Structurally Caused Freezing Point Depression of Biological Tissues

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ABSTRACT When investigating the freezing behaviour (by thermal analysis) of the glycerol-extracted adductor muscle of *Mytilus edulis* it was observed that the temperature of ice formation in the muscular tissue was up to 1.5°C lower than the freezing point of the embedding liquid, a 0.25 N KCl solution with pH = 4.9 with which the tissue had been equilibrated prior to the freezing experiment. A smaller freezing point depression was observed if the pH values of the embedding 0.25 N KCl solution were above or below pH = 4.9. Reasoning from results obtained previously in analogous experiments with artificial gels, the anomalous freezing depression is explained by the impossibility of growing at the normal freezing temperature regular macroscopic crystals inside the gel, due to the presence of the gel network. The freezing temperature is here determined by the size of the microprisms penetrating the meshes of the network at the lowered freezing temperature. This process leads finally to an ice block of more or less regular structure in which the filaments are embedded. Prerequisite for this hindrance of ideal ice growth is a sufficient tensile strength of the filamental network. The existence of structurally caused freezing point depression in biological tissue is likely to invalidate many conclusions reported in the literature, in which hypertonicity was deduced from cryoscopic data.

I. INTRODUCTION

The well known controversy concerning the question of isotonicity or hypertonicity between the intra- and extracellular spaces in tissues has been studied since 1901, when Sabbatani (19) claimed that the freezing point of mammalian skeletal muscle or mammalian liver tissue showed a significantly depressed freezing point when compared to mammalian whole blood *in vitro*. This was repeatedly confirmed by others; *e.g.*, reference 7.

In order to clarify further this problem numerous investigators repeated these experiments within the last 15 years and contradictory results were published. Opie (16), Pichotka (18), and others reaffirmed the earlier results of Sabbatani, whereas Conway and McCormack (5) and others (3), who in order to avoid decomposition subjected the tissue to a rather radical treat-

ment, claimed the contrary. According to Conway (5) the previous results were caused by chemical decomposition.

When the view was expressed by Pichotka (18) that a depressed freezing point might be specific for living tissue, experiments were undertaken in our laboratory to test the situation with artificial gels. This investigation led to the observation, with a number of these gels, of a freezing point difference (one or several degrees) between the solvent contained in the gel and the solvent constituting the embedding liquid with which the gel is in thermodynamic equilibrium. It was found that the phenomenon is due to the impossibility of the formation of intact macroscopic crystals inside the gel, because of the presence of a filamental network. This effect, the influence of intact structural elements of an intact gel system on the freezing point depression, had hitherto not been noticed or considered.

It was demonstrated (11) that a synthetic gel which is tested by thermal analysis (*i.e.* observation of the temperature of the gel as a function of the cooling time), shows formation of ice, not at the freezing point of the swelling fluid of the gel, but 1°–2°C below that temperature (See Fig. 1, Curve ϕ). This was, as mentioned, explained (9) by the assumption that the liquid contained in the gel is restricted by the network to a kind of microcrystalline growth. As is well known in the case of microdroplets, microcrystals have a higher vapour pressure than the corresponding macrophase. The resulting freezing point depression of a cube-shaped crystal is given by:

$$\Delta T = -\frac{4 \cdot \sigma \cdot T_0}{a \cdot l_0 \cdot \rho} \tag{1}$$

where σ signifies the interfacial tension between ice and water, T_0 the absolute freezing temperature of pure water, a the linear dimension of the cubic crystal, l_0 the specific heat of melting, ρ the density of ice. By inserting the values for water:

$T_0 = 273°C$
$\rho = 1 \text{ gm/cm}^3$
$l_0 = 80 \text{ cal/gm}$
$\sigma = 10 \text{ erg/cm}^2$

We get as an approximation:

$$\Delta T = -\frac{3.65 \cdot 10^{-6}}{a} \tag{2}$$

The above value of the interfacial tension between ice and water is an estimate based on the differences in density. A more detailed mathematical treatment is given elsewhere (1, 9–12, 15). The freezing point depression is therefore a function of the mesh size of the network. This was affirmed in reference 15.

There with aqueous gels of a mixture of polyacrylic acid (PAA) and polyvinyl alcohol (PVA) the mesh size of the network was evaluated from the experimentally determined values of the modulus of elasticity and the degree of swelling. The values obtained were in agreement with the size of the ice crystals calculated from the structurally caused freezing point depression of the same gel using Equation 2. The determination of the structurally caused freezing point depression is therefore a possible method of obtaining information about the microstructure of a gel (12).

In a recent paper (10) we referred to a further property of systems with a structurally caused freezing point depression: the temperature of *melting* of these systems is not considerably lower than that of an iso-osmolar solution (See Fig. 1, curve μ). In the case of gels containing a network of molecular filaments, it was shown with the aid of x-rays that the difference between the freezing and melting curves arises as follows. Instead of a conglomerate of microcrystals, the ice formed in the gel actually consists of larger single crystals. A small ice crystal in the gel, whose normal growth is hindered by the network, forms well oriented microprisms that grow between the filaments at the lower temperature, later embedding the filamental network that initially interfered with regular growth. The depressed temperature on freezing thus results from the small size of the microcrystals that are first formed while the nearly normal temperature of melting is characteristic for the larger single crystals. The paper mentioned (10) should be consulted for further details. It has been pointed out that the difference between the freezing and melting behaviour (as illustrated by the D region of curve ϕ and curve μ of Fig. 1 and explained in the way just mentioned) could be taken as specific for the influence of a filamental network structure on the freezing point of the system.

It has however to be stressed that not all gels show the structurally caused freezing point depression. Its occurence also depends on the strength of the cross-links. With the growth of the crystals the cross-links are exposed to a considerable stress. In the case of the PVA-PAA gels mentioned above, this results in a partial breakdown of the cross-links, which is shown by the fact that in a second freezing of a previously frozen gel the value of the depression is smaller; *i.e.*, the freezing temperature is higher than in the first experiment. In the case of gelatin gels where the cross-linking is only produced by weak hydrogen bonds, the destruction of the cross-links goes so far that no depression at all is observed (15).

As it can be assumed that in biological tissue a reasonable part of the liquid present is contained in a molecular network gel, it is reasonable to ask whether the structurally caused freezing point depression does not influence cryoscopic measurements in biological systems. The purpose of the experiments reported here was to find a biological gel, in osmotic equilibrium with its embedding fluid, which would show a freezing point depression as compared with the

embedding fluid. Any such depression would therefore have to be structurally caused.

Experimental Approach

In the experiments previously reported on synthetic gels (9, 11, 12, 15), thermal analysis was carried out by introducing the gels to be examined in a cooling bath of constant temperature. The rate of cooling could be regulated by the extent of insulation of the test piece. The temperature was measured by a mercury thermometer or a copper constantan thermocouple.

This procedure involved two disadvantages which we have avoided now by the following improvements. Keeping the temperature of the cooling bath constant results in a gradual decrease of the temperature difference between the sample and the cooling bath. Consequently the rate of cooling drops. In order to obtain a constant cooling a fixed temperature difference (*e.g.* 5°C) was maintained between the sample and its surrounding by an automatic regulator.

We furthermore eliminated an additional source of error in cryoscopic measurements on non-stirred systems. The temperature differences within a test piece turned out to be much higher than expected. In order to take this into account we measured in each case the temperature difference between center and edge of the samples. There were, therefore, three thermocouples used in the experimental apparatus: the first one measured the actual temperature of the test piece by recording the thermovoltage between the test piece and an ice water mixture on a microvoltmeter, multiflex, type Mg 0. (Dr. R. Lange, Berlin). The second one measured the temperature difference between center and edge of the sample by recording on a multiflex galvanometer type Mg 1 a. The third one regulated the fixed temperature difference between test piece and its surroundings by means of a third galvanometer, type miravi (Hartmann and Braun, Frankfurt/Main). A more detailed description of the experimental arrangement is given elsewhere (1).

Results of Preliminary Experiments

The experiments were started as part of a broader project concerned with the contraction mechanism of muscles (13). We therefore restricted our experiments to muscles. The smallest structural unit of the muscle fiber known until now is the myofilament. This is a gel thread with a thickness of a few hundred Angstroms, where the network consists of a system of polypeptide filaments. It was originally assumed by us that the contraction of a muscle consists in an increase of cross-links and therefore a decrease of the degree of swelling of the myofilaments. We assumed that as a result of this the mesh size of the polypeptide filaments might be within the range where structurally caused freezing point depression could be observed. Most of the results reported earlier on freezing biological tissue dealt with various muscles which most probably were in a state of cooling contracture.

Contrary to our expectations no structurally caused freezing point depression was found by testing the following muscles: rectus and sartorius of *Rana*

esculenta, diaphragm muscle of white Swiss rats, parts of the outer muscular system of *Lumbricus terrestris*. All of these muscles were tested under various biological conditions but no freezing point depression compared with an isotonic Ringer solution was ever detected.

There are several possible ways of explaining the negative outcome of these preliminary experiments: (*a*) even in a gel with a network of a suitable mesh size the occurrence of the structurally caused freezing point depression is dependent on the strength of the cross-links; (*b*) it is difficult to keep a muscle in a chemically induced contraction for sufficient time to carry out the freezing experiments, and (*c*) chemical decomposition of the tissue after removal from the organism may result in a change of the network to a mesh size that will not give structurally caused freezing point depression.

2. A BIOLOGICAL SYSTEM (TISSUE) WITH A STRUCTURALLY CAUSED FREEZING POINT DEPRESSION

The adductor muscle of *Mytilus edulis* is basically a paramyosin system (4) which offered improved possibilities of overcoming most of the difficulties referred to in the results of preliminary experiments on the skeletal muscle of *Rana esculenta* and the white Swiss rats, and the outer collagen-like musculature of *Lumbricus terrestris*. In order to fulfill its function the adductor muscle of the mollusc *Mytilus edulis* must be able to remain contracted for many hours, which it can (4, 8, 14, 17, 21). Szent-Györgyi (8) suggested conserving this muscle by extraction with glycerol. Muscles treated in this manner were reported to maintain their capability to contract for many days and even weeks after removal from the living organism. The same method of glycerol extraction has been applied also to skeletal muscle systems and the effects of EDTA, ATP, the relaxing factor, calcium and magnesium on the contraction-relaxation cycle of these actomyosin systems has been known for a long time (6).

Preparation of the Adductor Muscle of Mytilus edulis

The adductor muscle of *Mytilus edulis* (maintained in an aerated standard sea water bath at 20°C in a constant temperature room) was removed without ostensible damage to the tissue under the level of the embedding fluid and then transferred immediately to a 50 per cent mixture of glycerol and water kept at 0°C. This procedure required less than 60 seconds. (The purpose of the treatment with glycerol is, in part, the destruction of the enzyme systems which might cause a chemical decomposition of the tissue.) Following the basic method of Szent-Györgyi (8, 17, 20) the muscle was kept at 0°C for 48 hours and then transferred to a large volume of 50 per cent glycerol and water at 0°C and then gradually lowered to −20°C, where it was kept for 10 days. The muscle system left after extraction (which by some authors is still

considered as a muscle, *e.g.* reference 6, by others as a protein gel) was then washed thoroughly with decreasing concentrations of glycerol solution and eventually both washed and equilibrated with 0.25 N KCl at different pH's at 0°C, until all of the glycerol had been removed. The final, about 0.3 gm, muscle samples were equilibrated in the 0.25 N KCl at a specific pH with about 1 mg ATP per gm wet muscle added. They were kept at 0°C and at approximately the same ratio of muscle volume to embedding fluid volume. All weight measurements were made on a rapid, automatic balance in a constant temperature room. Each muscle sample (lightly blotted on ion-free, chemically inert filter paper immediately prior to the testing procedure) was tested immediately after its preparation for its freezing behaviour and in each specific case *compared* with the freezing behaviour of its embedding fluid. The time required for removal from the embedding fluid and placement in the final freezing compartment required, on the average, a few seconds. Standard physical-chemical procedures were followed to insure careful handling of the small quantity of biological tissue in order to avoid damage and outside contamination. The detailed procedure for the freezing of the synthetic gel systems, as reported elsewhere (1, 2, 9–12, 15), is essentially the same as for the *Mytilus edulis* muscle samples. For a detailed description of the preparation and behaviour of the *Mytilus edulis* samples at different KCl concentrations and pH's of the embedding fluid, see reference 8 and its related references.

Comparison of the Freezing Behaviour of a Synthetic Gel with the Behaviour of a Glycerol-Extracted Muscle

Fig. 1 shows the freezing behaviour of a system where the structurally caused freezing point depression is well established. The sample consists partly of water and partly of small pieces of the previously described PVA-PAA gel (Fig. 1). During the period *AB* the system undergoing cooling becomes supercooled. At *B* the limit of supercooling is reached, the temperature then rises and subsequently becomes constant during *BC*, all of the free embedding water freezes out during this period. During *CD* practically no freezing occurs. At *D* the swelling fluid of the gel begins to freeze. As the network is not homogeneous the fluid does not freeze at a sharp freezing point and the temperature therefore does not come to a sharp end point. At *E* all of the gel fluid is frozen, the rate of decrease in temperature becoming practically the same as before freezing the system. As shown in the second part of the curve the melting of the gel is by no means the freezing process in reverse. The temperature ranges of melting of the free water and of the gel fluid are not separated. The system melts as a whole at a temperature close to the melting point of free water. As noted above this difference in freezing and melting behaviour is characteristic of systems with structurally caused freezing point depression.

Fig. 2 shows freezing and melting behaviour of a glycerol-extracted muscle. The dotted horizontal line *B* shows the freezing temperature of the embedding fluid, a 0.25 N KCl solution at pH 4.9. The freezing temperature was found to be -0.8 ± 0.05°C (theoretically -0.84°C). The first freezing curve ϕ_1 of the

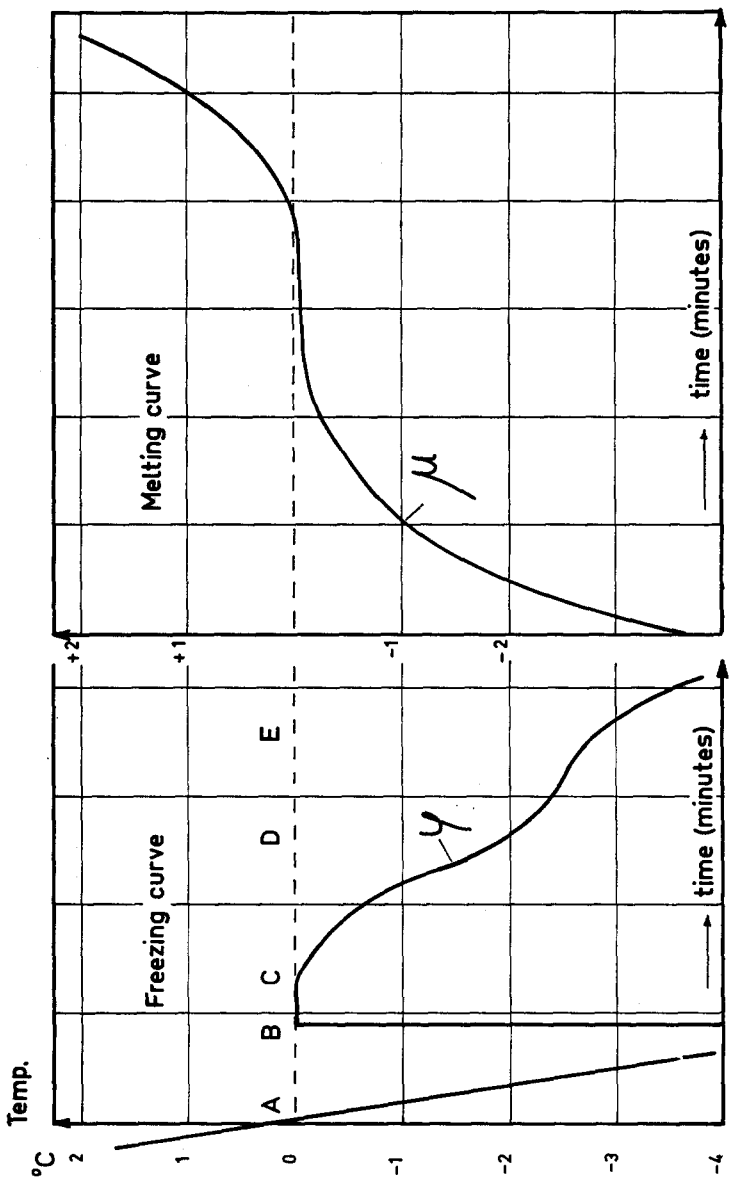

FIGURE 1. Freezing and melting behavior of a gel, consisting of equal parts of polyvinyl alcohol and polyacrylic acid, swollen in water.

ϕ = freezing curve; μ = melting curve; abscissa: time (in minutes); ordinate: temperature of sample observed.

sample shows a significant plateau[1] at −1.6°C, indicating a structurally caused freezing point depression of 0.8°C. The temperature differences between edge and centre of the sample occurring during freezing never exceeded 0.2°C. The melting curve μ of the same sample measured consecutively shows a less marked melting plateau, which however lies significantly higher than the freezing plateau. The temperature differences within the sample were of the order of 0.5°C at the end of the melting process. The difference between melting and freezing behaviour indicates that the freezing point depression is caused *structurally* and not osmotically. The second freezing curve ϕ_2 of the same sample shows an entirely different behaviour. Its freezing

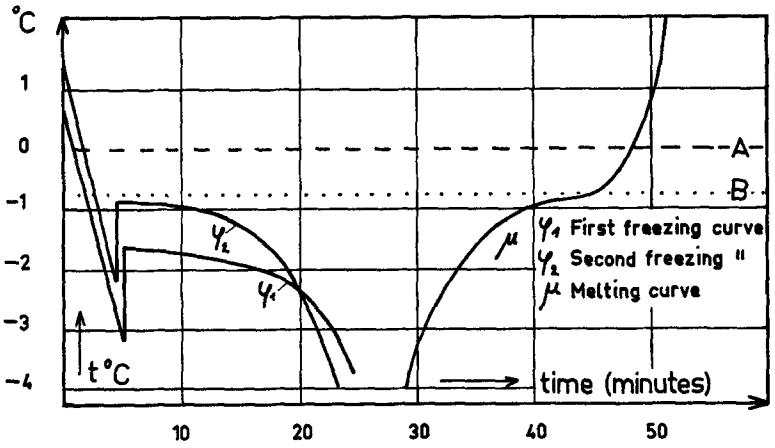

FIGURE 2. Freezing and melting behaviour of glycerol-extracted *Mytilus edulis* adductor muscle. pH = 4.9.

ϕ_1 = first freezing curve; μ = melting curve (warming up after first freezing); ϕ_2 = second freezing curve; abscissa: time in minutes; ordinate: temperature of sample observed. Dashed horizontal line *A*: freezing point of pure water; dotted horizontal line *B*: freezing point of 0.25 N KCl, pH = 4.9; liquid in which the muscles were embedded before being subject to the freezing experiment.

plateau is only slightly lower than the freezing temperature of the embedding fluid. Obviously the muscle now shows freezing damage as reported in reference 15 in the case of certain PVA/PAA gels. The extent of the freezing damage is surprising. It seems that during the first freezing the whole fluid is contained in a gel of rather homogeneous mesh size, whereas in the consecutive experiments nearly all of the fluid can freeze unhindered. The differences between the first and second freezing however indicate that systematic errors can be excluded, as such mistakes would have to occur during both experiments (Fig. 2).

[1] This plateau corresponds to part *DE* of Fig. 1. Part *BC* of Fig. 1 is missing in Fig. 2 because there was no free embedding solution in the muscle-freezing experiment.

The results on muscles were not as reproducible as those on artificial gels. In Fig. 3 we give a summary of all our measurements. The freezing point depression compared with that of the embedding fluid is given as a function of pH. It is seen that a certain number of experiments showed no significant freezing point depression. However the number of successful experiments is significant enough to prove that the muscle tested by us represents a *first reliable example of a biological tissue in which a structurally caused freezing point depression is clearly established* (Fig. 3).

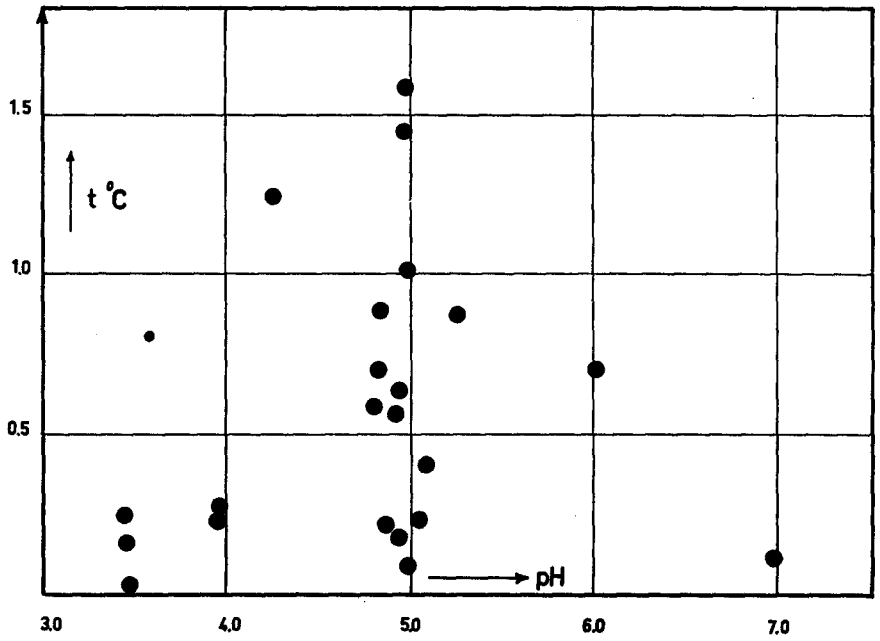

FIGURE 3. pH dependence of the freezing point depression of glycerol extracted *Mytilus edulis* adductor muscle. Synopsis of individual results. Abscissa: pH; ordinate: freezing point depression observed.

The non-appearance of the phenomenon sometimes observed under conditions where we found a large number of successful results remains unexplained for the moment. It may be mentioned in this context that in the comparable freezing experiments of Luyet and Gehenio (14) as well as in the work of Pichotka (18) it was reported that in a number of samples the accepted freezing point was not found.

Interpretation

To interpret the pH dependance of the structurally caused freezing point depression we summarize what is known about the adductor muscle of *Mytilus edulis* (4, 8, 14, 17, 21).

The tonus muscles of molluscs are called "catch" muscles and are marked by their capability of maintaining large tensions for prolonged periods of time at a very low rate of metabolic activity. The "catch" state is accompanied by a highly increased resistance against tension; *i.e.*, high values of the modulus of elasticity and high values of tensile strength. The muscle seems to be in rigor mortis (21). The muscle fibre consists of two kinds of myofilaments: filaments which are 50 to 60 A thick and filaments which are 250 to 270 A thick (17). Chemically the muscle consists of actomyosin and paramyosin, the latter being both the chief and characteristic constituent of "catch" muscles. The filaments show lengthwise, in periods of 725 A, passages of magnified density which are 125 A long. It is assumed that at these places temporary cross-linking of the different filaments occurs during contraction (4). Besides its "catch" state the muscle can behave as an ordinary muscle, its modulus of elasticity then does not show extraordinary values. It is a matter of pH whether the muscle is in its "catch" state or whether it is elastic. Szent-Györgyi (8) demonstrated that for these muscles the "catch" state occurs at a pH a little below 5 and the muscle shows normal elasticity within the pH range of 5 to 7. He explained the occurrence of the "catch" state by a crystallization of the paramyosin. It was shown elsewhere (17) that the solubility of the paramyosin showed a marked minimum at pH 5. He likewise attributed the formation of cross-links between different myofilaments to the crystallization of paramyosin.

It seems probable that the occurrence of the structurally caused freezing point depression at pH 5 can be related to the "catch" state occuring at the same pH. It seems that the crystallization of the paramyosin causes within the system of myofilaments cross-links of sufficient strength to hinder the growth of the crystals of the freezing fluid. It is therefore understandable that the structurally caused freezing point depression disappears at pH values diverging from 5, because the solubility of paramyosin increases both at higher and at lower pH values.

From the experimental results it is apparent that the structurally caused freezing point depression reaches values up to 1.5°C. Using the mathematical approximation (Equation 2) this value corresponds to a crystal or mesh size of 200 A. This agrees well with the values which have been described and which can be expected, at this time, in a network of myofilaments as described above (4, 8, 14, 17, 21).

3. CONCLUSION

The results obtained with the adductor muscle of *Mytilus edulis* prove that the structurally caused freezing point depression which had been observed with synthetic samples is by no means limited to artificial gels. Biological tissue

shows, depending on the stability of its structure, the same kind of non-osmotically caused freezing point depression. Information may be obtained from such data about the stability and eventually the limits of the width of the network structure; no conclusion can be drawn, however, from freezing point measurements as to the osmolality of biological tissue.

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