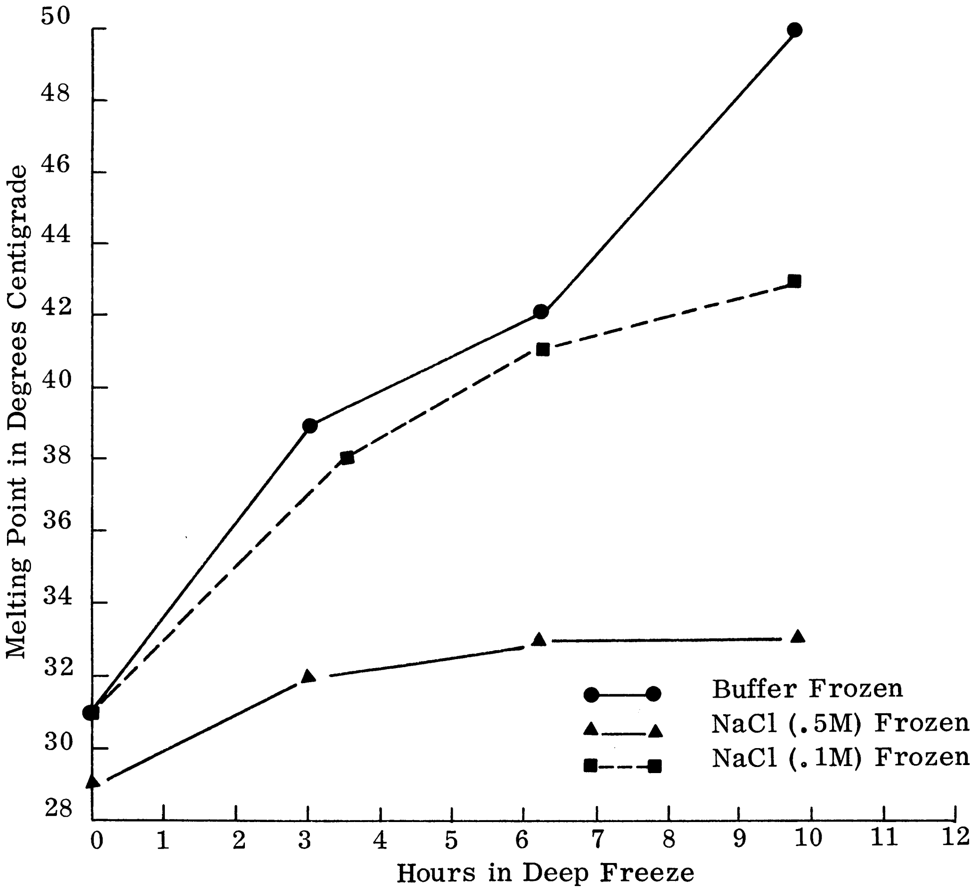


Figure 5: Rate of rise of melting point of Thiogel control, Thiogel with .5M NaCl and Thiogel with .1M NaCl when frozen at -8°C.

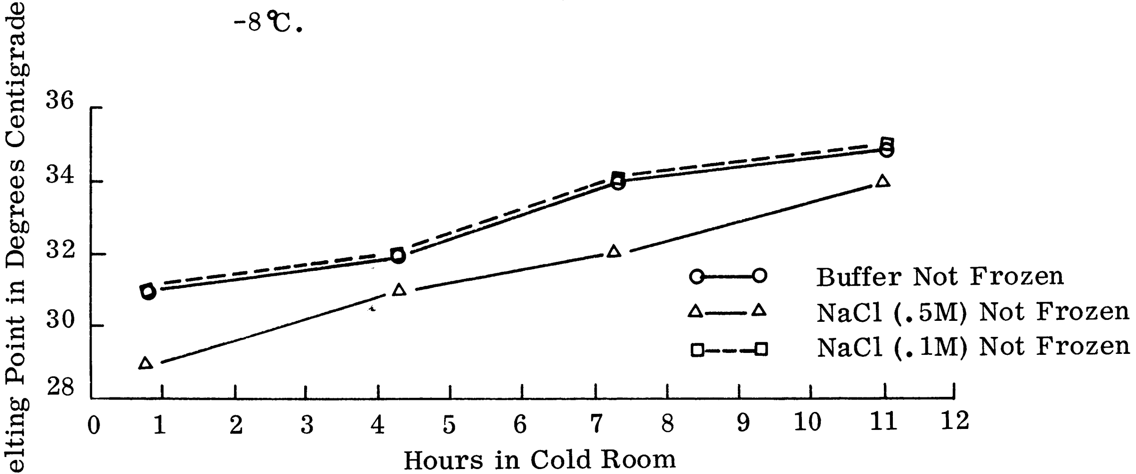


Figure 6: Rate of rise of melting point of Thiogel control, Thiogel with .5M NaCl, and Thiogel with .1M NaCl when unfrozen at 2°C.

of NaCl did not melt at temperatures as low as samples with .5M solutions of NaCl. Protection in this case was intermediate between the control gel and .5M concentration samples. Curves of samples with NaCl kept at 2°C were similar to those with other agents kept at 2°C (Fig. 6).

Melting points of samples stored at 2°C were tested over a period of 24 hours (Fig. 7 and 8). Control gels and those containing protective agents showed a gradual rise in the melting point for 12 hours. However, after 12 hours the rate of rise increased both in the control gel and in the samples containing DMSO. Samples with sucrose gradually rose to 36°C and rose no further during the 24 hour period. Glycerol samples rose to a 50°C melting point after 24 hours, but the rise was more gradual and lagged behind that of the control. (See discussion for explanation).

As stated earlier, the rise in the melting point of Thiogel is due to SS bonding. This was proven by putting samples of Thiogel which would not melt at 100°C into a .1M solution of glutathione buffered to a pH of approximately 7 (13). After 35 to 40 minutes the melting point was again at 33°C. If the glutathione solution was not buffered it had a pH of 3 or lower. Gels soaked in a non-buffered solution for 1 hour showed no change.

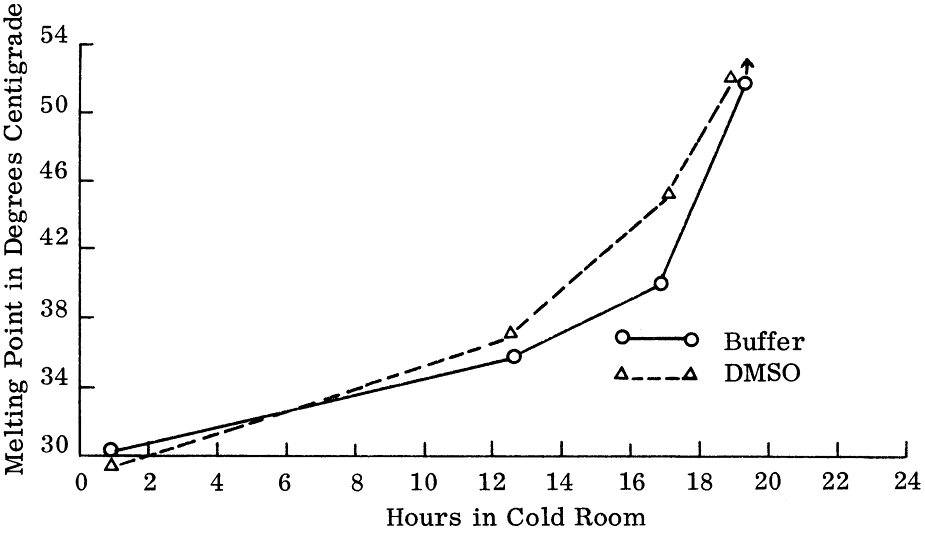


Figure 7: Rate of rise of melting point of Thiogel control and Thiogel with 1M DMSO unfrozen at 2°C for 24 hours.

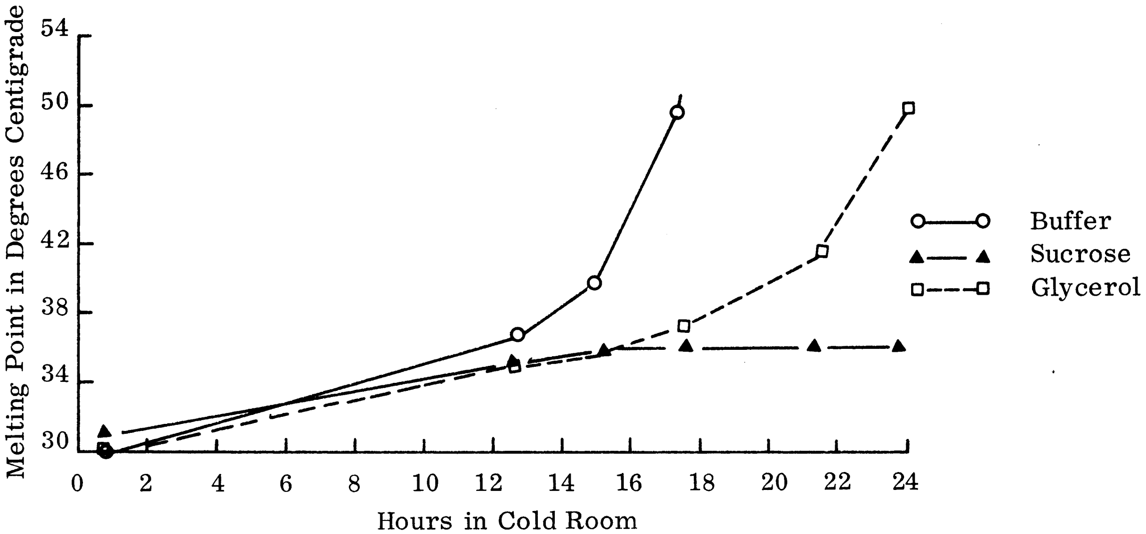


Figure 8: Rate of rise of melting point of Thiogel control, Thiogel with 1M sucrose and Thiogel with 1M glycerol unfrozen at 2°C for 24 hours.

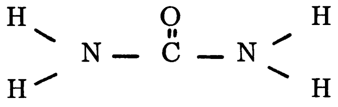
CHAPTER V

DISCUSSION AND CONCLUSIONS

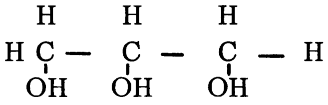
The cryoprotective agents sucrose, glycerol, and DMSO did give protection against intermolecular SS formation when used in 1M concentrations. There are several means by which these compounds could have protected.

1. It is possible that SS bond formation is prevented sterically. The naked molecules of sucrose, glycerol and DMSO, because of their size, could prevent the Thiogel molecules from coming together. If this were the case, sucrose, which is by far the largest molecule, should show the most protection (Fig. 9). It did not. Therefore, SS bond formation must be prevented by some other means.

2. Another possible mechanism of action is concerned with hydrogen bonding. In hydrogen bonding, a hydrogen atom forms a weak second bond with a strongly electronegative atom such as oxygen or nitrogen. Sucrose, glycerol, and DMSO have oxygen atoms which could bond with a hydrogen atom (Fig. 9). Hydrogen bonds forming between one of these agents and the hydrogen on a sulfhydryl group would conceivably protect the sulfhydryl group and prevent SS bonding. However, protection was obtained with a 1M concentration of urea which is a strong hydrogen bond breaker. Furthermore, protection was also obtained when using a .5M concentration of sodium chloride which does not form hydrogen bonds. In

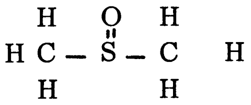


UREA



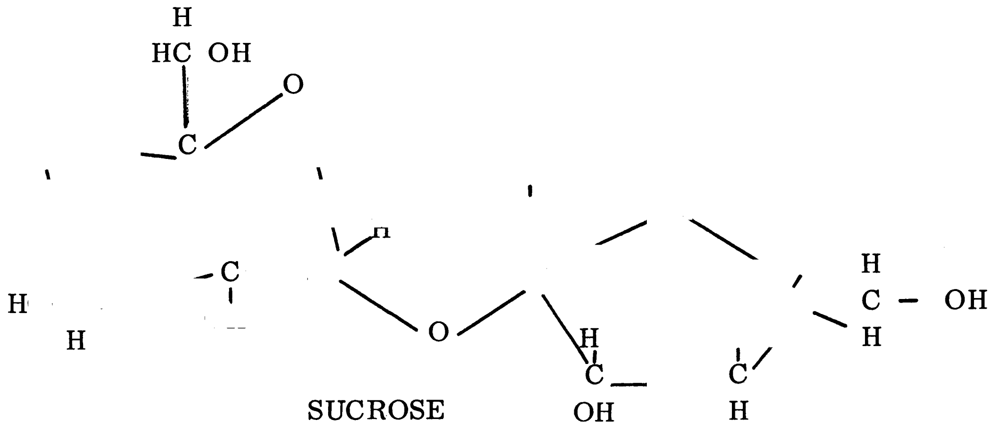
$$\mathrm{H}\,\underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}} - \underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}} - \underset{\mathrm{OH}}{\overset{\mathrm{H}}{\mathrm{C}}} - \mathrm{H}$$

GLYCEROL



$$\mathrm{H}\,\underset{\mathrm{H}}{\overset{\mathrm{H}}{\mathrm{C}}} - \overset{\mathrm{O}}{\overset{\shortparallel}{\mathrm{S}}} - \underset{\mathrm{H}}{\overset{\mathrm{H}}{\mathrm{C}}} \quad \mathrm{H}$$

DMSO



SUCROSE

Figure 9: Molecular Configurations of urea, glycerol, DMSO, and Sucrose.

addition there is little evidence of SH groups forming hydrogen bonds with oxygen and nitrogen atoms (2) (3).

3. Sucrose, glycerol, and DMSO bind water, but that this is the main mechanism of action of protection in the frozen samples is unlikely. There would be varying degrees of protection with these compounds since they do not all bind water to the same extent. All gave the same amount of protection in 1M concentrations.

4. Another explanation could be that these compounds are producing an osmotic effect which would decrease SS bonding. The agent lowers the free energy of the water causing the gels to be less dehydrated on freezing than the sample which does not contain any of these agents. This retained water would sufficiently separate the Thiogel molecules to decrease SS bonding. The fact that each agent was 1M in concentration and gave approximately the same amount of protection supports the osmotic suggestion for the mechanism of action. Further support comes from the fact that urea and sodium chloride, two compounds which are not known cryoprotective agents, also gave protection. Half molar NaCl was used to give the same osmotic effect as the 1M solutions of non-dissociating substances. The fact that .1M NaCl, a lesser concentration than others used, gave intermediate protection is in agreement with an osmotic effect hypothesis. It is concluded that the mechanism of action of protection in the frozen samples is an osmotic

effect.

Samples of Thiogel without protective agents, which were not frozen and therefore not dehydrated to any appreciable extent, had a melting point above 50°C after being stored at 2°C for 16 to 18 hours. Similar results would be expected for gels stored at the same temperature with a cryoprotective agent added since the osmotic effect due to freezing would not occur in this case. When DMSO and urea were used the expected results were obtained. In contrast, after 17 hours the sample with sucrose melted at 36°C and the one with glycerol at 37°C. Although the glycerol sample did rise to a melting point of 42°C after 21 hours, it still had a decreased rate of SS bond formation when compared to the sample without a protective agent.

Since sucrose and glycerol do bond water, it is thought this is the reason for the deceleration of rate of rise in the melting point of the unfrozen gels. An envelope of water around these molecules would certainly be a physical barrier which could decrease SS bonding by keeping the Thiogel molecules separated. Sucrose naturally would be a larger physical barrier than glycerol and this explains the difference in the rates.

The conclusions reached in this study agree with the SH-SS hypothesis. According to the hypothesis, the rate of SS bond formation is increased when water is removed from the cell, because the protein molecules are close enough to permit the SH$\longrightarrow$SS reaction. Levitt (13) has shown (as does this investigation) that intermolecular SS bonds are formed in Thiogel when water is removed by freezing. Upon the addition of cryoprotective agents, apparently enough water is retained osmotically to protect the protein molecules by decreasing their coming together and this is the cause of the decreased rate of SS bond formation.

Although Thiogel is a thiolated protein much like those found in living plant cells, it must be emphasized that there is a great difference between Thiogel and a living cell. For example, the protein molecules of Thiogel are denatures and those in the living system are not. Because Thiogel is a non-living system many experiments may be done with Thiogel, which if the same experiments were performed on a living organism, would result in death of that organism. Since Thiogel has no membrane, many problems of permeability due to the cell membrane are avoided. For example, sucrose will not permeate the living cell to the same extent as will DMSO and glycerol. A comparison of the protective effects of these agents when inside the cell is therefore impossible. Even though using Thiogel avoids many of the problems one has with living material, one should realize that the final test of a hypothesis must be performed with living material. A model system allows one to experiment with obstacles at a minimum, but this model system can not replace the living system for final proof of a theory.

This investigation, while being far from the final answer on cryoprotective agents, points the way toward further research. Further work on osmotically retained water in Thiogel would be useful, followed by experiments with living plants.

CHAPTER VI

SUMMARY

1. The results of this study show the first evidence that the model system Thiogel can be protected from intermolecular SS formation by cryoprotective agents.
2. Protection was found with the known protective agents sucrose, glycerol, and DMSO.
3. Protection was also found with urea and sodium chloride, compounds which are not cryoprotective agents.
4. It was concluded that these agents were protecting the frozen samples by an osmotic effect.

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APPENDIX

TABLE I

ACTUAL TIME AND MELTING POINTS FROM WHICH GRAPHS IN FIGURES ONE AND TWO WERE PLOTTED

Hours in cold room or freezer	Melting Points in Degrees Centigrade					
	Buffer		Sucrose		Glycerol	
	Cold room	Frozen	Cold room	Frozen	Cold room	Frozen
3/4	30		31.5		31	
2 1/2		37		34		33
3 1/2	32		33		32	
4 3/4		42		35		35
5 1/2	33		33		33	
8 1/2		46		36		35
9 1/4	34		34		34	
9 3/4		50^+		36		36
11	35		35		35	

TABLE II

ACTUAL TIME AND MELTING POINTS FROM WHICH GRAPHS IN FIGURES THREE AND FOUR WERE PLOTTED

Hours in cold room or freezer	Melting Points in Degrees Centigrade					
	Buffer		DMSO		Urea	
	Cold room	Frozen	Cold room	Frozen	Cold room	Frozen
1/2					29	
1	30		30			
3 1/4		39				31
3 3/4				33.5		
4					31	
4 1/4			33			
4 1/2	33					
8		47		35		33
9 3/4		50			33	
10			35	36		34
10 1/4	34					
10 3/4					34	
11 1/4	35		36.5			

TABLE III

ACTUAL TIME AND MELTING POINTS FROM WHICH GRAPHS IN FIGURES FIVE AND SIX WERE PLOTTED

Hours in cold room or freezer	Melting Points in Degrees Centigrade					
	Buffer		NaCl (.5M)		NaCl (.1M)	
	Frozen	Cold room	Frozen	Cold room	Frozen	Cold room
3/4		31		29		31
3	39		32			
3 1/2					38	
4 1/4		32		31		32
6 1/4	42		33		41	
7 1/4		34		32		34
9 3/4	50^+		33		43	
11		35		34		35

TABLE IV

ACTUAL TIME AND MELTING POINTS FROM WHICH GRAPH IN FIGURE SEVEN WAS PLOTTED

Hours in cold room or freezer	Melting Points in Degrees Centigrade	
	Buffer	DMSO
3/4		30
1	30.5	
12 1/2	36	37
16 3/4	40	
17		45
19 1/4	52^+	
19		52^+

TABLE V

ACTUAL TIME AND MELTING POINTS FROM WHICH GRAPH IN FIGURE EIGHT WAS PLOTTED

Hours in cold room or freezer	Melting Points in Degrees Centigrade		
	Buffer	Sucrose	Glycerol
3/4	30	31	30
12 3/4	36.5	35	35
15	40		
15 1/4		36	36
17 1/2	50^+	36	37
21 1/2		36	41
23 3/4		36	50