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Study of Gelatin Gels and the Effect of Urea on Their Formation

ALINA S. SZCZESNIAK and ROBERT V. MacALLISTER,* *Technical Center, General Foods Corporation, Tarrytown, New York*

Synopsis

The effect of urea on gelatin gels was studied by measuring gel rigidity at different gelatin and urea concentrations. Rigidity of gels was expressed as the force necessary to depress the gel with a standard plunger a distance of 4 mm. In agreement with the data in the literature, a straight-line relationship was obtained when gel rigidity was plotted against the square of gelatin concentration. This relationship held true in the presence and absence of urea and also for all urea concentrations tested. Urea had a marked effect on lowering the rigidity of gels and the ratio of rigidity to the square of gelatin concentration decreased with increasing molarity of urea. In order to gain some comprehension of the mechanism of gelatin gel formation, the rigidity values were interpreted in terms of the number of crosslinkages which would account for such rigidity values. The fraction of amino acid residues effective in crosslink formation was calculated from gel rigidity data by applying concepts developed by Flory and others for rubberlike elastic polymer networks. The state equation was modified in order to adapt it to the measurements of gelatin gel rigidities. Calculations indicate that about 1.7 amino acids per molecule were involved in crosslink formation in a 1.8% and 8.0 in a 1.0% gelatin gel set at 10°C. 1*M* urea inhibited the formation of 30–45% of crosslinks and 4*M* urea inhibited 90–100% of the bonds, the exact effect depending upon the concentration of gelatin.

INTRODUCTION

The prevailing theories on the mechanism of gelatin gel formation postulate the formation of intermolecular linkages after an initial period of intramolecular orientation. The intermolecular linkages occur at limited regions of the molecular chain and may have the nature of salt bonds or hydrogen bonds.[1–3]

Bello and Vinograd[4] investigated the role of functional groups of gelatin in relation to the gelation process and obtained some evidence of the participation of peptide groups from a study of the biuret type complex of copper ion. More recent work by Bello et al. showed that at low gelatin concentrations (0.6–0.7%) the charged groups act as inhibitors rather than as crosslinking sites in the early stages of gelation[5] and that at higher gelatin concentrations (5%), amino, carboxyl, guanido, and hydroxyl groups do not affect significantly the gelation process.[6] Grabar and Morel[7]

* Present address: Clinton Foods, Division of Standard Brands, Clinton, Iowa.

reported that blocking of free amino groups does not prevent gel formation, but that the ability to gel is lost when the guanido group of arginine is destroyed.

Croome,[8] from his work on the effect of temperature on formation and breakdown of gelatin gels, suggests a two-stage process of gelation. Primary intermolecular linkages are first set up at limited sites along the gelatin molecule. Their number is a direct function of the molecular weight of the polymer. Next, secondary bonding occurs at sterically suitable points within the primary gel structure. These linkages are highly temperature-dependent and give rise to gel rigidity. Croome obtained an empirical expression for gel rigidity indicating its dependence on molecular weight and a temperature-dependent factor called the rigidity factor.

Todd[9] attempted to relate the rigidity factor to the chain configuration by correlating the optical rotation of the gel with the rigidity modulus. She concluded that the rigidity factor defines the capacity of the gelatin molecule to fold in a regular manner forming orientated triple helices, and postulated that the rigidity factor is determined by the proportion of residues in sequences capable of forming the collagen fold.

Boedtker and Doty[10] attribute gelation of gelatin to the simultaneous growth and interlocking of aggregates arising from crystallite formation. They do not make any definite statements as to the types of bonds involved but favor hydrogen bonding in view of the lack of influence of ionic strength. Ferry and Eldridge[11] suggested that crosslinking in gelatin gels consists of multiple hydrogen bonds. Bello et al.[5] appear to agree with this but postulate that, especially in early stages of gelation and at low gelatin concentrations, some contribution may be made by single hydrogen bonds, salt links, or interactions between nonpolar groups.

Ferry,[2] Ferry and Eldridge,[11] Shephard and Sweet,[12] Naraynamurti[13] and others have reported that the rigidity of a given gelatin gel is closely proportional to the square of concentration up to a certain gelatin level. Ferry and Eldridge[11] explain this proportionality by assuming that the rigidity is proportional to the concentration of useful intermolecular crosslinks in the system and that the concentration of useful crosslinks is proportional to the square of total concentration. A number of workers[14–16] found that a change in optical rotation is closely associated with the development of gelatin gels rigidity. In contrast to rigidity, the change in optical rotation is proportional to the first power of gelatin concentration rather than to the square. Ferry and Eldridge[11] explain this difference by postulating that the change in optical rotation reflects the formation of intramolecular crosslinks or of some intramolecular rearrangement, while the change in rigidity reflects the formation of intermolecular crosslinks.

In this study urea was used to inhibit crosslinking between gelatin molecules. The "state" equation for flexible polymer networks was adapted to the measurements of gelatin gel rigidity and used to estimate the number of sites on the gelatin molecule involved in gel formation.

MATERIALS AND METHODS

Materials

Gelatin. The gelatin used in this work was a commercial material produced by the Atlantic Gelatin, General Foods Corp. It was a blend of type A and B gelatins; the properties of the component gelatins and the blend are given in Table I.

TABLE I

Gelatin	Bloom	Viscosity at 60°C., Mpoise	pH	Moisture, %
Type A	260	42.5	4.4	—
Type B	155	37.5	5.6	—
Blend (A + B, 1.0:1.3)	195	39.5	5.0	10.2

The following standard method was used for bloom determination. A $7^1/_2$-g. portion of gelatin was dissolved in 105 ml. distilled water with stirring and allowed to stand for $^1/_2$ hr. at room temperature and for $^1/_2$ hr. at 60°C. in standard bloom jars. The solution was then allowed to set at 10°C. for 17 hr. and bloom was determined with the Bloom Gelometer. The values were corrected to 6% solids. Viscosity was determined with a calibrated Oswald pipette on the solution prepared as above. Samples for pH determination were made up by dissolving 1 g. of gelatin in 50 ml. of hot distilled water (65–90°C.), cooling to room temperature and making up to 100 ml. The pH was determined with a Beckman pH meter, Model G. Moisture was determined by weight difference after drying at 100–105°C. for 17 hr.

Urea. Urea was Baker's analyzed reagent in crystal form.

Sample Preparation

Samples were prepared by mixing 2, 4, 6, 8, or 10 g. of the "as is" gelatin with 13.6 g. of granulated sucrose to facilitate dispersion, and heating in 100 ml. of water or in 100 ml. of urea solutions to about 80°C. with constant stirring until a clear solution was obtained. The concentration of urea varied from 0 to 5*M*. It was noticed that gelatin dissolved more easily in the presence of urea. The gelatin sols (80-ml. portions) were poured into glass dishes, 66 mm. in inside diameter and 50 mm. high, to give a gel depth of 23 mm. The gels were set at 10°C. in a constant temperature cabinet for 18 hr. Variations of temperature in the cabinet were less than ±0.5°C.

Measurement of Gel Rigidity

Rigidity measurements were taken at room temperature immediately after the samples were taken out of the setting cabinet. A Gel Character-

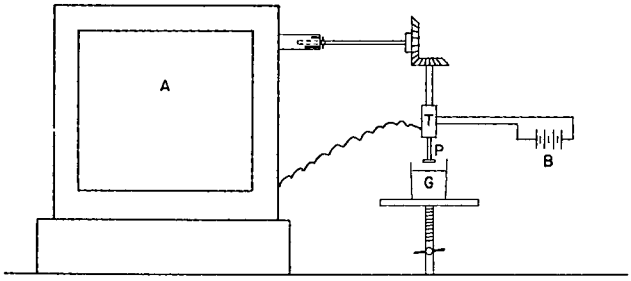

Fig. 1. Schematic drawing of the Gel Characterization Apparatus.

ization Apparatus,[17] developed at the research laboratories of General Foods Corporation, was used to measure gel rigidity. A schematic drawing of the instrument is presented in Figure 1. The chart drive of a recording potentiometer A is connected through a rigid mechanical drive to a carriage holding a Stratham transducer T. As the chart moves, the transducer moves downward at a slow constant rate. A flat, circular plunger P, having a surface area of 1.98 cm.^{2} and rigidly connected to the transducer, is forced against the gel G. The force exerted on the plunger as it moves against the gel unbalances the transducer bridge B. The extent of unbalance and the magnitude of the resultant e.m.f., which is fed back to the potentiometer, is proportional to the resistance of the gel against the deforming action of the plunger. Consequently, the pen on the recorder traces a load–deformation curve in which the ordinate (direction of chart movement) is directly proportional to the gel deformation, and the abscissa is directly proportional to the force or load on the gel.

The shape of the obtained load–deformation curves is characteristically different in different gel systems. Gelatin gels give essentially straight lines, the slopes of which are proportional to gel rigidity. In this study, rigidity R_4 was defined as the force in dynes necessary to make the plunger depress the gel a distance of 4 mm. With the selected size of the sample, no container wall or bottom effects on gel rigidity were observed. No appreciable stress relaxation occurred during the measurements.

Precision of Measurements

Precision of gel rigidity measurements was determined in a statistically designed experiment involving 60 measurements in duplicate in hourly intervals over a period of 10 days. The measurements were taken on 1.0% gelatin gels set as described under sample preparation. The errors involved in the measurements included, in addition to the instrument error, day effect, room temperature effect, errors in sampling, weighing, etc. Room temperature at the time of measurement (74–87°F.) and the time of day had no consistent effect on the absolute value or reproducibility of values. The least significant difference (95% confidence limits) was 5.8% for duplicate readings taken at the same time on the same day, 12.1% for two

readings taken at different times on the same day, and 13.8% for two readings taken at different times on different days. Since data presented here were obtained over a period of more than one day, the latter precision value is of importance.

RESULTS AND DISCUSSION

Figure 2 shows the relationship of gelatin gel rigidity to the square of gelatin concentration (dry basis) and its dependence on urea concentration. A series of straight lines was obtained. The decrease in slopes with increasing urea concentrations indicates an inhibiting effect of the additive on gel formation. The 5*M* urea effected almost a total inhibition in the gelatin range investigated, 7.2 and 9.0% gelatin samples showing only extremely weak gelation. This effect cannot be explained on the basis of pH differences, since the pH ranged from pH 5.0 for 0*M* urea–gelatin solutions to pH 5.4 for 5*M* urea–gelatin solutions. When the slopes of the straight lines in Figure 2 are plotted against the molarity of urea, the relationship shown in Figure 3 is obtained.

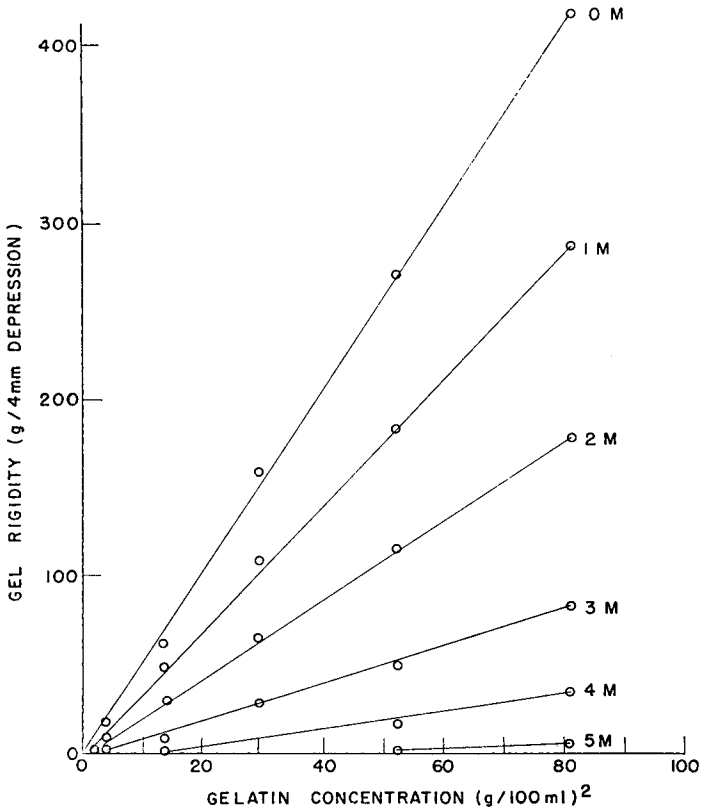

Fig. 2. Relationship of gel rigidity to gelatin concentration in the presence and absence of urea.

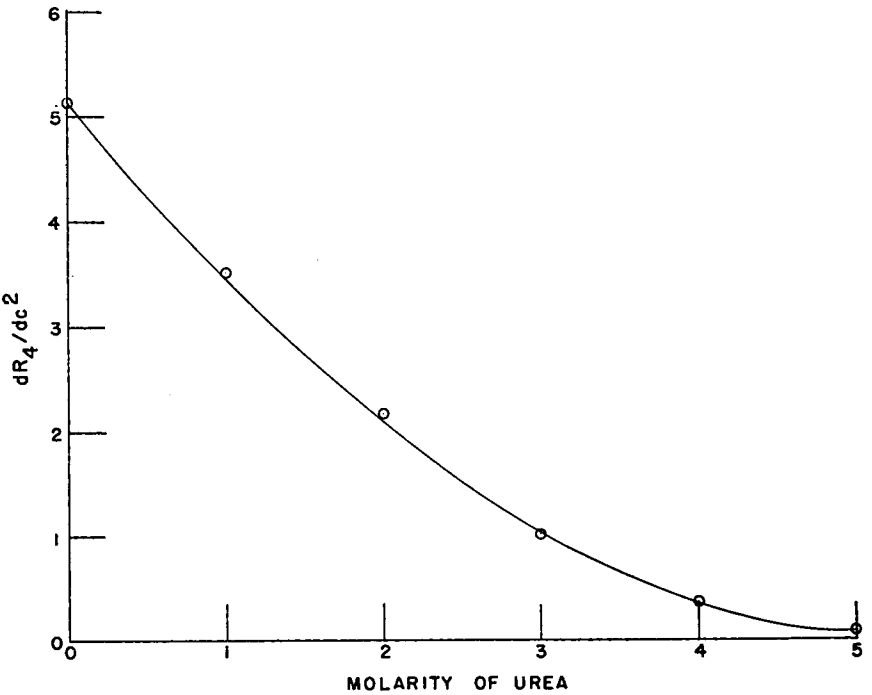

Fig. 3. Dependence of the inhibitory effect on the concentration of urea.

The effect of urea on rigidity of gelatin gels is apparently related to its inhibiting action on formation of crosslinks leading to gelation. By applying formulae available in the literature for rubberlike elastic polymer networks to the rigidity measurements on gelatin gels, one can calculate fractions of building units effective in crosslinkage formation.

Equation of State for Flexible Polymer Networks

By a consideration of the forces which resist the stretching of a solid polymer, the "state" equation for flexible polymer networks may be expressed after Flory and Rehner[18] as:

$$\tau = \left(\frac{\nu}{V}\right) RT \left[\frac{l}{l_0} - \left(\frac{l_0}{l}\right)^2\right] \tag{1}$$

where τ = tension (force per unit initial cross section) (in (dynes/cm.^{2}), ν = number of crosslinkages in the polymer (in g.-mole), V = volume of the polymer, R = molar gas constant (in dyne/cm. g.-mole K.°), T = absolute temperature, l_0 = unstretched length of the polymer, and l = stretched length of the polymer. The quantity ν/V is called the network activity. It may be determined, following the reasoning of Bardwell and Winkler,[19] by the relationship:

$$\nu/V = g(d/M_0)\rho \tag{2}$$

where g = a dimensionless proportionality constant = 1, d = density of the polymer (in g./cm.^{3}), M_0 = molecular weight of the structural unit, and ρ = fraction of structural units involved in crosslinkage formation. These two equations may be combined to yield the relationship:

$$\tau = \rho \left(\frac{d}{M_0}\right) RT \left[\frac{l}{l_0} - \left(\frac{l_0}{l}\right)^2\right] \tag{3}$$

In the derivation of the eq. (3), the degree of extension (l/l_0), the absolute temperature T, and the fraction ρ of structural units involved in crosslinkages are treated as being capable of independent variation. The dependence of gel rigidity and of crosslinking on the absolute temperature was discussed by Ferry,[2] Miller et al.,[20] and Eldridge and Ferry.[21] It is obvious that gelatin gels exist only over a certain temperature range and that their properties are thermodependent. Considerations developed in this work refer to gels at 10°C.

Adaption of the State Equation to Gelatin Gel Rigidity Measurements Obtained with the Gel Characterization Apparatus

We define d = weight of gelatin per unit volume of gel (in g./cm.^{3}), l = depth of the gel before compression = 2.30 cm., l_0 = depth of the gel after compression = 1.90 cm., R_4 = gel rigidity (in dynes/4 mm.), $\tau = R_4/1.98$ = compressive force in dynes per unit area eqerted by the plunger on the gel in deforming the gel from 2.30 cm. to 1.90 cm., M_0 = average molecular weight of the monomer units in gelatin molecule = 92.4,[22] T = 283°K. With these modifications, eq. (3) adapted to gelatin gels takes the following form:

$$\frac{R_4}{1.98} = \rho \left(\frac{d}{92.4}\right) (8.314 \times 10^7)\,(283)\left[\left(\frac{23}{19}\right) - \left(\frac{19}{13}\right)^2\right] \tag{4}$$

or

$$\rho = (R_4/d) \times 3.78 \times 10^{-9} \tag{5}$$

Thus, the fraction of the structural units involved in gel formation may be estimated from the rigidity and the volume concentration of the gels.

It is acknowledged that this application of Flory's theory to gelatin gels involves a number of approximations. It is believed, however, that the calculated values are sufficiently accurate to establish the order of magnitude of gel particle associations. Some of the approximations involved are: (*a*) the compression of the gel from depth l to depth l_0 is mechanically equivalent to the stretching of a gel from l_0 to l; (*b*) the effective area of compression is assumed to be that of the plunger. The actual effective area is probably somewhat larger than this.

Fractions of amino acids involved in crosslink formation ρ at different urea concentrations are plotted in Figure 4. They are directly proportional to gelatin concentration at a given urea concentration. Density of the

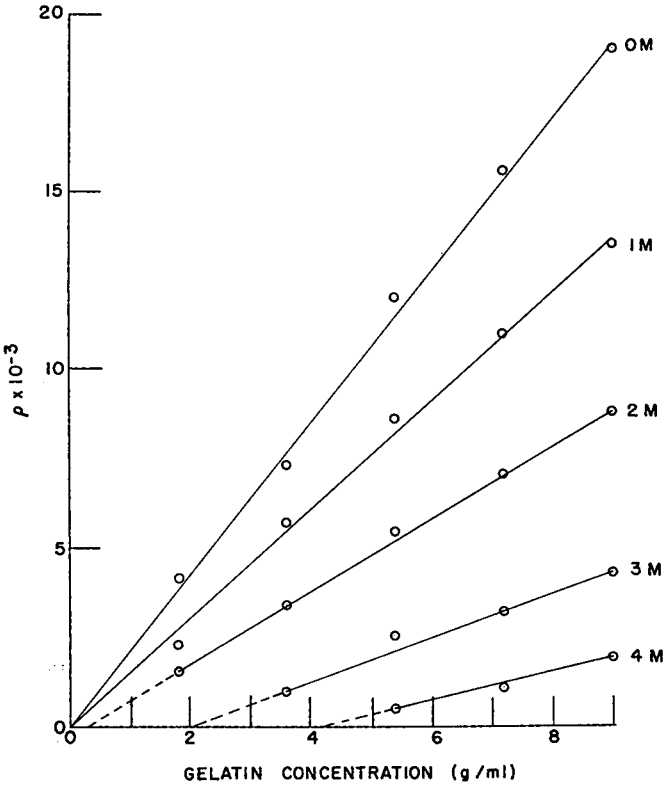

Fig. 4. Fraction of amino acid residues involved in crosslink formation in the presence and absence of urea.

polymer d has been calculated from the volume of solutions measured at room temperature and from the weight of gelatin, dry basis. The use of solution volume rather than gel volume is justified, since the theoretical loss in volume on cooling from room temperature to 10°C. and during the sol–gel transformation does not amount to more than a fraction of one per cent.

The calculated numbers indicate that only a very small percentage of amino acids comprising the gelatin molecule form crosslinks building the gel structure. If the number of amino acid residues per molecule of gelatin of the molecular weight of 38,700 is taken as 419,[22] then an approximate average of 1.7 amino acids per molecule would be involved in crosslinkage formation in a gelatin gel containing 1.8% of the protein ($4.2 \times 10^{-3} \times 419$). For a gel containing 9.0% of gelatin, this number would be raised to about 8.0.

Urea lowered significantly the number of useful crosslinks formed in gelatin gels. Figures 4 and 5 show that the total number of linkages inhibited by urea increased with the concentration of gelatin and with the concentration of urea. The differences between the fraction of amino acid residues

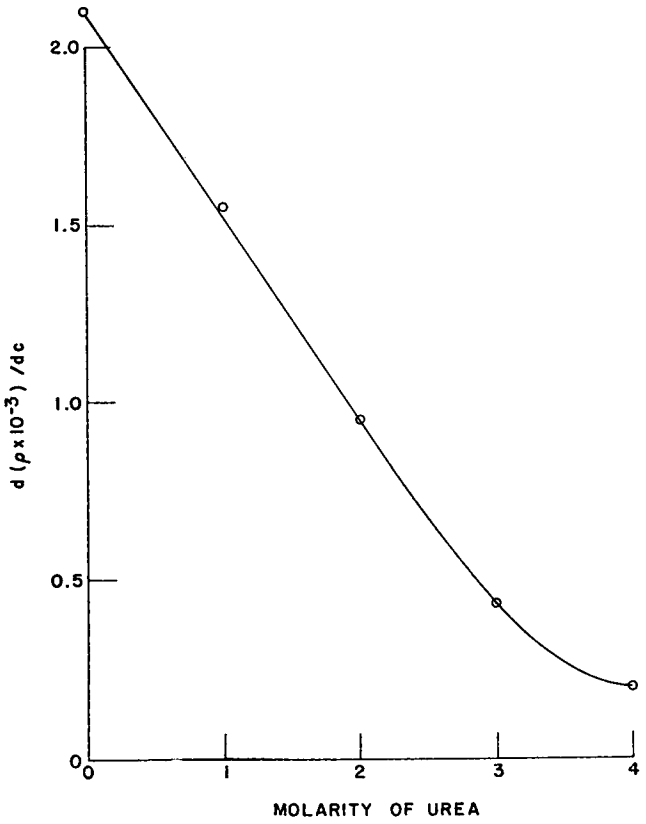

Fig. 5. Effect of urea concentration on fraction of amino acid residues effective in gelation.

effective in crosslinkage formation in the absence of urea, and the corresponding value in the presence of urea, represents the fraction of crosslinks inhibited by the given concentration of urea. Calculations of these values from Figure 4 indicate that the percentage of linkages inhibited increased with the concentration of urea, but decreased somewhat with the concentration of gelatin. Approximate calculations indicate that 1*M* urea inhibits the formation of 45–30% crosslinks, 2*M* urea inhibits 60–55%, 3*M* urea inhibits 100–70%, and 4*M* urea inhibits 100–90%, in the order of increasing gelatin concentration.

Urea is a well known protein-denaturing agent and causes unfolding of the protein molecule.[23,24] It increases the solubility of certain amino acids[25] and brings about softening (*in vivo*)[26] or dissolution (*in vitro*)[27] of collagen tissue. The exact mode of action of urea is not known. It is generally believed that the effect of urea on proteins,[28] polyvinyl alcohol,[29] polyacrylic acid,[30] and other similar structures is due to breaking of hydrogen bonds. Kauzmann[31] advanced the possibility that, in addition to breaking hydrogen bonds, urea may have a small direct effect on weakening hydrophobic

bonds. Levy and Magoulas have shown recently[32] that urea does not break hydrogen bonds in dicarboxylic acids and suggested that this evidence be applied to the interpretation of the role of urea in protein denaturation. In light of conflicting views on the type of linkages affected by urea, no firm conclusions can be drawn from this study regarding the type of bonds involved in gelatin gel formation, although most of the evidence would seem to point to hydrogen bonds.

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Résumé

On a étudié l'effet de l'urée sur des gels de la gélatine en mesurant la rigidité des gels à des concentrations differentes de la gélatine et de l'urée. La rigidité des gels était exprimée par la force nécessaire de déprimer la surface du gel avec un plongeur étalon pour une distance de 4 mm. On a trouvé un rapport linéaire, en accord avec les données dans la littérature, quand la rigidité des gels était tracée vers le carré de la concentration de la gélatine. Ce rapport tenait même dans la présence ou l'absence de l'urée et aussi

pour toute les concentrations essayées. L'urée montrait l'effet éclatant d'un abaissement de la rigidité des gels et le rapport de la rigidité au carré de la concentration de la gélatine diminuait avec l'augmentation de la molarité d'urée. Pour comprendre le mécanisme de la formation des gels de la gélatine, les valeurs de la rigidité étaient présentées par la quantité des ponts qui pourrait expliquer de telles valeurs de la rigidité. Les auteurs ont calculé la fraction des résidus des acides aminés effective dans la formation des ponts des valeurs de rigidité des gels en appliquant des théories développées par Flory et al des cadres de polymères élastiques. L'équation d'état était modifiée pour l'adapter aux mesures des rigidities des gels de la gélatine. Les calculs indiquent que ca 1,7 acides aminés par molécule étaient responsables pour la formation des ponts dans un gel de la gélatine d'une concentration de 1,8% et 8,0 acides aminés dans un gel de la gélatine d'une concentration de 9,0% gelé à 10°C. Une concentration d'urée de 1*M* inhibait la formation des ponts de 30 à 45% et l'urée de 4*M* inhibait de 90 à 100% des ponts, l'effet exact qui dépend de la concentration de la gélatine.

Zusammenfassung

Zur Untersuchung des Einflusses von Harnstoff auf Gelatine-Gele wurde die Steifigkeit des Gels bei verschiedenen Gelatine- und Harnstoffkonzentrationen gemessen. Die Gel-Steifigkeit wurde durch die Kraft, die nötig ist, um das Gel mit einem Standardkolben um 4 mm herunter zu drücken, charakterisiert. In Übereinstimmung mit Literaturangaben besteht eine lineare Abhängigkeit der Gelsteifigkeit vom Quadrat der Gelatinekonzentration. Diese Abhängigkeit bestand sowohl in Gegenwart von Harnstoff bei allen untersuchten Konzentrationen als auch ohne Harnstoff. Harnstoff setzte die Gelsteifigkeit stark herab und das Verhältnis der Steifigkeit zum Quadrat der Gelatinekonzentration nahm mit zunehmender Harnstoffmolarität ab. Um den Mechanismus der Gelentstehung zu verstehen, wurden die Steifigkeitswerte zu der Zahl der dafür verantwortlichen Vernetzungsstellen in Beziehung gebracht. Die Zahl der Aminosäurereste, die beim Entstehen von Vernetzungsstellen wirksam waren, wurde mit Hilfe der von Flory und anderen für kautschukartige elastische Polymerwetze aufgestellten Theorien aus den Steifigkeitswerten errechnet. Die Zustandsgleichung wurde in eine für die Auswertung von Messungen von Gelsteifigkeiten geeignete Form gebracht. Die Berechnungen zeigen, dass in einem bei 10°C erstarrten, 1,8 prozentigem Gelatinegel 1,7 Aminosäuren, und bei einem 9 prozentigem Gel 8,0 Aminosäuren pro Molekül an der Entstehung von Vernetzungsstellen beteiligt sind. Eine 1-molare Harnstofflösung verhinderte die Bildung von 30–45% der Vernetzungen und eine 4-molare Harnstofflösung von 90–100%, wobei die genaue Prozentzahl von der Gelatinekonzentration abhängt.

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