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Studies of the Agarose Gelling System by the Fluorescence Polarization Method. II.*

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ABSTRACT: The sol—gel transformation of agarose and other polysaccharide fractions from agar were investigated by the fluorescence polarization method in order to study the gelling mechanism of agarose. The results showed that the gelation in dilute systems was a cooperative process of a helix segment with a kink segment in the agarose. In the process, the role of the helix segment was interpreted on the basis of Flory's mechanism for the transition and phase equilibria of asymmetric polymers. That is, the helix segment folded into a rodlike helical chain at the helix point to form an isotropic solution. At the gel point, the isotropic solution was separated into the tactoidal concentrated solution phase (coacervate) and the isotropic more dilute solution phase. The coacervate droplets were joined to each other with the non-helical kink segment to form a three-dimensional network structure. The rodlike helical chain tended to aggregate into the crystalline phase, though the rate was fairly slow just below the helix point because of the nucleation controlled process. Some of the helical chains still tended to aggregate into the crystalline phase in the coacervate. The melting point of the gel corresponded to that of the crystalline phase, and was higher than the gel setting point. The hysteresis of the agarose sol—gel transformation was thus interpreted by the difference between the melting point and the setting point.

KEY WORDS Fluorescence Polarization Method / Agarose / Gelling Mechanism / Phase Separation / Hysteresis /

The gelling mechanism of agarose has been investigated by many researchers. Among them, Rees, *et al.*, carried out the most active work as a part of their studies of polysaccharide gels.[1,2] According to their conclusion, the agarose chain is almost completely helical in the gel state, and the helices aggregate to form the gel framework. They also explained the hysteresis phenomenon in terms of the free energy surface with two valleys appearing in the transition of the helices.[3]

In a previous paper,[4] the authors studied the agarose gelling system by the fluorescence polarization method and found that the method effectively followed the sol—gel transformation including the hysteresis. In the course of our study, a new transition point was detected at around 50°C in the decreasing temperature pathway from sol to gel, and it was assigned as the coil—helix transition point.

In order to study the gelling mechanism of agarose, including the newly found coil—helix transition point, it is necessary to reexamine in detail the phase changes occuring in the sol—gel transformation. The examination of the roles of helix and kink segments is especially important. Here, the term helix segment means an alternating copolymer chain of galactose and anhydrogalactose in an agarose molecule, and the kink segment denotes a chain, substituted by charged groups, of the same backbone as the helix segment.[2]

In the present paper, the phase changes in dilute aqueous solutions of agarose, fluorescein isothiocyanate (FITC)-conjugated agarose, and some fractions of agar polysaccharide are studied mostly by the fluorescence method.

In a previous experiment,[4] uranine was used as an extrinsic probe, not chemically combined with

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agarose macromolecules. If the fluorescent probe is chemically combined with the macromolecules as an intrinsic probe, the fluorescence polarization measurement of the probe will provide more direct information on the micro-Brownian motion of the polymer segments.[5] FITC-conjugated agarose was prepared as such a test specimen for the study of the micro-Brownian motion.

Besides the fluorescence polarization method, some supplemental examinations of the solution properties were carried out. These were measurements of the electric conductivity, sedimentation volume by moderate centrifugation, and adsorption of uranine on agarose.

MATERIALS AND EXPERIMENTS

Materials

The samples of pure agarose used were "Agarose A-37" (Nakarai Chemicals Ltd., Kyoto, Japan) and "Agarose Standard Pure Powder" (Marine Colloids Inc., Rockland, Maine, U.S.A.). The former was used in our previous investigation,[4] and contained 0.30% of sulfate. The latter was used in Rees' work and was essentially free of substituents.[1] Unless otherwise noted, Agarose A–37 was used. "Difco Bacto Agar" was also used to obtain fractionated samples, which were the same samples as used in Yaphe's work.[6]

FITC-conjugated agarose was prepared according to the usual method.[7,8] Since it is known that the fluorescence characteristic of the conjugated FITC is nearly the same as that of uranine, the concentration of the conjugated probe was determined spectrophotometrically at 490 nm. The result showed that the aqueous FITC-conjugated agarose solution of 0.05 g/d*l* contained $1.06\times10^{-6}\,M$ of the probe as uranine. To obtain the agarose solution of 0.05 g/d*l*, the conjugated agarose was simply dissolved in water. More concentrated solutions were prepared by adding nonconjugated agarose to the conjugated agarose solution of 0.05 g/d*l* (for example, a 0.3 g/d*l* solution contained conjugated agarose of 0.05 g/d*l* and nonconjugated agarose of 0.25 g/d*l*).

The fractionation of Difco Bacto Agar was carried out by the successive water extraction method according to Yaphe's report.[6] The fraction F–1 was the cold water extractive, corresponding to the agaropectin rich in substituents. The fraction F–2 was the warm (55°C) water extractive which contained less substituents than F–1. The fraction F–3 was the extractive free residue and corresponded to the crude agarose.

The segmented agarose was prepared according to Rees' method[9] by the kink-splitting reaction with periodate from Marine Colloids' Agarose. This sample was composed only of the helix segment in the agarose macromolecule.

Table I. Sulfate contents and calculated chain lengths of helix segment in agar polysaccharide fractions

Samples	SO_4, %	$(C_{12}H_{13}O_9)_nSO_4$
Bacto Agar	2.96[5]	$n=10$
F–1 (cold water ext.)	7.00[5]	4.2
F–2 (warm water ext.)	4.10[5]	7.3
F–3 (crude agarose)	0.95[5]	33
Agarose A–37	0.30[4]	104
Marine Colloids' Agarose	trace[1]	—
Segmented Agarose	0[9]	—

In Table I are listed the sulfate contents of the samples quoted from the literature and the average lengths of helix segment calculated from the sulfate contents.

Fluorescence Polarization Measurements

Measurements were carried out with a Hitachi Fluorescence Spectrophotometer 204.[4] The measurements of the hysteresis phenomenon were continued for two days: The setting process from sol to gel was examined on the first day and, after an overnight standing of the sample, the melting process was examined on the second day. The fluorescent probe used was uranine (unless otherwise stated, the concentration was $10^{-6}\,M$).

RESULTS

Scanning Curves within the Agarose Hysteresis Loops

In a previous paper,[4] it was reported that the changes in the fluorescence polarization in the sol—gel transformation of agarose with temperature showed a clear hysteresis loop as shown in Figure 1 by the heavy curves. Here, p represents

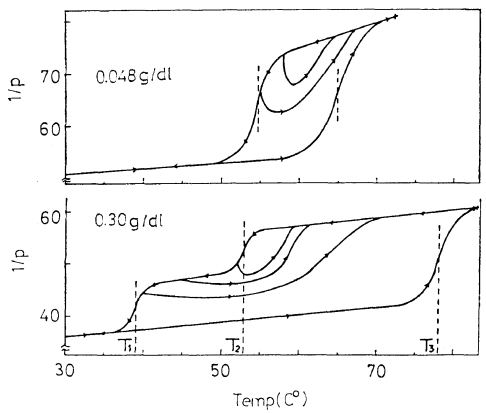


Figure 1. Hysteresis behavior in the gelling and melting of agarose, shown by the $1/p$ changes: T_1, gel point; T_2, coil—helix point: T_3, melting point.

the degree of fluorescence polarization which is defined by $p=(I_{//}-I_{\perp})/(I_{//}+I_{\perp})$ in terms of the polarized components of fluorescence intensity, $I_{//}$ and $I_{\perp}$, parallel and perpendicular to the incident polarized light. T_1 correspondes to the gel point, T_2 the coil—helix point, and T_3 the melting point, respectively. In this type of hysteresis phenomena, some partial hysteresis curves often traverse the loop when the direction of the temperature change was reversed in the course of measurements. The partial hysteresis curves thus obtained are called the "scanning curve," which should give information on the detailed mechanism of the phenomena.

Figure 1 shows typical results of the scanning curves, in which the curves of the gelling concentration (0.3 g/d*l*) and non-gelling concentration (0.05 g/d*l*) are included. The scanning curve was observed when the reversion of temperature was started from the range of T_1 to T_2 in the setting process. No scanning curve was observed in other part encompassing the melting point T_3. This means that the hysteresis of agarose gel is not caused by the gel formation itself but by the helix formation.

For the 0.3-g/d*l* agarose solution, an anomalous hysteresis, unexpected increase of $1/p$ above T_1, was observed in the temperature increasing pathway as shown in Figure 2, provided the concentration of uranine was higher than 2×10^{-6} *M*. The concentration of uranine does not usually affect the fluorescence polarization, unless a too concentrated solution is employed. The gel structure could not be changed by such a slight change of the uranine concentration. Therefore, the anomaly would be a reflection of some change of fine structure in the gel.

Change of Fluorescence Polarization in the FITC-Conjugated Agarose Systems

Figure 3 shows the change of $1/p$ of the FITC-conjugated agarose solution with the concentration at 25°C. Figure 4 shows the $1/p$ changes of 0.3 g/d*l* and 0.05 g/d*l* solutions with temperature.

The appearance of the curve in Figure 3 was essentially simillar to that of the non-conjugated agarose system reported previously[4] (*cf.* Figure 5 in this paper) except for the lower value of $1/p$. The lower value seems to reasonably reflect the longer rotational relaxation time of the probe. In Figure 4, however, the changes of $1/p$ at critical points, T_1, T_2, and T_3, were much less than those of non-conjugated agarose (*cf.*, Figure 1). The change at T_1 was particularly faint, and a decrease of $1/p$ by standing overnight was observed. The difference in behavior between the two agarose systems will be discussed later.

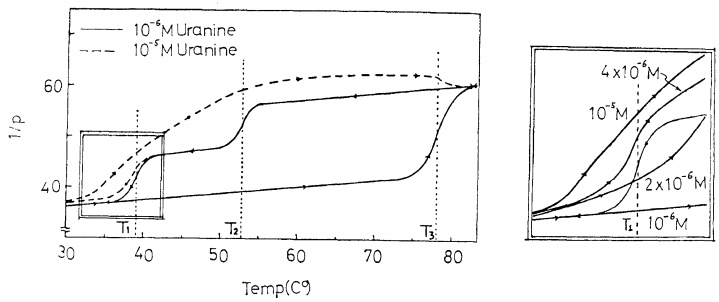


Figure 2. Anomalous hysteresis curves by uranine more concentrated than 2×10^{-6} *M*.

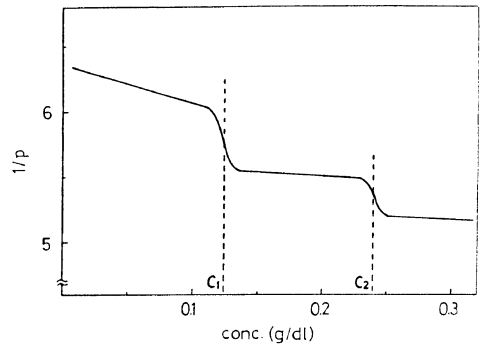


Figure 3. $1/p$ changes with concentration of FITC-conjugated agarose, at 25°C: c_1, concentration at which agarose domain occupies whole solution volume; c_2 gelling concentration.

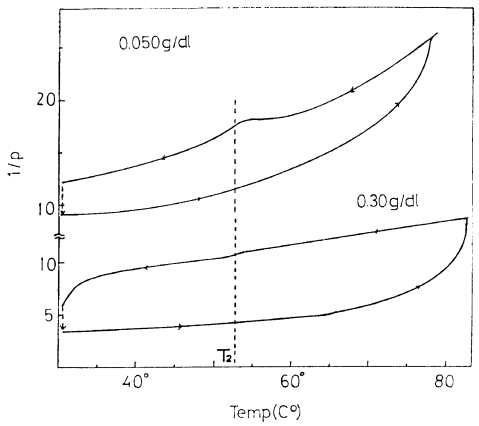


Figure 4. $1/p$ changes of FITC-conjugated agarose with temperature.

Gelation of Some Agar Polysaccharide Fractions

As polysaccharide samples, three fractions of Difco Bacto Agar, two pure commercial agarose powders, and the segmented agarose from Marine Colloids' Agarose were used. Table I gives the sulfate content of each and the average respective length of the helix segment.

Figures 5 and 6 show the $1/p$ changes with the polysaccharide concentration at 25°C, and those with the temperature at 0.3 g/d*l*, respectively. In Figure 5, an abrupt change in the $1/p$ at a lower concentration corresponded to the concentration at which the polysaccharide domain occupied the whole volume of the solution, and the second change at a higher concentration corresponded

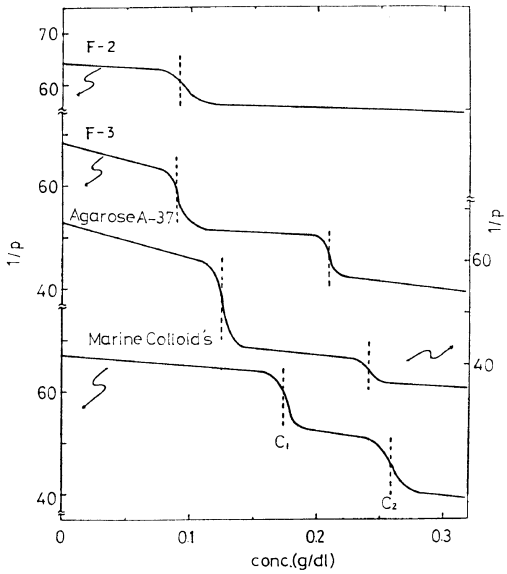


Figure 5. $1/p$ changes with concentration of some agar fractions, at 25°C.

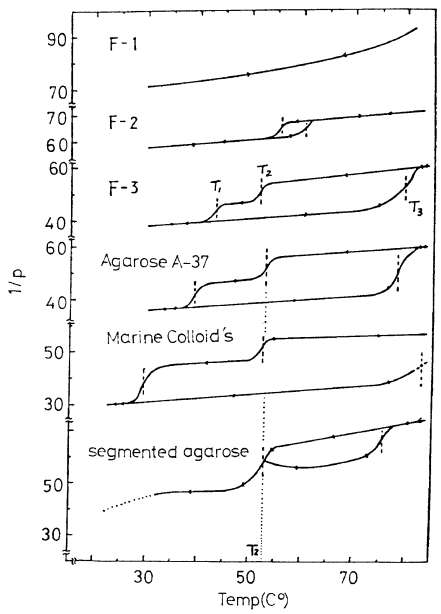


Figure 6. $1/p$ changes of some agar fractions with temperature, at 0.3 g/d*l*.

to the gelling concentration. Both concentrations became higher with decreasing sulfate content in the sample. This means that the ionization of the sulfate substituent promoted the

expansion and overlapping of the polysaccharide domains in the solution.

In Figure 6, it is seen that F–1 and the segmented agarose did not gel. The F–1 was a sulfate-rich agaropectin and formed no helix and no gel, and, therefore, had no hysteresis. The segmented agarose had no substituent and showed a sharp change at helix point, and could not form a gel because of the lack of kink segment to make a gel framework in such a dilute solution as 0.3 g/d*l*. The segmented agarose became a little turbid at 40°C and precipitated at temperatures below 35°C. In more concentrated solutions up to 1.0 g/d*l*, only the precipitation was observed without any gelation. Therefore, it appears that the helix segment cannot make a gel without the coexistence of a kink segment in a very dilute system. The complete hysteresis loop of the segmented agarose could not be obtained because of the precipitation, but the scanning curves were drawn at the temperatures above 45°C as shown in Figure 6. Other samples showed a behavior in the middle of F–1 and segmented agarose. The F–2 formed a helix only slightly and showed a little hysteresis, but no gel could form even at concentrations above 1.0 g/d*l*. The helix segment in F–2 seemed too short to make gel, though it made helix barely.

An interesting result that explains the roles of the helix segment and the kink segment was obtained from a mixture of segmented agarose with F–2. Both samples could not form gel individually as mentioned above, but the mixture was a viscous, transparent liquid like an agar solution. The mixture of 0.24 g/d*l* segmented agarose solution and 0.06 g/d*l* F–2 solution geled at room temperature.

Aggregation in Dilute Agarose Solutions

Agarose A–37 geled at 40°C at such a low concentration of 0.24 g/d*l* (*cf.*, Figure 5). The macroscopic observation of the agarose gelling process showed no remarkable change at the helix point T_2 but the turbidity changed suddenly at the gel point T_1. From this observation, it seems reasonable that the aggregation of the helices, proposed by Rees, *et al.*,[3] may occur at the gel point. Such an aggregation was also expected to occur even in a nongelling dilute system.

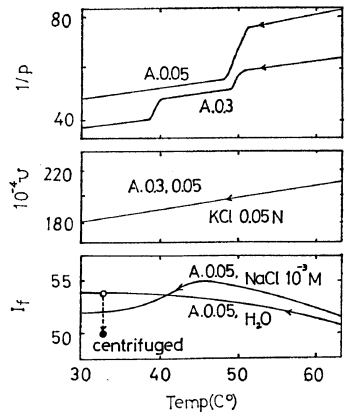


Figure 7. Changes of $1/p$, electroconductivity (υ), and fluorescence intensity (I_f) of agarose solution with temperature: *A*, agarose concentration (g/d*l*).

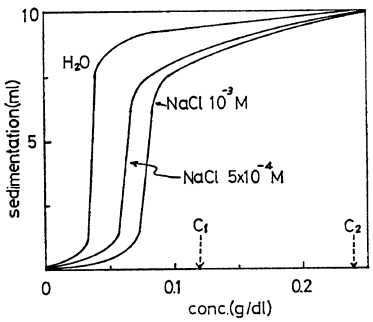


Figure 8. Sedimentation volume changes with concentration from 10 m*l* of agarose solutions, with or without containing NaCl.

In order to check the aggregation in such a dilute agarose solution, various experiments, such as the determination of electric conductivity, fluorescence intensity, and sedimentation volume by moderate centrifugation, were carried out. The results are shown in Figures 7 and 8. In Figure 7 are given the changes in the $1/p$, electric conductivity, and fluorescent intensity (I_f) with temperature.

The electric conductivity of the agarose solution containing 0.05-*N* KCl did not show any remarkable change at 40°C even at a gelling concentration of 0.3 g/d*l*, and there was no detectable difference in the conductivity between the gelling concentration (0.3 g/d*l*) and the nongelling concentration (0.05 g/d*l*). The I_f (at 520 nm) of 0.05 g/d*l* solution containing 10^{-6} *M*

uranine showed no abrupt change at 40°C, but some fluctuation of the values of I_f during the measurement was noticed at temperatures below 40°C. In the figure, the averaged value of the several measurements was plotted. The fluctuation suggested that the system became somewhat heterogeneous. If the solution was centrifuged for 10 min at 3,000 rpm, the I_f showed an apparent decrease compared with that before centrifugation. When NaCl of 10^{-3} M was added to the system, the I_f decreased without any treatment at temperatures below 45°C. The 0.3 g/d*l* agarose solution containing 10^{-3}-M NaCl geled at 45°C.

These results mean that, even in the dilute solution which did not gel, aggregation occurred at the gel point and the aggregated particles were suspended in the solution. When the solution containing the suspension was centrifuged, or salt was added to it, the suspended particles sedimentated to the bottom. At that time, if the uranine was positively adsorbed on the agarose chains in the aggregate, the uranine concentration in the supernatant solution becomes lower so as to decrease the I_f.

Figure 8 is the result of the sedimentation volume measurements from the 10-m*l* dilute agarose solutions at 25°C, after 10 min centrifugation at 3,000 rpm. The centrifugation was made to separate the coarse particles of the aggregate from the solution. The abrupt change of the sedimentation volume was not observed at 0.12 g/d*l*, where the agarose domains occupied the whole volume of the solution, but at 0.03 g/d*l*. Within the concentration range from 0.04 to 0.12 g/d*l*, the sedimentation volume hardly changed and occupied almost the entire volume of the solution, although the net agarose domain could not do so. This suggests that the aggregated particles were joined to each other to make a three-dimensional structure, and that each individual particle was a coacervate. In dilute solutions below 0.03 g/d*l* the coacervate droplets were precipitated to the bottom showing a small sedimentation volume. These considerations lead us to the conclusion that the gel point, or aggregation point, of agarose solution may correspond to the phase separation point in the liquid—liquid phase equilibrium. This will be discussed later in detail. The addition of salt suppressed the dissociation of charged groups.

Adosorption of Uranine on Agarose Macromolecule

In our previous paper,[4] the abrupt change of the $1/p$ at the transition points was interpreted as due to the adsorption of the extrinsic probe, uranine, on agarose molecule. Some qualitative proofs were obtained in the course of the present study: for example, in dilute agarose solutions, the decrease of I_f by centrifugation was observed at room temperature as described above.

In order to obtain quantitative proof of the adsorption, the freeze-thaw procedure of agarose gel[9] was employed: The agarose gels of 0.3 g/d*l* containing the varied amount of uranine from 10^{-7} to 10^{-4} M were frozen at −20°C for 1 hr, and then thawed at room temperature. The agarose gel was broken and precipitated as a transparent hard particles with a small volume by thits procedure. After centrifugation, uranine in the supernatant solution was determined spectrophotometrically, and the adsorbed uranine

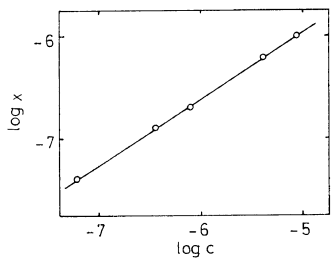


Figure 9. Adsorption of uranine on agarose chains in the gel, at 25°C: agarose, 0.3 g/d*l*; inintial concentration of uranine, 10^{-7}—10^{-5} M; x, decreased concentration of uranine from initial, after centrifugation: c, residual concentration of uranine in supernatant, after centrifugation.

on agarose was calculated. Figure 9 shows the results. It is seen, in the concentrative region from 10^{-7} to 10^{-5} M, that uranine was positively adsorbed on agarose chains according to Freundlich's equation, $x=ac^{1/n}$. Here, x is the adsorbed uranine, c is the equilibrated uranine concentration in supernatant, a is a constant, and $1/n$ is the adsorption factor. The adsorption factor, 1/6.1, showed a moderate but undoubted adsorption. At higher concentrations than 10^{-4} M, Freundrich's equation could not be applied.

DISCUSSION

The characteristic features of the agarose gel outstanding from other gels are the strong ability to gel even in dilute solutions such as 0.3 g/d*l*, the remarkable hysteresis in sol—gel transformation, and the unusual gel properties such as a big porosity for the gel filtration. The agarose macrmolecule is composed of an alternating chain of the helix segment with the kink segment. The helix segment is an uncharged, regular copolymer chain, and behaves as an asymmetric, rodlike chain at the temperatures below the helix point T_2. Therefore, the agarose system is regareded as a specific system in which the asymmetric helix segment coexists with the randomly coiled, negatively charged kink segment.

The behavior of the helix segment will be considered first. The helix segment in agarose is expected to behave similarly to segmented agarose. In such a low concentration as 0.3 g/d*l*, the double helix formation at T_2 should be mainly intramolecular. At T_1 another kind of interaction among the helical chains may be expected. However, the DSC thermogram[4] showed no change at T_1. This may suggest that, at T_1, there might be no detectable change in the intermolecular forces among the helical chains. The agarose gel prepared by the cooling procedure hardly showed any analyzable crystal patterns by the X-ray diffraction method,[1] indicating also that the major gelling reaction at T_1 was an aggregation accompanied with no regular orientation such as crystallization.

The kink segment, on the other hand, is composed of the substituted galactose residues with the neighbouring several repeating units excluding from the helix segment owing to the steric and electrorepulsive hinderance by the substituents. The behavior of F–1 in Figure 6 may be regarded as a representative of the behavior of the kink segment. The nonhelical kink segment in agarose may always take a random coil, or random-coil-like conformation, and will not contribute directly to the change of $1/p$, shown in Figure 1.

Though midway in the consideration of the kink segment, the role of the fluorescent probe in the fluorescence polarization method will be considered here. The abrupt change of $1/p$ at the transition point corresponds to the change of the rotational relaxation time of uranine in the system. For the more concentrated polymer system, it was suggested that the change of the relaxation time depended upon the change of local viscosity.[6] In a dilute system such as studied here, the adsorption of uranine on the agarose chain was suggested to be more important in determining the value of $1/p$,[4] and this was confirmed quantitatively, as shown in Figure 9. Although adsorption sites on agarose chain by uranine were not confirmed in detail at this point, these should not be negatively charged kink segments, but the neutral helix segments. Therefore, the apparent relaxation time of the adsorbed uranine may depend upon the movement of the helix segment. When the movements of the helix segment change at the transition points, therefore, the $1/p$ of adsorbed uranine will changed abruptly. At the same time, however, free uranine and adsorbed uranine on the kink segment will not contribute to the $1/p$ change directly.

When the fluorescent probe was chemically combined to the helix segment, a more abrupt change of $1/p$ was expected. Contrary to the expection, however, the result of FITC-conjugated agarose shown in Figure 4 exhibited only a mild, or slight change. Since the conjugated agarose was prepared at room temperature, the helix segment folded into a helical chain, and it may be expected that the reaction with FITC dominated on the kink segment rather than the helix segment. Moreover, if FITC is conjugated to the helix segment, the conjugated segment will not be able to fold again into a helix because of steric hinderance. The result shown in Figure 4 may thus reflect the micro-Brownian movement of the kink segment, or nonhelical segments. The micro-Brownian movement of the kink segment itself could not change so abruptly at T_2, but the helical folding of the adjacent helix segment might be influenced indirectly the movement of the kink segment. This is an interpretation of the mild change at T_2 given in Figure 4, and also that of the difference in behavior between F–1 in Figure 6 and conjugated agarose in Figure 4.

As shown in Figure 4, there was no change in $1/p$ at T_1. This may suggest that the change of the helix segment at T_1 will not affect the movement of the kink segment. On the other hand, a distinct decrease of $1/p$ during overnight standing

at room temperature was observed. In the case of $1/p$ determination with extrinsic probe, the value of $1/p$ became stable within 10 min after attainment of the temperature.[4] These facts mean that another kind of slow process, detectable only by the conjugated probe, is included in the sol—gel transformation. This process does not attain equilibrium within several days, and may be considered a crystallization process of the helical chain to be discussed in the following.

A more detailed consideration will be requested regarding the aggregation of the helical chains at the gel point T_1, in order to discuss the gelling mechanism. The aggregation in the non-gelling dilute solution such as 0.05 g/d*l* could not be followed by the change of the $1/p$ as shown in Figure 1. However, the results shown in Figure 7 may suggest that the aggregation still proceeded in such a dilute concentration. Moreover, the 0.05 g/d*l* solution of the segmented agarose became turbid and separated an anisotropic phase at T_1. The changes in the sedimentation volume by the moderate centrifugation in Figure 8 showed that the aggregated phase was separated at 0.03 g/d*l* as a concentrated liquid phase, that is, a coacervate droplet. A similar coacervate should also be separated from the solution at concentrations above 0.03 g/d*l*. In the concentrated solution, the coacervate droplet should be connected to each other through the kink segment to make a three-dimensional structure. Therefore, the apparent sedimentation volume became greater.

The more developed the three-dimensional network structure of the coacervate, the more could it resist effectively the flow and deformation of the system. This state should be the gel of agarose! That is, most of the helix segment is concentrated into the coacervate droplet and the total volume of the coacervate is not enough to fill the whole volume of the gel. Since the kink segment was dissolved both in the coacervate and the dilute liquid phase, the intercoacervate junction was formed. The interstices of the coacervate network are filled by the dilute liquid phase. Thus, the agarose gel system at dilute concentration would be a heterogeneous, dual structure of the coacervate with the dilute isopropic phase. This proposed structure for the gel could well interprete the electric conductivity of agarose gel (Figure 7), the big porosity of the agarose gel filtration, and the turbidity of the dilute gel.

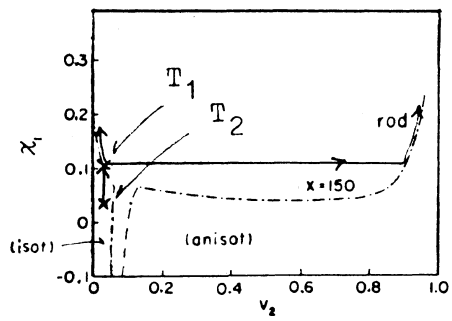
Another kind of aggregation of the helical chains, crystallization, will be proposed as the slower change observed in FITC-conjugated agarose. The double helical chain was regarded as an one-dimensional crystal, and was expected to aggregate into a three-dimensional crystalline phase as soon as the helical folding occurred at T_2. The crystallization of macromolecule is usually a kinetic controlled process. Therefore, the rate of the crystallization seemed fairly slow in the course of the temperature decreasing process. The gelation at T_1 was a liquid—liquid phase separation and the rate of the crystallization was not influenced directly by the gelation itself. The DSC thermogram which revealed no substantial change at T_1[4] supports this view.

The slow crystallization in the coacervate may be considered to continue with the gel standing at room temperature. The movement of the helix segment should almost stop both in the coacervate and crystalline phase. Therefore, the extrinsic probe method, as shown in Figure 1, will not be able to detect the course of the crystallization so well. On the other hand, the movement of the kink segment may still be allowed in the gel state. The orientation of the helical chains in the coacervate caused a shrinkage of the coacervate volume, which influenced the movement of the kink segment. The fairly slow crystallization was thus detected by the experiment of FITC-conjugated agarose.

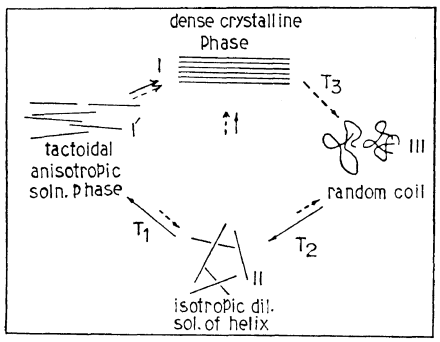
The crystalline phase, thus formed, melted at a higher temperature than the individual helical chain, and the size of the crystalline phase modified the melting point. This is one interpretation of the hysteresis, or scanning curves of Figure 1.

From the above consideration, the behavior of the helix segment may be interpreted by combining Flory's mechanism of the phase equilibria in asymmetric polymer systems[10] with Nakajima's phase diagram for rodlike polymers.[11]

Flory's mechanism dealt with the phase changes of the synthetic polypeptide as is shown by the following scheme. When the polymer molecule in the scheme is replaced with the helix segment of agarose, the interpretation of sol—gel transformation of agarose is as follows. The setting process from sol to gel is shown by an arrow (→) in the scheme. The phase of the solution



(Nakajima's Diagram)
"Phase diagrams for systems of rodlike polymers with axial ratio $x=150$ in solution."[11] In the diagram, the supposed pathway of agarose gelation from T_2 to room temperature *via* T_1 is indicated.



(Flory's Scheme)
"Schematic representation of transitions and phase equilibria involving polymer chains in ordered conformation."[10] In the scheme, the direction of each process is indicated by the arrow, on the basis of proposed mechanism for agarose gelation. The length of arrow represents the weight of the process qualitatively. →, gelling; ---→, melting.

at temperatures above T_2 corresponds to (III) in the scheme. At T_2, the phase (III) is transformed into (II) by the helix formation. At T_1, (II) is separated into two phases, (I') and an isotropic more dilute phase. This phase separation corresponds to the gelation of agarose, mentioned above. Some of the helical chains in (II) or (I') may aggregate into (I) at temperatures below T_2 or T_1, respectively, as depicted in the scheme.

The phase separation will be interpreted well by Nakajima's phase diagram as follows. In their diagram, the thermodynamic interaction parameter, χ_1, of the system of rodlike polymers is plotted against the volume fraction of polymer, v_2, and regions of the isotropic and the anisotropic phase are located separately. In the dilute agarose solution such as 0.3 g/d*l* ($v_2 \fallingdotseq 0.003$), the T_2 point is in the region of isotropic solution phase in the diagram. The χ_1 is proportional to the reciprocal of temperature from its definition, and does increase with decreasing temperature in most polymer solutions. In the agarose system, χ_1 is also suggested to increase with decreasing temperature. Therefore, the T_1 point would be on the phase separation line as shown in the diagram, and the tactoidal anisotropic solution phase (I') or coacervate will separate at this point.

The melting process of the gel is shown by a dotted arrow (←--) in the Flory scheme. By heating up to T_2, some of the uncrystallyzed helical chains (I') will be newly transformed into (I), and others, which are located in the position unsuitable for orientation, would be melted into (III) *via* (II). Therefore, the recovery of the phase (III), as the whole system, will not be reached until the melting point T_3 of (I). Anomalous increase of $1/p$ at temperatures above T_1 shown in Figure 2 can provide some support in the formation of (I) during the melting process. The formation of (I) by heating resulted in a decrease in apparent surface of the helix segment in the coacervate. Since uranine was adsorbed on the surface of the helix segment, a considerable concentration of the adsorbed uranine is caused in such a limited space as small coacervate droplets. The apparent increase of $1/p$ shown in Figure 2 is thus thought to be due to the excess concentration of uranine, which causes concentration depolarization.[12] The anomalous increase of $1/p$ disappeared at T_3 by the disappearance of (I). Under the standard condition of our experiments, in which 10^{-6} *M* of uranine was used, the concentrared uranine was below the critical point for the concentration depolarization. Since the critical concentration is about 10^{-4} *M*, the result of Figure 2 may suggest that at least a ten times' concentration (from 10^{-5} to 10^{-4} *M*) occurred on the agarose chains at the concentration of 0.3 g/d*l*. At this point, however, the above interpretation seems to be rather speculative, but it well explains the observed sol—gel transformation.

The behavior of the agar fractions in Figures 5 and 6 provided information on the cooperative

work of agaropectin with agarose in agar. Agaropectin should play a more effective part in the intercoacervate junction than the kink segment in agarose. Since the agaropectin macromolecule also dissolved both in the isotropic dilute phase and in the coacervate droplet, the coexistence of agaropectin may be considered to control not only the gelling condition but also the gel properties.

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