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# Study of cryostructuration of polymer systems: XIII. Some characteristic features of the behaviour of macromolecular thiols in frozen aqueous solutions

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**Abstract**

Thiol-containing derivatives of linear poly(acrylamide) (SH-PAAm) were synthesized through the free-radical copolymerization of acrylamide with bis-*N*-acryloyl-cystamine followed by the reduction of SS-bridges in the formed gel and separation of the solubilized polymeric products from the low-molecular admixtures. The polymer thus prepared, was used to study the peculiarities of the processes of cryotropic gel formation of water-soluble macromolecular thiols. It was shown that cryogenic treatment (freezing—frozen storage—thawing) of SH-PAAm water solutions containing soluble oxidants results in the formation of polymeric cryogels cross-linked with intermolecular disulphide bonds. The dependence of the oxidation rate of thiol groups on the temperature of a frozen system showed an extreme character due to the competition between the accelerating and decelerating factors; the major accelerating factor being an increase in the concentration of solutes in the unfrozen microphase after the crystallization of most part of the solvent. The position of the extreme points on the temperature axis and the absolute magnitude of the reaction rate were controlled, along with other factors, by the freezing mode used, i.e. by the thermal history of the system during its transition to the frozen storage conditions. The implementation of the low-temperature quenching procedure to the freezing of a polymer solution altered the respective kinetic curves giving rise to the appearance of lag-periods, whose duration depended on the particular temperature of frozen storage. With the use of $^{2}H$ NMR spectroscopy, the amount of unfrozen liquid microphase in macrofrozen solid samples was evaluated, and the time-dependent distinctions in the characteristics of these unfrozen inclusions were demonstrated for samples frozen using different methods. 

*Keywords*: Macromolecular thiols; Aqueous solutions of SH-bearing poly(acrylamide); Cryotropic gel formation

## 1. Introduction

Such natural polymers as proteins often contain available reactive thiol groups of cysteine in the structure of their macromolecules [1,2]. In many cases, these groups are essential for the manifestation of catalytic activity in respective enzymes [1–3]. As a rule, the SH-groups in proteins are rather sensitive to various external influences, i.e. they can be easily affected by chemical attack (oxidation, alkylation, acylation, thiol-disulphide exchange, etc.) and physical influence (such as heating, irradiation and change of the protein conformation due to the variation of thermodynamic quality of a solvent). Among similar processes, which are capable of affecting the intactness of SH-groups, cryogenic treatment (freezing—storage frozen—thawing) is known to act under certain conditions on such polymeric thiols causing, for instance, variation of the properties of some SH-enzymes and even their complete inactivation [4,5]. Furthermore, for some proteins, the freeze–thaw treatment causes intermolecular cross-linking via interchain disulphide bridges with the formation of soluble aggregates [6,7] or insoluble cryostructurates [8–10]. Such a drastic modification of protein characteristics is the reason for the formation of chimerical enzymes [11], cryochemical deterioration of food systems during their frozen storage [7], and freeze-induced structuration of protein solutions or colloidal dispersions, giving rise to cryogelled products like "koritofu" soy curd [12,13] or fish fillet-like texturates [9,14].

Unfortunately, apart from a qualitative description of similar phenomena, there are very limited data about the molecular mechanisms of these processes in frozen polymer systems, and the kinetic features of the chemical transformations of the corresponding thiol-bearing pendant chains. One of the obvious reasons for this situation is the complexity of the proteins due to their chemical polyfunctionality and strong dependence of conformational state on the

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microenvironment conditions, as such a state, as a rule, is unknown for the protein macromolecules in a frozen object. In addition, other events, for example, cryodenaturation, cryoprecipitation and cryocracking, can proceed in frozen protein solutions and dispersions, thus increasing considerably the sophistication of the process as a whole. Therefore, to elucidate the peculiarities of the behaviour of the proper SH-groups of biopolymer molecules in various frozen systems, it is necessary (as one of the possible ways) "to simplify" the object under investigation.

In our earlier works [15–17], much more simple (as compared to proteins) model macromolecular thiols were created in order to study the behaviour of such reactive polymers in aqueous solutions and dispersions both at positive and negative temperatures. It was shown that in the presence of dissolved atmospheric oxygen, the oxidative reactions in frozen systems caused a decrease in SH-content. At a polymer concentration higher than the critical concentration of gelation, the cryogenic treatment resulted in the formation of insoluble SS-linked gels (the so-called cryogels, the term introduced for the final "products" of such a cryotropic gel formation) [18]. In contrast, in anaerobic media, or in the presence of low-molecular reductants, no changes for the high molecular thiols were observed. It was also found [17] that no variation of the molecular-weight characteristics of soluble SH-polymers used was induced by the freeze-thaw influence, thus showing the absence of cryocracking phenomena under the conditions of moderate freezing.

The aim of the present work was to investigate in greater detail the influence of freezing regimes on the kinetics of the aforementioned cryotropic gel formation and to establish the main characteristic features inherent in the behaviour of macromolecular thiols in frozen aqueous solutions. These problems were studied using the specially synthesized SH-polymer: a water soluble thiol derivative of linear polyacrylamide—SH-PAAm, which was the copolymer of acrylamide and a small amount of *N*-acryloyl-cysteamine. The polymer did not contain any other reactive groups except for thiol ones. Hence, the only type of chemical transformations, which could take place during the cryogenic influence on the polymer under the employed conditions, was the oxidation of SH-functions either with atmospheric $O_2$ dissolved in the initial system or with the deliberately added external oxidant. Also, as this polymer did not include in its covalent structure either the charged, or the hydrophobic residues (usually existing in the proteins), the conformation of the SH-PAAm chains in aqueous medium was nearly the same as that for the linear non-substituted poly(acrylamide), i.e. the conformation of a statistical coil. The study of a similar derivative was, along with the modelling of some of the protein functions, also of interest, inasmuch as the freeze–thaw behaviour of such reactive flexible-chain polymers has not been previously investigated in detail.

The freeze-induced formation of both physically and chemically cross-linked cryogels has been studied for the past two decades. The most well known among these materials are the thermoreversible non-covalent poly(vinyl alcohol) cryogels [19–21]; considerably less investigated are the covalent cryogels, which, in turn, may be prepared in the frozen solutions either through the branched polymerization of respective monomers [22–24] or via the cross-linking of macromolecular precursors with reactive cross-agents [16,18,25,26]. The SS-cryogels, whose kinetics of formation were the target for study in the given work, relate to the covalent-type cryogels.

## 2. Experimental

### 2.1. Materials

The following reagents were used in the work without additional purification: acrylamide (AAm), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMED) and ammonium persulphate (APS) (all from Serva, Germany), L-cysteine (Cys) and 2-mercaptoethanol (2-ME) (Merck, Germany), bis-*N*-acryloyl-cystamine (BAC) (Pierce, USA), and 2.2′-dipyridyldilphide (DPDS) (Fluka, Switzerland).

Iodoacetamide (Calbiochem, USA) was recrystallized three times from dry heptane.

Dimethylsulfoxide (DMSO) (chemical pure grade, Reakhim, Russia) was initially purified by freezing-out, and then distilled at reduced pressure (10 mm Hg) in the stream of dry argon.

### 2.2. Methods

#### 2.2.1. Synthesis of SH-PAAm

The initial solution of AAm (9.88 g) and BAC (0.12 g) in 70 ml of deionized water, which was preliminarily distilled in a stream of oxygen-free argon, was prepared by heating the suspended monomer mixture at 50–52°C. The resultant solution was cooled to room temperature, 0.39 ml of TMED was then added, and the volume of the solution adjusted to 99 ml with water. Further, 1 ml of 5% water solution of APS was introduced, quickly stirred, and the system allowed to stand at room temperature for 0.5 h (during this time, a weak transparent gel was formed; more prolonged incubation was found to cause difficulties for the subsequent complete reductive dissolution of such a SS-gel). Then, 10 ml of 2-ME was added and the dissolution of the SS-gel continued for about 40–50 min. The obtained very viscous polymer solution was diluted fivefold with pure water and passed in 50 ml portions through the 1 l chromatographic column filled with Sephadex G-25 (coarse) molecular sieve resin (Pharmacia Fine Chemicals, Sweden). Oxygen-free 0.05 M water solution of acetic acid was used as eluent. After the separation of the low-molecular-mass fractions and the excess of reductant from the macromolecular fraction with similar gel filtration, the solution of SH-PAAm was freeze-dried. The polymer obtained was stored in a

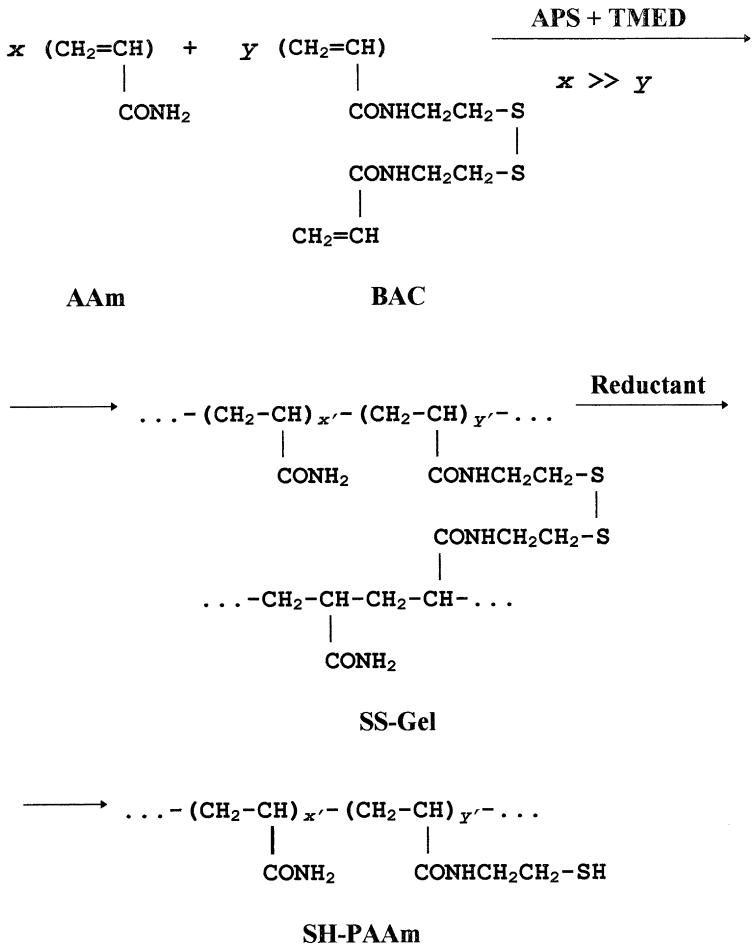


Fig. 1. The scheme for the synthesis of SH-PAAm.

vacuum-dessicator over active charcoal and dry $CaCl_2$. A conventional yield was about 5 g. The SH-group content in this product, as determined by spectrophotometric titration with DPDS by the method of Grassetti and Murray [27], was found to be 40 ± 2 μmol/g. The molar extinction coefficient of 2-thio-pyridone liberated upon the thiol-disulphide-exchange during the titration was taken as 8080 [28].

*2.2.2. S-carboxyamido-derivative of SH-PAAm*

One gram of SH-PAAm was dissolved in 100 ml of oxygen-free 0.1 M Na-phosphate buffer, pH 8.3. Then, 0.03 g of iodoacetamide was added, and the reaction mixture was stirred for 1 h at room temperature. The low molecular mass admixtures were separated from the polymeric matter by gel filtration, as in the case of SH-PAAm purification. The final S-carboxyamidated polymer was freeze-dried. The product did not contain any detectable SH-groups, thus demonstrating the exhaustive alkylation of the thiol functions in SH-PAAm.

*2.2.3. Cryotropic gel formation of SH-PAAm*

The freeze-dried polymer was initially dissolved in pure water, and the solution obtained was then quickly diluted with 0.1 M Na-bicarbonate until the SH-PAAm concentration of 2 g/dl and the buffer concentration of 0.01 M, the final pH value was equal to 7.7–7.8. One millilitre aliquots of the solution were dispensed into the 10 ml vials and placed either in a thermostat (U10 model, MLW, former GDR) for the incubation at positive temperatures, or immersed in a coolant (ethanol) of cryostat FP 45 HP (Julabo, Germany) for freezing and frozen storage according to procedure A (see Section 3), or put for 1 h into a Dewar vessel with liquid nitrogen and only then immersed in the cryostat (low temperature quenching technique—B freezing mode). After certain time intervals, the frozen samples were quickly thawed (at a rate of about 0.25 degree per second). Later, an equal volume of 1.5 mM solution of DPDS in the same, but air-free, buffer was added, and the system consisting of a soft sponge-like polymeric cryogel and free liquid was carefully stirred with a glass rod and left to stand for 30 min at room temperature. Further, the insoluble material and the free liquid were separated by filtration through the sintered glass filter, and optical absorption of the filtrate was measured at 343 nm with the aid of a Model-557 UV–VIS spectrophotometer (Hitachi, Japan) for the determination of the amount of residual unreacted SH-groups.

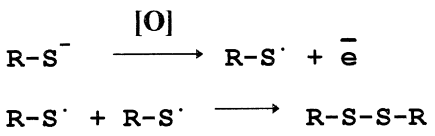


Fig. 2. The scheme of SH-compounds oxidation in neutral and weakly alkaline aqueous media [2,35].

### 2.2.4. $^2H$ NMR studies

These were accomplished with a WP 200 SY spectrometer (Bruker, Switzerland) with an operating frequency of 30.7 MHz. The relative amount of unfrozen solvent ($D_2O$) was estimated by comparative integration of the NMR signals recorded under identical conditions before and after the freezing, using the instrument software in the absolute intensity mode ($AI = 1$). The procedure was essentially the same as described elsewhere for the studies of frozen solutions of poly(acrylamide) [29].

## 3. Results and discussion

### 3.1. Synthesis of the thiol derivative of linear poly(acrylamide)

The synthesis of SH-PAAm was carried out essentially (with minor modifications) in accordance with the earlier described procedure [17], which, in turn, was based on the method by Hansen [30]. The latter technique is employed in biochemistry for the preparation of the so-called 'solubilizable poly(acrylamide) gels' by free-radical copolymerization of AAm and BAC with subsequent reduction of the SS-bonds in the gel formed (Fig. 1). Recently, the same approach was also implemented by Lee and Park [31] in the preparation of thiol-derivatives of thermoresposible poly(*N*-isopropyl acrylamide).

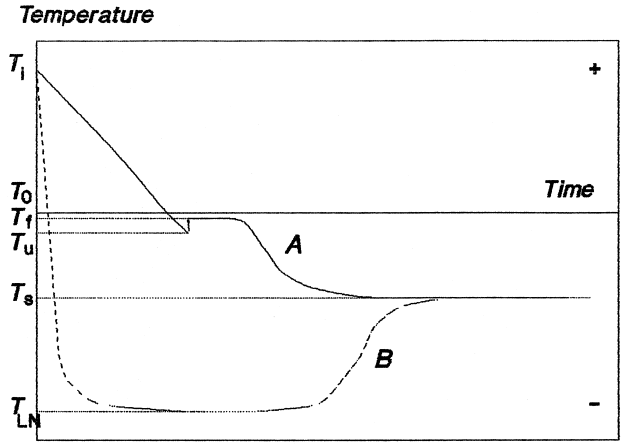


Fig. 3. The appearance of thermograms, which correspond to different procedures of the sample freezing: $T_i$: initial temperature; $T_0$: freezing point of a pure solvent; $T_f$: freezing temperature of the particular polymer solution; $T_u$: the lowest temperature of undercooling; $T_s$: frozen storage temperature; $T_{LN}$: the temperature of liquid nitrogen; (A) ordinary freezing mode; (B) low-temperature quenching mode of freezing.

The key stage in the isolation of the desired SH-containing polymer was the exhaustive separation of SH-PAAm from the excessive reductant (2-ME). This was accomplished with the preparative gel filtration under anaerobic conditions followed by freeze-drying of the polymer solution. SH-PAAm, thus prepared, contained 40 ± 2 μmol of thiol groups per 1 g of dry matter. Such an amount corresponded to the ratio of about 1 pendant SH-residue per 350 amide residues in the macromolecules under discussion and answered to $\bar{M}_c$ values around 25 000 in the SS-gel which acted as a precursor for SH-PAAm assuming a virtually uniform distribution of SH-bearing units along the core chains. Inasmuch as the molar ratio BAC/AAm in the initial monomer solution was equal to 1:150 (or 1:75 accounting for the moles of sulphur per one mole of acrylamide), one may conclude that the efficiency of the insertion of BAC molecules into the growing PAAm chains was rather low, namely around 21.4%.

As the aforementioned extent of substitution of $CONH_2$ groups with $CONHCH_2CH_2SH$ groups in the chains could not affect the molecular weight characteristics and conformation of poly(acrylamide) macromolecules in solution significantly, it was reasonable to suggest that the coefficients $K$ and $\alpha$ in the Mark–Kuhn–Houwink equation reported for PAAm ($3.73 \times 10^{-4}$ and 0.66, correspondingly) [32] would also be valid for the approximate evaluation of the viscosity-average molecular weight of SH-PAAm. In this respect, for the prevention of thiol oxidation during the viscometric analysis, the SH-groups in the pendant chains of the polymer were preliminarily alkylated with iodoacetamide (see Section 2), to give stable $SCH_2CONH_2$ groups instead of the SH-ones. The procedure used for such S-carboxyamidation was based on the method [33] reported for the same type of modification of SH-proteins. The measurements with capillary viscometer ($[\eta] = 1.70 \pm 0.03$ dl/g, 1 M $NaNO_3$, 25°C) gave the value of 349 500 ± 9400 for the viscosity-average molecular weight of SH-PAAm. This, along with the data about this particular soluble polymer, virtually showed the average size of macromolecular chains constituting the network of SS-gel and testified that the amount of pendant SH-groups in the polymer was about 10 per a chain.

### 3.2. Cryotropic gel formation of water solutions of SH-PAAM in the presence of dissolved oxygen

When studying the freeze-induced oxidation of SH-PAAm by the $O_2$ dissolved, solutions of the polymer were prepared in a medium of 0.01 M Na bicarbonate buffer (pH 7.7–7.8). The solvents used preliminarily for 24 h, in turn, were brought into an equilibrium with the ambient atmosphere at 25°C. Similar storage provided a concentration of dissolved oxygen of the order of 0.04 g/l or 1.25 mmol/l [34]. Taking into account the aforementioned content of SH-groups in SH-PAAm, one may easily calculate that at the polymer concentration, for instance equal to 2 g/dl, the

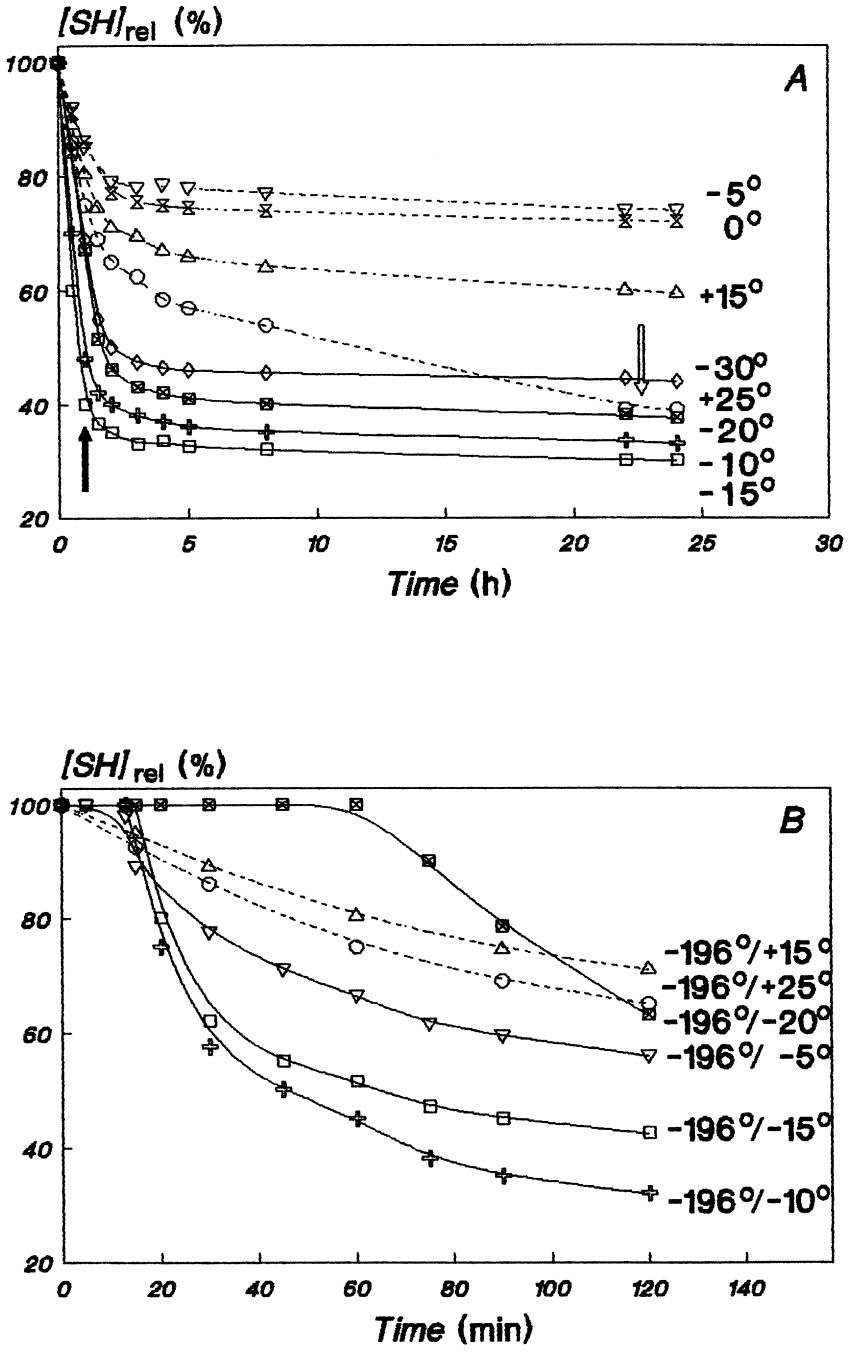


Fig. 4. The variation in time of the relative amount of residual thiol groups in the 2 g/dl water solutions of SH-PAAm unfrozen and frozen with different freezing procedures. A: ordinary freezing mode; B: low-temperature quenching mode of freezing.

initial concentration of thiol groups in such an initial solution was about 0.8 mmol/l, that is, only a slight molar excess of the oxidant existed in the system before its freezing. Also, it is well known that, according to the recognized mechanisms [2,35], the general route of the oxidative transformation of thiols to disulphides in aqueous neutral and weakly alkaline media, where a certain portion of sulfhydryl groups are in the ionized state, is described by the two-stage reaction (Fig. 2), in which 1 mole of the electron acceptors is consumed per 1 mole of thiol, and the only final product of the reaction, when the water-dissolved $O_2$ performs as the oxidant, is the corresponding disulphide.

In the course of experiments, two different modes of freezing the samples were employed:

1. Tightly stoppered vials with the polymer solution under study were placed into the liquid coolant (ethanol) in the cryostat chamber, where the pre-assigned temperature was maintained constant throughout the required time. A general pattern of the temperature variation in similar

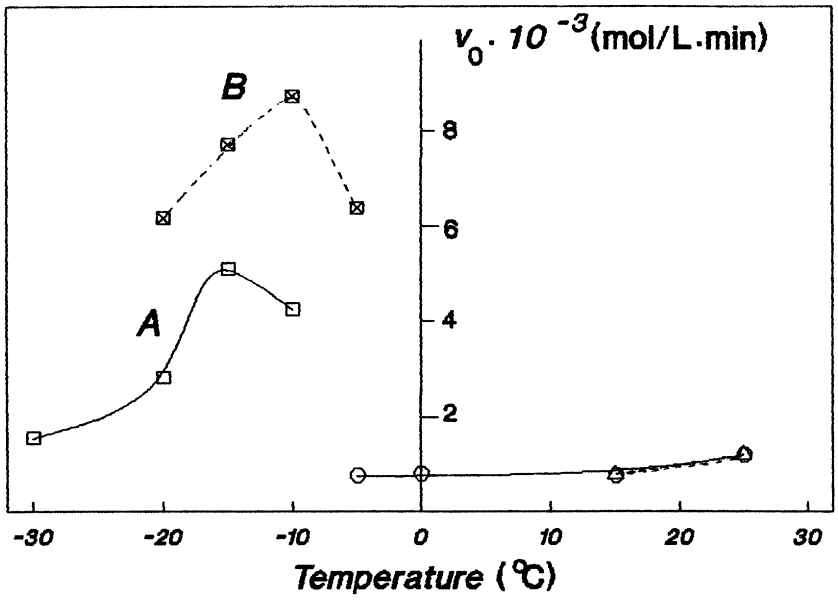


Fig. 5. The temperature dependence of the initial rates of decrease in SH-titre in 2 g/dl water solutions of SH-PAAm unfrozen and frozen with different freezing procedures.

sample is depicted by the curve A in Fig. 3. Further, such freezing is referred to as an "ordinary" freezing procedure. In this case, because of undercooling effects, the temperature of a sample during the cooling stage can decrease somewhat below (to $T_u$) the thermodynamic freezing temperature ($T_f$) of a particular solution, the solvent then crystallizes (plateau of crystallization), and the temperature eventually reaches the level of the frozen storage ($T_s$) preassigned by an explorer.

2. Analogous samples were initially flash-frozen by immersing into liquid nitrogen, kept here for 1 h, and only then were they put into the cryostat and processed in the same way as described for variant A. This second procedure is depicted in Fig. 3 as curve B, which describes the so-called "low-temperature quenching" two-step mode of freezing, which is commonly used [36] in cryochemical studies, when it is necessary to minimize the duration of the freezing stage itself. In this case, the ice crystallization plateau is not recorded in the real-time scale because of the very high freezing rate, and the frozen storage temperature is reached on warming the sample from $T_{LN}$ to $T_s$.

Using these freezing procedures, we studied the kinetics of variation of the amount of free thiol groups in SH-PAAm as dependent on the duration of frozen storage at negative temperatures, and, for the sake of comparison, unfrozen exposure to certain positive temperatures. The results obtained are shown in Fig. 4.

At +25°C, a slow decrease in the concentration of SH-groups in time was recorded, and approximately after one day storage, the formation of a very weak transparent gel was observed (pointed with a hollow arrow in Fig. 4(A). Naturally, yet slower oxidation of SH-PAAm took place at +15 and, all the more, at 0°C. In the latter case, no gel formation was registered throughout at least three days observation. Almost the same pattern was obtained at −5°C, as this polymer solution did not freeze under these thermal conditions, apparently because of the undercooling phenomenon. At the same time, when the freezing occurred (temperatures $\leq -10$°C), a considerable acceleration of both the SH-oxidation dynamics and the gelation process were observed. For example, at −15°C, the 20% loss of the initial thiol-titre took place soon after ~10 min frozen storage, the 50% loss was observed for 30–40 min, and to the elapse of the first hour a soft sponge-like polymeric cryogel was formed (indicated with a filled arrow in Fig. 4(A). Thus, in the frozen bulk at this negative temperature the gel phase was formed more than 20 times faster than at room temperature; and, even at −30°C, the chemical reaction under discussion also occurred somewhat faster than in the solution at a temperature of 55°C higher (i.e. at +25°C). In other words, the pronounced speed-up effects were inherent in the cryotropic gelation of SH-PAAm, when the freezing procedure of A type was employed.

Theoretically, for the sol/gel transition of similar reactive polymers, the participation of more than two pendant groups is required. But this is an ideal case, which does not take into account the intramolecular cross-linking, formation of soluble cross-linked associates and small microgel particles, which do not constitute the macrogel network, but in the course of their formation SH-groups are consumed. For instance, when three (say) SH-groups from the ~10 initial ones per chain react, 30% of the titrable thiols disappear; but it is very unlikely that this will result in gel formation within the total bulk of the system. The investigations revealed that

for the SH-PAAm, the formation of macrogels was observed, when 40–50% (depending on the temperature) of the initial thiol functions (that is, 4–5 groups per a chain) reacted. It could also be noted that the intramolecular cross-linking, as well as the formation of soluble SS-linked aggregates, as an intermediate state from a solution to a gel upon the oxidation of SH-bearing macromolecules, are well known for both proteins [2,6,11] and synthetic thiol-containing polymers [31].

In the case of the B freezing mode, the character of respective kinetic curves was more sophisticated. Fig. 4(B) demonstrates the kinetic curves for the first 1.5–2 h of the reaction, assuming that the start of the process corresponded to the transfer of the samples from liquid nitrogen into the cryostat chamber. One may see the existence of a certain delay (lag-period) in the propagation of the reaction, followed by a rather quick decline of the SH-content, when the rate of the SH-titre diminishment was again considerably higher than at room temperature.

It could be supposed that such an induction period was a consequence of a low temperature in the course of the slow warming of the reacting system after its transfer from liquid nitrogen into the cryostat. However, direct measurements accomplished with the thermocouple frozen in the samples (their volume was 1 ml) have shown that the time required for the temperature to reach the coolant thermal level was only 2–4 min, that is, the major portion of the lag-period extended over the constant temperature conditions. Hence, a low enough temperature was not the reason for the existence of this delay region on the kinetic curves. The particular duration of the induction period depended on the cryostat temperature (i.e. frozen storage temperature, $T_s$): the lower the $T_s$ value, the longer the lag-phase. When the reaction vials, after their storage for 1 h in liquid nitrogen, were transferred into a thermostat at +15 or +25°C, the kinetic curves obtained were identical to those recorded for samples at the same temperature, but without the low-temperature quenching treatment. This suggests that such a quenching treatment has no effect on the process under consideration in a liquid medium at room temperature.

From the linear regions of respective kinetic curves of Fig. 4(A) and (B), the values of initial rates ($\nu_0$) of the reaction of oxidation of SH-PAAm were computed. These values are presented in Fig. 5 as a function of the process temperature. As a result, the following tendencies were found:

1. The rates of SH-titre decrease, as well as of the gel formation, were always markedly higher in the systems frozen non-deeply, if we compare the $\nu_0$ values for temperatures which were equal in their absolute magnitude, but above and below the freezing point (~0°C) of the 2 g/dl SH-PAAm water solution. For example, at −15°C and the A freezing mode, the rate was about seven times faster, and for the case of the B freezing mode it was more than 10 times faster, than at +15°C. The main reason for this was due to an increase in the concentration of solutes, whereby the major portion of a pure solvent was initially crystallized during the freezing of the system, and all the soluble components were displaced to the so-called “unfrozen liquid microphase” [36–38], where the interactions of reactants occurred in a considerably more concentrated medium than the initial one. The prefix micro- in the term “microphase” implies that the volume of this liquid-like phase is small compared with the total volume of a frozen sample.
2. The temperature dependencies of $\nu_0$ values showed a maxima for both the freezing procedures used. The ascending, with the decrease on temperature, branches of the curves A (from −10 to −15°C) and B (from −5 to −10°C) reflected the trend which seemed inverse to the Arrhenius law, i.e. the growth of the reaction rate with the reduction in temperature was observed in our experiments. However, such an extreme shape of the rate vs. temperature curves is well known and is typical of the kinetics of numerous chemical reactions of low-molecular substances in multicomponent frozen solutions, provided the order of a reaction is higher than the first [37]. Here, we would like to emphasize the commonness of similar extreme temperature dependences not only for the cryochemical processes of low-molecular reactants, but also for the reactive macromolecular compounds, including not only the polymeric thiols, but also the various other systems capable of cryotropic gel formation [39]. This extreme shape of the curves A and B points to the competition between certain accelerating and decelerating factors that affect the kinetic features of the processes under discussion. The accelerating factors are known [36–38] to be the formation of regions of structural and phase inhomogeneity (crystalline phase and liquid microphase) and, as noted before, the concomitant increase in concentration of solutes in the residual unfrozen fluid, as well as the increase in its polarity with a decrease in temperature and, perhaps, the increase in pressure caused by ice crystallization [40]. The decelerating factors are rather obvious, namely, the slowing of thermal motion of particles with a decrease in temperature, increased viscosity of unfrozen inclusions, and in the case of gelation phenomena, the formation of spatial gel network inducing certain steric hidrances for the intermolecular interactions.
3. From the data of Figs. 4 and 5, a rather important conclusion of practical interest may also be drawn concerning the frozen storage of water solutions of the thiol-containing polymers, biopolymers and likewise SH-proteins. In particular, moderate freezing of similar solutions (when the dissolved $O_2$ is present) does not only suppress the chemical transformation of these compounds, but also facilitates the occurrence of these reactions. Hence, for the protection of such SH-macromolecules against freeze-induced oxidation, it is required prior to the cryogenic treatment to remove even minor amounts of an

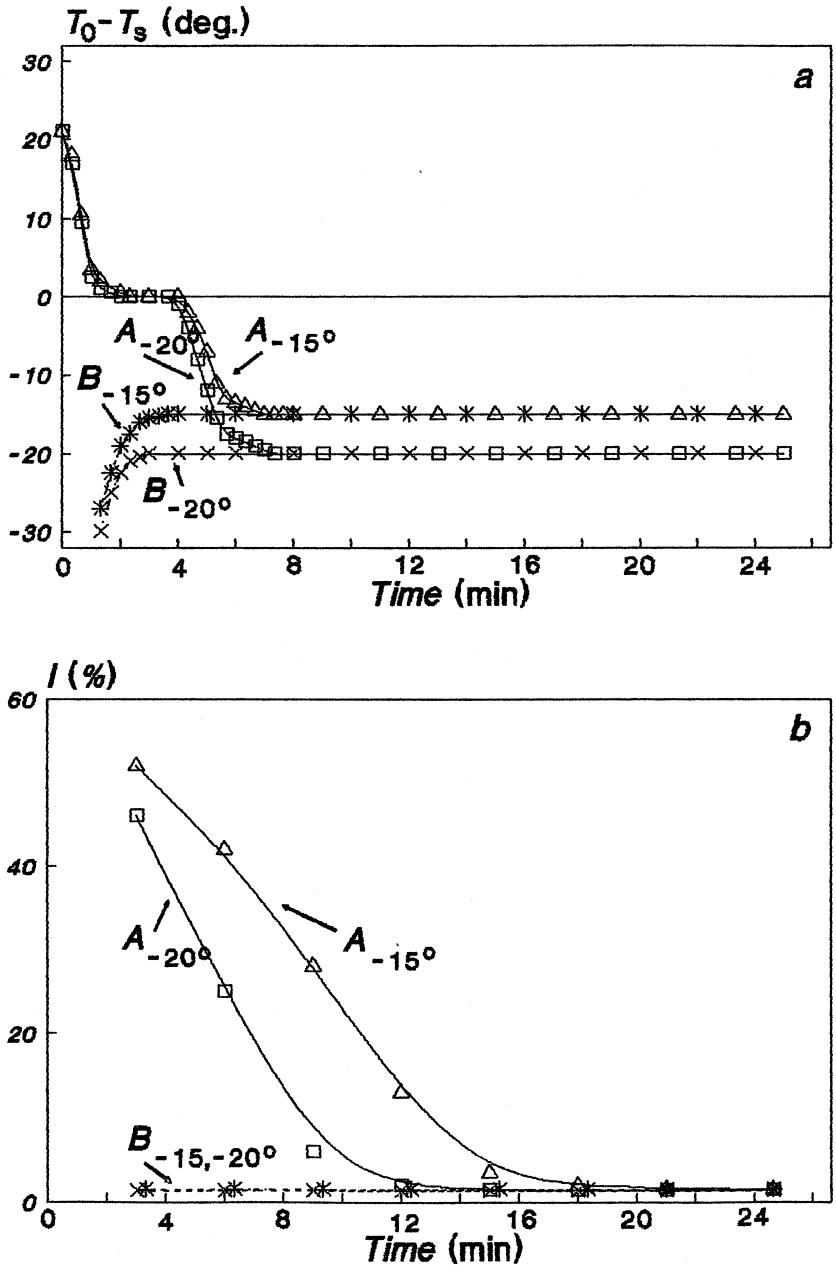


Fig. 6. The processes of the freezing of 2 g/dl $D_2O$ solution of S-carboxyamidated derivative of SH-PAAm at various temperatures and freezing procedures: (a) the variation in time of the temperature of samples; (b) the variation in time of the integral intensity of $^2H$ NMR signals.

oxidant or to add the corresponding reductants (e.g. low molecular thiols).

The existence of induction periods on the kinetic curves in Fig. 4(B), as well as the non-equivalence of the rate/temperature dependences A and B in Fig. 5 and distinct temperatures of these extreme points require a separate discussion.

We have supposed that the basic reason for these differences could be the unequal content of the components in a reaction system at the initial stages of its residence at $T_s$ depending on the freezing mode, that depends on how the system was brought to such a temperature (thermal prehistory of a sample). Indeed, when the initial solution of SH-PAAm is frozen according to procedure B at −196°C (i.e. under essentially non-equilibrium conditions), no liquid microphase should exist, however, upon subsequent heating of the samples from $T_{LN}$ to $T_s$ and thermostating, the system tends to reach phase equilibrium. Thereafter, because of the slow rates of mass-transfer processes in a highly viscous cooled polymer-containing phase, the amount of unfrozen liquid at some instant, while the systems travels to equilibrium, could, obviously, be less than in the sample that resided for the same time at $T_s$, but had been

Table 1
$^2$H NMR spectral characteristics of the 2 g/dl $D_2O$-solution of S-carboxyamidated derivative of SH-PAAm crystallized with different freezing mode

| $T_0-T_s$ (°) | Integral intensity of $^2$H signal (%)[a] | | Signal width (Hz) | |
|---|---|---|---|---|
| | A | B | A | B |
| −15 | 1.55 | 1.52 | 1150 | 1350 |
| −20 | 1.40 | 1.38 | 1600 | 2000 |

[a] In per cent from the integral intensity of $^2$H NMR signal of the unfrozen solution; error <10%.

chilled through "pathway" A. In turn, the differences in the amount of liquid solvent (its volume, as determined elsewhere [41,42] for the water–PVA frozen systems, depended on the thermal prehistory of the samples of identical initial composition) are equal to the differences in the concentration of solutes. In other words, the higher initial rates of the cryogenic oxidation of SH-PAAm in the case of freezing procedure B (the descending lines of the plots in Fig. 4(B) and $\nu_0$ values of the curve B in Fig. 5) could be the result of the initially higher extent of cryo-concentration of the reactants compared with the A-mode-frozen samples at the same temperatures $T_s$. The quantitative data on the amount of unfrozen solvent in these systems were obtained with NMR measurements (Fig. 6), which were carried out with the solutions of *S*-carboxyamidated derivative of SH-PAAm (the same derivative as was used for capillary viscometry). As the polymer was dissolved in heavy water, the temperature scale in the subsequent discussion was presented relative to the freezing point of $D_2O$ ($T_0$ + 3.8°C) [43], i.e. as the differences $T_0 - T_s$.

The graphs in Fig. 6(a) show the variation of temperature in the samples frozen by the procedures A and B and stored frozen at $T_0 - T_s$ equal either to −15, or −20°C. These data were obtained with a microthermocouple positioned directly in the polymer solution poured into the polytetrafluoroethylene ampoule. Then, the ampoule was placed (in the case of freezing mode A) in the NMR spectrometer probe, where the desired temperature was maintained. When the freezing procedure of the B type was employed, such an ampoule was initially immersed in liquid nitrogen for 1 h, and then replaced in the spectrometer probe cooled to the required $T_0 - T_s$ level. Although the conditions for the heat transfer

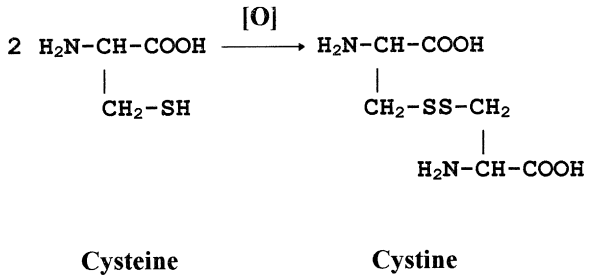


Fig. 7. The scheme of the oxidation of cysteine to cystine.

in these measurements (Teflon ampoule with the solution tested) were somewhat different from those used in the kinetic experiments (a glass vial with the polymer solution), the freezing times (A procedure) differed by only 2–3 min, and in the case of method B the differences between the times required to heat the samples from $T_{LN}$ to $T_0 - T_s$ were even smaller. Therefore, one might assume that the time-dependent variations of the amount of unfrozen solvent found by NMR analysis (this value is in proportion with the integral intensity ($I$) of the $^2$H resonance peak in the corresponding spectra) were very close to the values for the samples frozen in the glass vials. The instrumental conditions for these NMR measurements were such that the spectra were registered every 3 min, as is shown by the corresponding points on the curves in Fig. 6(B).

In the case of ordinary freezing, it was found that the temperatures of −15 and −20 degrees were reached in 8 and 7 min, respectively, whereas the amount of the unfrozen mobile (on the $^2$H NMR frequency scale) solvent varied for a significantly longer period, as the $I$ values stopped varying only after the elapse of the 19th or 23rd min, correspondingly. The higher the $T_s$ magnitudes, the more prolonged the time taken for an additional amount of water to freeze out. This obviously meant that during this time, the concentration of both the polymer and the $O_2$ dissolved in the unfrozen liquid microphase was less than during the subsequent incubation of the samples frozen at the temperatures indicated. It also meant that the oxidation reaction with SH-PAAm during this time proceeded in changing the volume of the unfrozen portion of the system. Hence, the values of the initial reaction rates $\nu_0$ should be considered only as the apparent ones, and should not be identified with the initial rates routinely determined in the classical kinetic investigations, namely, under the conditions of non-variable constant volume of a liquid system.

At the same time, when technique B for the cryogenic treatment was utilized, $I$ values reached their constant level (1.5–1.4%, see later) virtually in parallel with the stabilization of temperature, and this required only 3–4 min. In other words, this, in principle, meant that the concentration of reactants, when the oxidation process began to occur in the unfrozen fractions of a system, was higher than the concentration of the solutes in the unfrozen microphase at the onset of the cryochemical process under study in the samples crystallized by means of freezing method A. It is thought that similar differences in the concentration of the reactants may account for the differences in $\nu_0$ values (Fig. 5), observed for the water solutions of SH-PAAm frozen with A and B procedures.

As regards the solvent itself, the curves $A_{-15}°$ and $A_{-20}°$ in Fig. 6(b) testified a certain slowing of the rate of "liquid–solid" phase transition for a certain portion of the solvent: to the 7th–8th min, the temperature stopped varying, but $I$ values continued to vary for an additional 12–15 min. This, obviously, was the consequence of the effect of

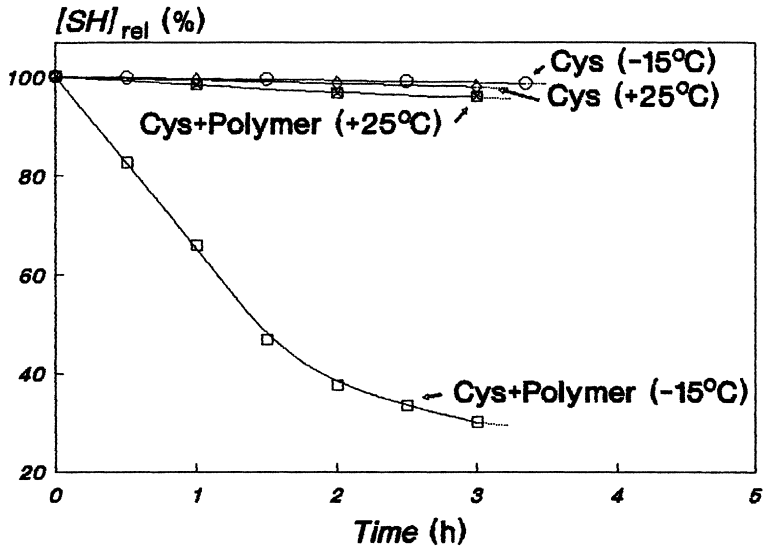


Fig. 8. The variation in time of the relative amount of titratable thiol groups in the unfrozen and frozen at −15°C water solution of Cys in the presence and absence of S-carboxyamidated derivative of SH-PAAm dissolved.

polymer presence in the solution to be frozen on the diffusion rates of water molecules.

It is of interest that even after the stabilization of $I$ values at every particular $T_0 - T_s$ value, the complete equivalence of NMR spectral characteristics of the samples frozen by means of procedures A and B was not reached for, at least, the 30 min (−15°) or 1 h (−20°) storage time (Table 1). The values of $I$ were small, but not zero, thus providing obvious evidence for the existence at these negative temperatures of the unfrozen liquid microphase or, in the terms usually used in the description of frozen polymer systems, of the non-freezable solvent. In the temperature range studied, the content of mobile solvent in the B samples was slightly less than in A samples. Besides, a certain difference in the width of $^2H$ NMR signal was detected, pointing to the somewhat higher mobility of $D_2O$ molecules in the polymer solution frozen via method A. Such a difference, in principle, could be one of the possible reasons for the existence of lag-periods on the kinetic curves in Fig. 4(B).

### *3.3. Cryogenic oxidation of low-molecular thiol in the absence and presence of non-reactive polymer*

From the analysis of all the discussed experimental data, the following rather natural question arises: could a considerable acceleration of the oxidation of thiol groups of SH-PAAm in the liquid microphase of moderately frozen water solutions of the polymer be observed in a somewhat different case, namely, when the macromolecular component of the system is non-reactive and plays a role only of the phase-formation ingredient (the "carrier" of the non-freezable solvate liquid), and the reactants are the low-molecular-weight compounds? In order to elucidate this problem, we studied the kinetics of freeze-induced oxidation of SH-containing amino acid cysteine by the dissolved atmospheric oxygen in the absence and presence of the *S*-carboxyamidated derivative of SH-PAAm. As such a polymer did not contain neither oxidative, nor reductive groupings, its macromolecules should not participate in the reaction of transformation of cysteine into cystine (Fig. 7). The latter statement is illustrated by the curve "Cys + Polymer (+25°C)" in Fig. 8, where the variations in time of the concentration of thiol groups in liquid and frozen systems are depicted. The initial Cys concentration was 0.8 mmol/l, that of the polymer was 2 g/dl, i.e. the concentrations were the same as the concentrations of SH-groups and SH-PAAm in the experiments, whose results were described in Figs. 4 and 5.

As is seen, the oxidation of cysteine was rather slow under these conditions at room temperature, both in the absence and presence of polyacrylamide derivative used. In fact, only negligible decrease in the SH-titre was registered for the solution of this amino acid frozen for 1–3 h at −15°C, evidently showing that at such a temperature we dealt with the system being under the eutectic temperature for this not very well water-soluble substance (solubility of Cys, as it was observed in our experiments, progressively decreased with a decrease in temperature). At the same time, in a moderately frozen solution of Cys, in the presence of non-reactive soluble polymer the amount of unfrozen liquid microphase was, obviously, large enough for the dissolution of the SH-amino acid (the dissolution practically within the solvate water of the polymer), and, as a consequence, its oxidation occurred at a high rate (curve "Cys + Polymer (−15°C)" in Fig. 8). This result testifies to the promising potential of this mode of carrying out various chemical


Fig. 9. The scheme of the oxidation of thiols with DMSO [44,45].

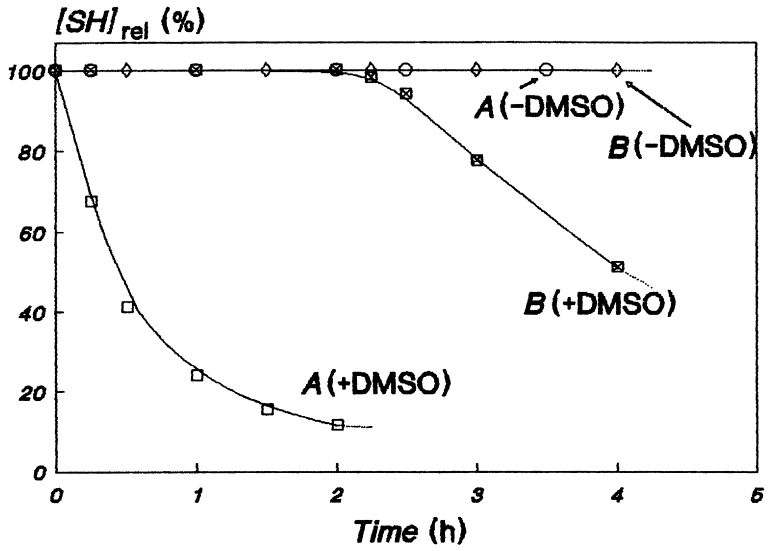


Fig. 10. The influence of the freezing mode and foreign oxidant (DMSO) on the variation in time of the amount of residual thiol groups in SH-PAAm.

reactions with low-molecular substances provided they or respective products possess low temperature stability and insufficient solubility. One may perform such a reaction in a moderately frozen system (in order to increase the efficiency (rate) of the process and protect the substances against the temperature damage) in the presence of non-reactive dissolved polymer (in order to increase the volume of unfrozen liquid microphase so that a greater amount of the reactants could be dissolved). Certainly, the net thermal effect of a similar "hypothetical" reaction should be exothermic. Otherwise, in the case of endothermic reaction, the cooling of the system will shift the thermodynamic equilibrium towards the initial reagents.

### *3.4. Cryotropic gel formation of water solutions of SH-PAAM in the presence of foreign oxidants*

As regards the presence of induction periods on the kinetic curves describing the propagation of the oxidation of SH-PAAm in its water solutions frozen with the use of a low-temperature quenching technique (Fig. 4(B)), it was also supposed that yet another possible explanation could be some specific behaviour of the oxidant, i.e. the dissolved atmospheric oxygen, upon very fast solidification of the reaction bulk immersed in the liquid nitrogen. This assumption was based on the known phenomenon of the formation of gas-derived blisters, when a molten metal is cooled and solidified rapidly. If such an analogy is correct, very fast freezing of the polymer solution could result in gas phase segregation with the formation of air microbubbles entrapped within a frozen solid. Further, upon warming the system to a pre-assigned $T_s$, a certain time is required for the oxidant to diffuse from similar immobilized microbubbles to the reaction zone, i.e. into the liquid microphase. If this is really so, no lag-periods should be observed in the case of well-soluble foreign oxidants.

In order to check such a statement, we carried out the experiments, in which the carefully deoxygenated aqueous buffer (0.01 M sodium carbonate, pH 9.5) was used as the solvent for SH-PAAm, and the calculated amount of DMSO was introduced into the system as an oxidant. DMSO is completely miscible with water and is known to be capable of oxidising the thiol groups to the disulphide ones (Fig. 9) [44,45] in weakly basic media under mild conditions. DMSO was added at a concentration of 1.25 mmol/l, that is, almost the same as the initial oxygen concentration in the experiments discussed in the previous sections.

The results of the corresponding kinetic studies are summarized in Fig. 10, where the variants in the presence (+DMSO) and absence (−DMSO) of the oxidant are depicted and the distinctions between the curves A(+DMSO) and B(+DMSO) are more than evident. Under the temperature conditions implemented ($T_s$ = −20°C), the duration of the lag-period for the freezing mode B turned out to be around 2 h. Hence, these experiments showed that the presence of these induction periods on the respective kinetic curves is inherent not only in the SH-PAAm oxidation by the dissolved $O_2$, but also by other oxidants. Therefore, this phenomenon is controlled by the specific microscopic physical and chemical phenomena during the low-temperature quenching treatment of the system of interest rather than by the specific features of the physical macroscopic behaviour of gaseous reagent during the fast freezing of the initial system.

The patterns of curves B in Fig. 4(B) and B (+DMSO) in Fig. 10 look like the kinetics of the known autocatalytic processes [46], which proceed at varying growing concentrations of a catalyst, the latter commonly being one of the reaction products formed. However, in the case under consideration, such a similarity apparently bears only a superficial resemblance, as no final product of both the reactions discussed (Figs. 3 and 9) can serve as a catalyst here. It

is more probable that, when the B freezing mode is employed, the conditions for the "onset" of the oxidation process are, on the one hand, markedly distinct from those existing in the course of ordinary freezing (we have already discussed this problem before), and, on the other hand, these conditions give rise to a situation for the initial accumulation of active particles until their critical concentration is reached, and only afterwards (when the rate of the generation of these active particles exceeds the rate of their disappearance) the oxidation of SH-PAAm begins to propagate. However, it is thought that the presence of the lag-phase in the kinetic curves of the B type could possibly be explained in a simpler way from the viewpoint of the dissolution behaviour of the polymer during the heating step of the two-stage freezing procedure B. Indeed, during the deep freezing of the polymer solution in liquid nitrogen, a very strong dehydration of the macromolecules occurs. Further, upon the subsequent heating of the system up to moderate negative temperatures, the dynamics of the de novo rehydration (including obligatory swelling phenomena) should depend on the thermal conditions. Inasmuch as certain mobility of, at least, segments of the chains is essentially required for the effective chemical interactions of the polymeric reactants, the formation of intra- and intermacromolecular SS-bridges will begin only after the repeated swelling of sufficiently long ($\geq M_c$) segments, and the time necessary for such a swelling (or even dissolution) will be longer with the decrease in $T_s$ value, thus, in turn, determining the duration of the induction period. The latter situation is in fact observed in the experiment.

## 4. Conclusions

The studies of the processes of cryotropic gel formation of water solutions of SH-PAAm have allowed us to draw the following general conclusions:

1. The cryogenic treatment (freezing—frozen storage—thawing) of water solutions of SH-PAAm results in the formation of spatial cryogels cross-linked with intermolecular disulphide bonds, provided that the initial system contains an oxidant, for instance, dissolved atmospheric oxygen, and the SH-polymer concentration exceeds the critical concentration of similar cryo-structuration.
2. The dependence of the oxidation rate of thiol groups in SH-PAAm on the temperature of frozen system is of extreme character due to the competition between the accelerating and decelerating factors, the major accelerating factor being an increase in the concentration of solutes in the unfrozen microphase upon the crystallization of most part of the solvent.
3. The position of extreme points on the temperature axis and the absolute magnitude of the reaction rate are determined, along with other factors, by the freezing mode used, i.e. by the thermal history of the system during its transition to the conditions of frozen storage.
4. If the low temperature quenching procedure is implemented for the freezing of initial polymer solution, the corresponding kinetic curves of the reaction show lag-periods, whose duration depends on the particular temperature of frozen storage.
5. The freezing of water solutions of SH-containing polymers in the presence of oxidants, for instance, the dissolved oxygen, does not only suppress (at least, until the temperatures is 30 degrees lower than the crystallization point of the system) the transformation of macromolecular thiols into the corresponding disulphides, but also promotes such a process.

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