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Differential scanning calorimetric study of heat-set gels of globular protein

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Abstract

A calorimetric study of concentrated solutions of globular protein lysozyme has been carried out in the temperature range 20–140°C. The effects of pH, ionic strength of the solution, and protein concentration on thermotropic gelation have been studied. Calorimetric studies were complemented by the visual control of solution state and properties in identical heating conditions. The results obtained have shown that two types of gel can be formed within the pH range 1.7–9.0: a clear meltable gel (type I) and a turbid one (type II), which does not melt. The thermograms of the type I gel heating demonstrate that, in addition to the denaturation heat absorption, there also exists a high temperature maximum (HTM), which is the calorimetric manifestation of cooperative transformation accompanying the melting of this gel. The thermodynamic melting parameters of heat-set lysozyme gel are obtained. The incipient stage of type I gel formation is observed at critical values of pH, ionic strength and protein concentration. It is shown that by adjusting the protein aggregation in solution by the use of these parameters, one can expand considerably the pH range in which heat-gel is formed, and provide such changes in the structure of branched gel which bring up its melting. This results in the appearance of HTM in the thermograms of such a gel.

Keywords: Calorimetry; Globular protein; Thermotropic gels; Irreversible denaturation; Aggregation; Melting of gel

1. Introduction

The present paper is aimed at presenting the calorimetric study of aggregation structures which originate in globular protein concentrated solutions after their denaturation. It is well known that after denaturation, along with the loss of nativity of protein molecules, the formation of a gel network can occur. These processes are related to sharp structural change, in the former case of an individual protein molecule, and in the latter, of the solution as a whole. Gels of fibrillar protein collagen were the first gels formed in polymer solutions which have been studied in detail. This type of gel is formed when cooling collagen (gelatin) denaturated solution below its denaturation temperature (cold-set gel). Gels of gelatin are of the thermoreversible type. Their structure is rather well established [1–3]. The nodes of a gelatin gel network are composed of short sections of ternary helix (of the poly-L-proline type) which is similar to the one of the collagen native molecule. However, the structure of a gelatin gel network is less thermally stable

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than that of native collagen. This is manifested calorimetrically by the fact that the gel melting temperature is always lower than that of collagen denaturation.

Unlike fibrillar protein gels, those of globular protein — which are the subject of present study — are not formed upon cooling, but upon heating the solutions above their denaturation temperature. These are the so-called thermotropic or heat-set gels. Even the fact of their formation at a temperature higher than the temperature of globule denaturation implies that the thermostability of gels of such proteins is higher than that of a native globule in solution. In recent years, the structure and properties of heat-set gels have been investigated by modern physico-chemical techniques such as electron microscopy, X-ray analysis, infrared spectroscopy, and the method of circular dichroism [4–7]. These studies have shown that the forces binding the protein into the thermotropic gel network are of the same origin as the forces stabilizing the native structure of the protein molecule (hydrogen, hydrophobic, Van der Waals) [6]. The difference in thermostability is evidently connected with the structure peculiarities of gel networks for globular protein. It has been shown in [4] and [5] that this structure is based on bound globules (as a whole), which do increase to some extent in volume upon denaturation (but do not completely unfold).

In this paper we present the results of an additional method, namely, the differential scanning calorimetric (DSC) method. As our preliminary studies have shown [8–10], the DSC method provides valuable new thermodynamic information on the structure and stability of heat-set gels. This method reinforces the existing understanding of heat-set gels as a state with an ordered spatial arrangement throughout the concentrated solutions of globular protein.

In tradditional calorimetric studies of intramolecular melting of globular proteins, the main experimental problem has been to eliminate the intermolecular interaction in the solution [11]. On the contrary in similar studies of the denaturation in concentrated solution, which is followed by gel formation, primary attention should be paid to the aggregational protein interaction. Gelation during the solution heating is closely connected with the denaturation process which, in this case, always proceeds irreversibly. On the one hand, as a result of the denaturation, a material appears which can be the basis of subsequent gel formation. On the other hand, due to the extension of denaturation in some temperature range, a gel can be formed during temperature scanning before the unfolding process of the whole globular ensemble is completed. Thus, while studying the formation and stability of heat-set gel in the scanning regime, it is worthwhile to consider the denaturation process as well.

A detailed systematic study of globular protein denaturation in concentrated solutions using the DSC method has already been conducted and is described in the authors' review [12]. It has been shown that if the denaturation in the concentrated protein solutions is reversible, the intermolecular interaction does not essentially change the thermodynamic parameters of denaturation transition, as is the case for diluted solution. Quite the reverse, in the case of irreversible denaturation, the intermolecular interaction leads to considerable variation of transition parameters, which are determined experimentally. The regularities of these parameters variations in the scanning regime have been established. The most important result is the discovery, in the heating thermograms of some globular proteins, of an additional endothermic maximum at temperatures higher than temperature of the maximum of denaturation heat absorption [8–10].

In the present paper we try to show that the appearance of the high-temperature maximum (HTM) in the course of the irreversible thermal denaturation of globular protein is connected with structural transformations in the heat-set gel, and therefore is related to the new phase transition, not observed earlier in these systems.

2. Materials and methods

The study has been performed by using a differential scanning calorimeter DSC-III, Setaram, with on-line computer IN-50 (France). Globular protein lysozyme (Sigma production),

widely spread in nature, has been studied. It belongs to the so-called small globular proteins with single-stage denaturation transition and demonstrates only one peak of denaturation heat absorption on heating curves of diluted solutions [13]. The determination of denaturation thermal effect (ΔQ_d) and of the temperature of maximum heat absorption (T_d) have been described in detail in [12] for both reversible and irreversible denaturation processes. Heat-set gel can be formed in lysozyme concentrated solutions after denaturation. The parameters of the protein environment affecting the gelation process include the protein concentration, the pH and the ionic strength of the solution, as well as temperature of the process. The protein concentration in the studies performed has varied in the range 1.5–25%. Lysozyme solution with a given pH have been prepared by means of the traditional method of dialysis of aqueous solutions of the sample with respect to a suitable buffer solution. Throughout the pH range 1.7–9.0 the glycine buffer was used. The pH region from 4 to 8, where this buffer loses its properties has been specially controlled by simultaneous pH measurements at protein denaturation temperature. The role of the buffer in this region reduces solely to setting the required pH value and to maintaining the ionic strength of the solution approximately identical for the whole pH range. The effect of ionic strength on lysozyme gelation has been studied with the addition of different NaCl quantities (0–0.5 M) to protein solution in glycine buffer (0.25–1.0 M) at pH 2.0 and 4.2 as well as to its aqueous solution (pH 6.4).

Temperature is a variable parameter in scanning calorimetry, in contrast to the other physical methods mentioned; therefore the gelation process is non-isothermal. As the ampoules with the solutions being studied are hermetically plugged and can withstand high pressure, the temperature range of calorimetric studies suffers no methodical limitations from above. The limitation depends only on thermal destruction of protein molecules themselves. The duration of the solution hold at any given temperature above T_d also affects the gelation process. This parameter can be varied in calorimetric experiment by changing

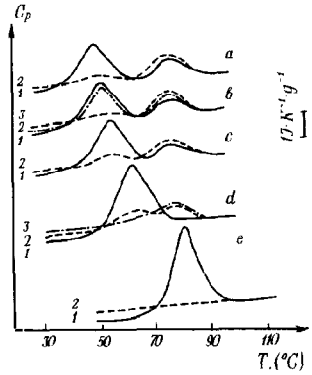

Fig. 1. Temperature dependence of heat capacity of the 10% lysozyme solution in 1.0 M glycine buffer, in the pH range 1.7–9.0: (a), (b), (c), (d) are for pH 1.7, 2.0, 2.4 and 2.7, respectively; (e) is for pH 5.3. Heating after quenching of the sample is indicated by the dashed line in (b). (1), (2) and (3) denote the succession of heating; $v_h = 5$°C/min.

the heating rate of the solution. We have studied the lysozyme solutions in the temperature range 20–140°C at the heating rates $v_h = 5$°C/min and 1.5°C/min.

Along with DSC measurements the visual observations of the state of all the solutions have been made in special transparent glass ampoules under identical heating conditions. If necessary, the heating could be stopped, the ampoule withdrawn and later opened at room temperature. This enables us to estimate qualitatively the gel's elasticity, homogeneity and ability to retain water.

3. Results and discussion

3.1 Dependence of lysozyme gel formation and melting on pH solution

In this section we consider the results for lysozyme solutions in 1 M glycine buffer with protein concentration $c = 10\%$, only one environment parameter (pH) being varied (Figs. 1–3). All the thermograms present the temperature dependences of lysozyme heat capacity, normalized to the protein mass in the solution and the temperature, given for the individual experiment most characteristic for the conditions considered. The lysozyme denaturation has proved to be irreversible over the whole pH range for all the

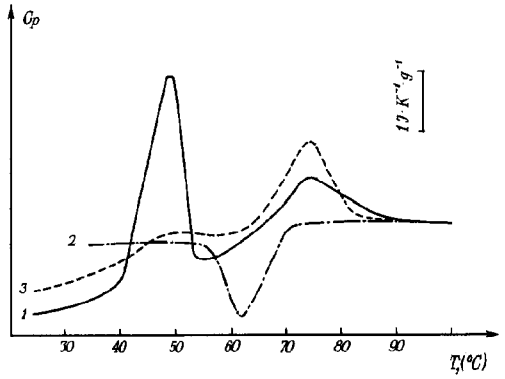

Fig. 2. Heat capacity of 14% lysozyme solution in 1.0 *M* buffer versus temperature during the cycle of (1) heating, (2) cooling and (3) reheating: (pH 2.0; $v = 1.5$°C/min).

solutions studied. This means that the maximum, corresponding to protein denaturation is missing at the repeated heating in the plots of protein heat capacity versus temperature. One should consider as the most important result the appearance of an additional endothermic high-temperature maximum (HTM), besides the one of denaturation heat absorption, in the heating curves of lysozyme solutions with pH = 1.7–2.7. At pH >

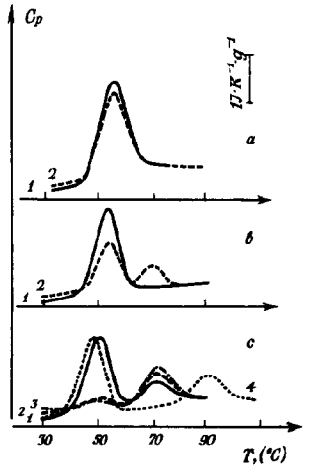

Fig. 3. Plot of heat capacity versus temperature for lysozyme solutions; one of the parameters (pH, buffer molarity, protein concentration) passing through its critical value; (a) reversible, (b) partially reversible, and (c) irreversible denaturation. (1), (2), (3) denote the succession of heating, and (4) incipient heating at pH 2.0 of 8.0% lysozyme in 0.5 *M* glycine buffer with 0.5 *M* NaCl.

Table 1

Effect of solution pH on the temperature and heat of conformational transitions in lysozyme during its denaturation and gel melting ($v_h = 5$°C/min, 10% protein in 1.0 *M* glycine buffer)

pH	T_{HTM} (°C)	ΔQ_{HTM} (J/g)	T_d (°C)	ΔQ_d (J/g)
1.7	75.1	8.8	48.6	13.9
2.0	76.3	9.6	51.2	15.1
2.4	76.0	8.8	55.6	16.4
2.7	75.0	5.5	62.1	26.5
3.4	–	–	70.8	31.5
3.8	–	–	78.0	34.9
5.3	–	–	80.0	34.4
6.5	–	–	78.0	33.6
8.0	–	–	75.0	31.7

2.7 only one peak appears on thermograms, which is related to protein denaturation. The results obtained are given in Fig. 1 and Table 1.

The complementary visual observations of the solutions up to 140°C have shown that as a result of aggregation after protein denaturation three different states arise, depending on the pH value. In the narrow region of pH 1.7–2.7 just after the denaturation, the clear elastic, weak gel is formed, which does not repell water under mechanical action (type I gel). At pH 2.8–5.0 after denaturation a weakly turbid viscous solution occurs. And finally, at pH > 5.0 a gel arises again (type II), being quite different from type I gel in its appearance and mechanical properties.

The comparison of DSC results with the visual observations shows that the curves with HTM refer to the solutions, in which type I gel is formed during heating. HTM is absent in the thermograms of lysozyme solutions in which either type II gel is formed after the denaturation or no gel at all. The correlation performed has shown that at heating of the native solution in aggregation conditions immediately after denaturation, the clear heat-set gel (type I) is formed. Upon further temperature increase the sample becomes liquid again which corresponds to gel melting. The temperatures of gel formation and melting have been shown to coincide with the ones of the denaturation and HTM, respectively. In such a way it has been proved that, firstly, the heat-set gel is formed during the denaturation

process as early as inside its temperature range and, secondly, that the observed HTM is closely related to the gel's melting. On the thermograms of the second time heating in these conditions (see, for example, Figs. 1 a–d) HTM always appears; the denaturation process is either absent or insignificant. The existence of HTM in the curves of repeated heating is evidence that gel, formed upon initial heating is thermally reversible. Measurements at the heating rates of 5 and 1.5°C/min have shown that at v_h = 1.5°C/min the time span is sufficient to complete gelation. Thermodynamic HTM parameters at slow heating characterise thermostability of the gel, which is the new form of protein molecular arrangement, arising under protein transition into the denaturated state (see Table 2).

The thermoreversible character of the gel formed manifests itself not only by the presence of HTM upon repeated heating. The cooling of the melted lysozyme gel under these conditions has been shown to lead to gel restoration even at temperatures above T_d; in this case an exothermic effect is observed in the thermograms, which closely matches the value of the heat of gel melting during the subsequent heating (Fig. 2, Table 2). It should be stressed, that overall an equilibrium gel structure, controlling its own thermostability, is formed at $T > T_d$. This refers to both processes of gelation under initial heating of native solution and of gel restoration from the melt. Further cooling down to room temperature introduces no essential changes into the heat-set gel structure. This distinguishes gels of globular proteins from gels of fibrillar proteins in which both gelation and gel structure variation exist well below the denaturation temperature. It appeared unexpected and interesting that by quenching (sharp cooling) the melt one could avoid gel restoration down to room temperature (T_{room}) and obtain again the renaturated protein solution. The denaturation maximum appears again in the thermograms of subsequent heating (Fig. 1b).

It has also been shown that the gel's thermostability and, hence, its structure depend noticeably on the temperature to which the melt is heated and on its hold period at this temperature. The gel's thermostability decreases with the increase of both factors.

As one can see in Fig. 1 and Table 1 the thermal effect of high temperature maximum decreases as pH increases; HTM vanishes at pH > 2.7. It has been observed that T_{HTM} does not depend on pH in the pH-range from 1.7 to 2.7, while T_d increases with the increase of pH, as it should be expected. The constancy of T_{HTM} and the increase of T_d lead to the overlapping of both maxima and their temperature coincidence at pH about 3.4. This means that the thermostability of part of gel at first and then of the complete gel becomes equal to the thermostability of the globule. The aggregation bonds arising under these conditions seem to have short life times and the denaturation process by now should have acquired its practically reversible character. This is in good agreement with the visual observations of the solutions in glass ampoules which have shown the reappearance of the solution after denaturation for pH > 3.5. However, protein aggregation increases with pH and at pH > 5.0 a gel is formed again which does not melt up to 140°C (gel of type II). In the respective heating thermograms, HTM is absent. As the temperature increases this

Table 2

Effect of solution pH on the temperature and heat of conformational transitions in lysozyme during denaturation, gel melting and restoration (v_h = 1.5°C/min, 10% protein in 1.0 *M* glycine buffer)

pH	T_d (°C)	ΔQ_d (J/g)	T_{HTM} (°C)	ΔQ_{HTM} (J/g)	$T_{restor.}$ (°C)	$\Delta Q_{restor.}$ (J/g)
1.7	47.6	14.3	74.2	9.7	62.2	7.6
2.0	49.0	14.7	74.0	9.6	62.0	8.4
2.4	53.6	16.4	75.0	9.2	62.3	9.2
2.7	60.5	22.3	73.0	9.6	62.0	8.4

gel gradually repells water whilst becoming more and more opaque and elastic. Hence, it follows that the absence of HTM in the heating thermograms of the type-II gels is the direct consequence of their thermo-irreversibility. It should be particularly emphasized that the formation of type-II gel as well as the aggregates in the solution obtain their calorimetric features (the decrease of the thermal effect in the denaturation region) as a result of aggregational molecular interaction. This aggregation thermal effect can be determined as the difference between the denaturation thermal effects of reversible and irreversible processes (for example, at pH < 5).

Thus, the DSC studies and visual observations of the changes of the sample rheological properties have shown that two possible types of heat-set gels can be formed in the pH 1.7–9.0 range. They are the meltable, transparent gel and the turbid (non-transparent) gel which does not melt. The high temperature maximum is the calorimetric manifestation of cooperative conformational transition, which proceeds in type-I gel upon melting.

A similar classification of the heat-set gels of globular proteins, based on structural investigations, has been presented in [5–7]. It has been shown there that the homogeneous clear gel which arises far from the isoelectric point, has regular linear structure, formed by successive attachment of disc-shaped globules, partially unfolded after the denaturation. The non-transparent gel has a heavily branched and/or cross-linked structure, based also on bonded, partially unfolded protein molecules. For the clear gel, a uniform protein density distribution is characteristic of the network structure over large distances; for the non-transparent gel the densified regions are randomly distributed in the network.

The results of the present research provide an additional confirmation of these concepts. On the one hand the existence of the rather narrow HTM in the thermograms of the clear gels, which manifests the cooperative conformation transition, is in favour of an ordered arrangement of protein molecules throughout the bulk during the formation of heat-set gels far from the isoelectric point. On the other hand, the absence of a HTM in the thermograms of the turbid gels is evidence of the absence of the transition, which in turn indicates the lack of long-range order in their structure.

3.2 Effects of solution ionic strength and protein concentration on formation and melting of different types of lysozyme gels

First let us turn to the pH-region where, as we have shown, a regular thermoreversible gel can be formed. For this purpose we have studied solutions of different lysozyme concentration (c = 1.5–25%) at glycine buffer molarities of 0.5 and 1.0 M with, in some cases, the addition of 0.5 M NaCl.

Consider primarily the incipient gelation. We have found that lysozyme solution denaturation, during the scanning over the temperature range, can be either reversible (Fig. 3a) or irreversible (Fig. 3c) depending on the protein concentration, the buffer molarity and pH. In the reversible process the denaturation thermal effect ΔQ_d is equal to the denaturation enthalpy and is related to the conformational variations, proceeding at a level of individual molecules. In an irreversible process the thermal effect is provided by structural variations in molecules in the course of their interactions. The transition from reversible to irreversible denaturation appears to proceed sharply in a narrow range of each parameter variation; the two others being kept unchanged. A critical value exists for each parameter at which denaturation is reversible at first heating and only partially reversible with the appearance of a HTM at reheating (Fig. 3b). The latter case is related to the initial gelation and the corresponding magnitudes of protein concentration, molarity and pH of the solution define their critical values. For completely irreversible denaturation HTM appears in the heating curves even at the first heating. As it follows from Fig. 3 the decrease of the denaturation thermal effect, the appearance of HTM and the growth of ΔQ_{HTM} are observed at the transition from reversible to irreversible denaturation. The decrease of ΔQ_d is caused by the increase of aggregational molecular interaction. Aggregation in this case is realized in the

form of the regular heat-set gel. As the experiment has shown, the irreversible lysozyme denaturation, for example at pH 2.0, can proceed at the following magnitudes of parameters: for 0.5 M buffer solution, $c \geqslant 10\%$ and for 1.0 M buffer solution, $c \geqslant 3\%$. Here $c = 10\%$ and $c = 3\%$ are the critical lysozyme concentrations of gelation under these particular conditions.

The heat of the reversible gel melting ΔQ_{HTM} is the thermodynamic parameter of gel–melt transition, which characterizes the depth of gelation and depends on all variables in the set of parameters: pH, molarity and lysozyme concentration. There exists a definite set of these parameters for which the heat of gel melting reaches its maximum, and which indicates the optimum gelation conditions. In our experiments $\Delta Q^{max}_{HTM} = (10.9 \pm 1.3)$ J/g. The plots of the gel melting heat in glycine buffer versus buffer molarity and protein concentration at constant pH (for example pH 2), are given in Table 3. One can see that the decrease of both, buffer molarity and lysozyme concentration result in a decrease of the thermal effect. This may be connected with the possibility of overlap of the melting and denaturation temperature regions. As has been shown above, the variation of the pH leads also to overlap of the denaturation and gel melting maxima. But while the pH affects the denaturation temperature (T_d), the ionic strength and lysozyme concentration influence primarily the temperature of gel melting (T_{HTM}). Hence it follows that their overlap can occur at the increase of the pH as well as at the decrease of the ionic strength and protein concentration.

The temperature of gel melting maximum (T_{HTM}) is the second thermodynamic characteristic of this transition. As has already been mentioned, the experiment has shown that the melting temperature of the regular linear gel is practically independent of pH. On the contrary, the experiments with solutions of variable ionic strength at constant pH and protein concentration have demonstrated a significant increase of T_{HTM} with ionic strength (Table 3). This effect can be seen, in particular, if one compares the variation of gel thermostability at different values of buffer molarity. From the data presented it also follows that the increase of the solution ionic strength due to NaCl addition also increases the thermostability of clear gel. (One should note here that T_d, unlike T_{HTM}, decreases only slightly.)

For the solutions with different ionic strength the high temperature maximum persists in all cases on the thermograms for the repeated heating, and gel which occurs at $T < T_{HTM}$ always turns to the melt at $T > T_{HTM}$. The identical phase states of the systems after gel melting for all magnitudes of ionic strength, enable us to assume that the increase of gel thermostability with ionic strength should be of energetic rather than of entropic origin. The increase of the protein solution concentration from 3 to 16% at constant pH and ionic strength leads also to T_{HTM} growth (Table 3). Unlike the ionic strength, the role of concentration in T_{HTM} increase is mainly of entropic origin. The increase of protein concentration, while enhancing the probability of protein–protein bonding, does not change the energies of these bonds. Moreover, the larger

Table 3

The dependence of temperature and heat of conformational transitions in lysozyme during its denaturation and the melting of type-I gel on the ionic strength of the solution and protein concentration ($v_h = 5$°C/min, pH 2)

[Glyc. buf.] (M)	[NaCl] (M)	[lysozyme] (%)	T_{HTM} (°C)	ΔQ_{HTM} (J/g)	T_d (°C)	ΔQ_d (J/g)
0.5	0	10.0	68.6	5.9	53.6	21.0
1.0	0	10.0	76.3	9.6	51.2	15.1
0.5	0.5	8.0	90.0	6.7	49.0	16.8
1.0	0	1.5	–	–	54.0	23.5
1.0	0	3.5	71.5	6.3	53.0	19.3
1.0	0	6.0	74.0	9.2	51.9	15.5
1.0	0	10.0	76.3	9.6	51.2	15.1
1.0	0	16.0	81.0	10.9	51.0	15.1

concentration reduces conformational variation of entropy, which takes place at gel melting.

The fact that HTM exists in the heating curves of lysozyme solution, with NaCl added, and that it is reproducible after repeated scanning, points out that in this case too the type-I gel is formed after denaturation. However, all the data obtained give evidence that aggregates can originate in solution in the presence of NaCl. These aggregates enter the network of regular thermoreversible gel after denaturation. Notably this fact seems to explain for the relatively low heat of gel melting in the presence of 0.5 *M* NaCl, in spite of the high ionic strength employed in these experiments (Table 3).

As it has already been noted, the hindrance to clear gel formation from lysozyme solution in glycine buffer at pH > 2.7 is the fast growth of globule thermostability with pH. Due to this fact the processes of denaturation, gelation and gel melting should occur in the same temperature range. The addition of 0.5 *M* NaCl to glycine buffer increases T_{HTM} of type-I gel up to 90°C, which exceeds the maximum lysozyme denaturation temperature ($T_d \sim 80$°C). In this connection one could hope that the addition of NaCl will broaden the range of the existence of this gel up to the higher pH values. As it follows from Fig. 4, the addition of salt really results in HTM appearance at thermograms of lysozyme solutions at pH values for which the HTM was not found in case of pure glycine buffer. The peculiarity of this maximum is that its temperature position, shape and heat absorption value undergo strong changes upon repeated heating. This means that the process providing this maximum is not completely reversible. To increase the reversibility of this process the concentrations of solutions studied were decreased (Fig. 4). In fact, it has appeared that lowering the concentration to 3.6% yields total reproducibility of HTM in repeated heating, i.e. for the solutions with the pH 4.2 we obtained a similar calorimetric HTM behaviour as obtained in the case of clear, homogeneous, and meltable gel, in the pH range from 1.7 to 2.7.

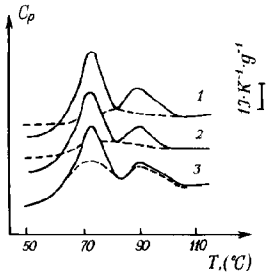

Fig. 4. Temperature dependence of heat capacity of lysozyme solutions in glycine buffer at pH 4.2 in the presence of NaCl. Variable protein concentration and 0.5 *M* NaCl; (1), (2), (3) are 15.0%, 6.3% and 3.6%, respectively. The successive heating is indicated by the dashed line.

In conclusion we would like to emphasize that the present results confirm our interpretation of the reasons which cause the formation of transparent melting gel in a relatively narrow range of pH. In addition, a method is provided for the essential broadening of the pH range of existence of such a gel through the increase of its thermostability, viz. through addition of NaCl.

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