THERMODYNAMIC COMPATIBILITY OF GELATIN WITH SOME D-GLUCANS IN AQUEOUS MEDIA

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ABSTRACT

The thermodynamic compatibility of gelatin with a number of D-glucans (amylopectin, glycogen, dextran) in aqueous media has been studied. Particular attention was given to the effect of the nature of electrostatic interaction of gelatin macro-ions on the compatibility with D-glucans. It has been established that all the systems investigated, namely water–gelatin–D-glucan, are thermodynamically unstable under isoionic conditions at sufficiently high concentrations of polymers. On centrifugation ($\sim 1000\,g$), they separate into two liquid phases. The phase separation is accompanied by almost complete separation of the gelatin and the corresponding D-glucan. However, when the pH was shifted towards the acid or alkaline region in relation to the pI of gelatin, as well as when the ionic strength was sufficiently increased, these systems undergo a reversible phase transition from a two-phase to a single-phase state. This suggests that the thermodynamic incompatibility of gelatin with D-glucans in isoionic conditions is determined by self-association of gelatin macro-ions due to interaction of charge fluctuations.

INTRODUCTION

The interaction between proteins and uncharged polysaccharides has been extensively investigated. Attention has been focussed on the thermodynamics of dilute, single-phase systems of water–protein–polysaccharide (excluding the volume effect)[1], as well as on the formation of soluble and insoluble protein–polysaccharide complexes[2–9]. However, until recently, comparatively little attention has been given to the problem of the thermodynamic compatibility of proteins and uncharged polysaccharides.

The first information about the compatibility of proteins and uncharged polysaccharides was obtained by Beijerinck[10], in studying the systems water–gelatin–agar and water–gelatin–soluble starch. At concentrations above 1%, the systems separated into two liquid phases. The phase separation resulted in nearly complete separation of protein and polysaccharide. A similar phenomenon was observed[11] with mixtures of gelatin solutions and autoclaved starch. Phase separation in mixtures of gelatin solutions with sols of various starches (grain and potato) were studied by

Ostwald and Hertel[12]. The systems containing potato starch were noted for their behaviour in the acid and alkaline pH regions; under such conditions, they were single-phase. Doi[13] investigated the phase state of the water–gelatin–amylopectin system in connection with the crystallization of amylopectin in gelatin gels. He plotted the cloud-point curves of this system at pH 5.2 and 7.0, and two temperatures (30°, 37°). A rise in temperature and pH somewhat displaced the binodal toward the region of higher concentration.

The above data are of a special and descriptive character, and a number of interesting points touching upon the generality and nature of the phenomenon of incompatibility in the systems water–protein–uncharged polysaccharide remain unclear. We have now investigated the compatibility of gelatin (G) with several D-glucans (DGL): amylopectin, glycogen, and dextran. Particular attention was given to the influence of the nature of electrostatic interaction of gelatin macro-ions on its compatibility with DGL. Preliminary experiments pointed to a sufficient generality of behaviour of systems containing different DGL, and this detailed investigation was therefore confined to the system water–gelatin–dextran (D).

RESULTS AND DISCUSSION

All the H_2O–G–DGL systems under investigation are thermodynamically unstable under isoionic conditions* when the concentrations of polymers are sufficiently high. On centrifugation, they separate into two liquid phases. The phase separation is accompanied by nearly complete separation of gelatin from the corresponding DGL. This follows, for example, from the phase diagram (Fig. 1) of the isoionic system H_2O–G–D, which is typical of all the systems investigated. The diagram shows that the equilibrium solubility of gelatin in a sufficiently concentrated dextran solution, like the solubility of dextran in a concentrated gelatin solution, does not exceed 1×10^{-2} g/g. Hence, according to our findings, the incompatibility of

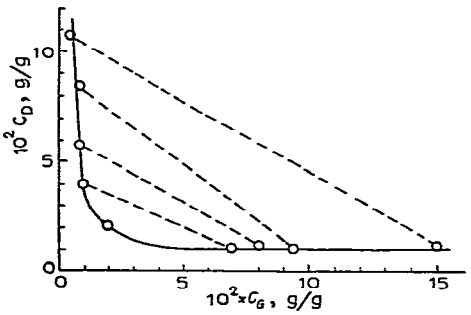


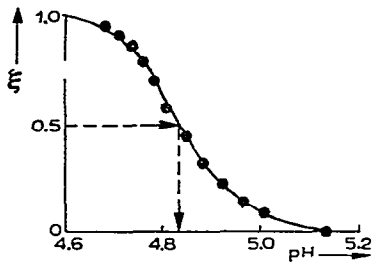
Fig. 1. Phase diagram of the H_2O–G–D system at 42.5 ±0.1°. ——, binodal; – – – –, tie lines.

*According to definition, the isoionic system H_2O–G–DGL does not contain any ions other than gelatin macro-ions and protons. As a rule, it has pH 4.9, but with an excess of DGL, the pH increases by 0.1–0.3 unit. However, it still may be assumed that, in this case too, the gelatin phase has pH 4.9.

gelatin with DGL appears to be a sufficiently general phenomenon. The substantial differences in the chemical and physical structure of the indicated DGL do not noticeably affect the compatibility with gelatin.

All the H_2O–G–DGL systems investigated undergo a reversible phase transition from a two-phase to a single-phase state when the pH shifts to the acid or alkaline region in relation to the pI of gelatin, as well as when there is a sufficient increase in ionic strength. The phase transition is accompanied by a characteristic change in light scattering. This made it possible to apply nephelometric titration to determine the points of phase transition. It is possible to consider the ratio $(I_{90}^{max}-I_{90})/(I_{90}^{max}-I_{90}^{min})$ as the co-ordinate of the transition (ξ), where I_{90}^{max} is the light scattering of the initial heterogeneous system, I_{90}^{min} is the minimum light-scattering corresponding to the absolutely stable, single-phase state of the system, and I_{90} is the light-scattering of the system in an intermittent state.

Figs. 2 and 3 present the curves of phase transitions ξ(pH) for the system H_2O–G(1.6×10^{-2} g/g)–D(6.4×10^{-2} g/g) in an acid and alkaline region in relation to the pI of gelatin. The curves are S-shaped. Within the accuracy of the experiment, the inflection points of the curves coincide with the midpoints of transition ($\xi=0.5$). For the given system, $\xi=0.5$ corresponds to pH 4.83 in the acid and pH 5.27 in the alkaline region in relation to the pI of gelatin. It is difficult to determine experimentally the stability of the system at these points. It may, however, be assumed that they belong to the spinodal of the system, *i.e.* to the boundary between the region of absolutely unstable states and that of states stable to small fluctuations in composition. At pH 4.6 and 5.4, corresponding to $\xi=1$, the system is thermodynamically stable and single-phase.



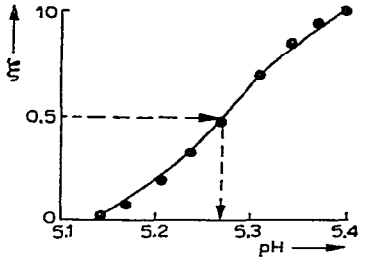
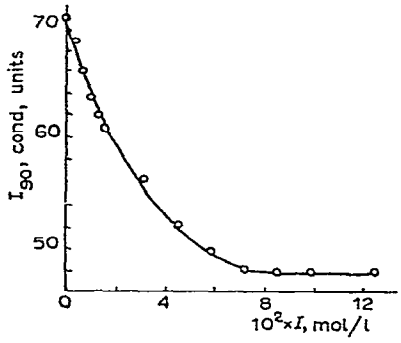


Fig. 2. Curve of phase transition for the H_2O–G(1.6×10^{-2} g/g)–D(6.4×10^{-2} g/g) system in the pH region that is acid in relation to the pI of gelatin.

Fig. 3. Curve of phase transition for the H_2O–G(1.6×10^{-2} g/g)–D(6.4×10^{-2} g/g) system in the pH region that is alkaline in relation to the pI of gelatin.

Phase transition in the case of a sufficiently high ionic strength is accompanied by a somewhat different change in light-scattering (Fig. 4). As the ionic strength increases, light-scattering monotonically diminishes to a minimum value with $I=0.1$ mole/l. At this ionic strength, the system is thermodynamically stable. Unlike

ξ(pH), the curve $I_{90}(I)$ has no particular points. This is probably linked with methodological peculiarities of titrating with salt solutions (see below).



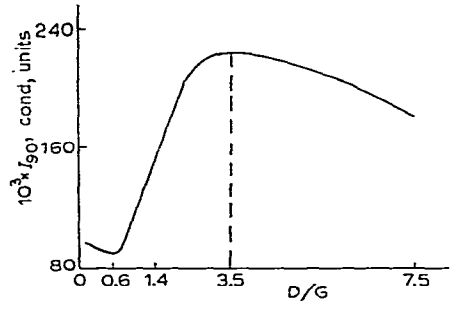


Fig. 4. Dependence of the light scattering of the H_2O–G(1.6×10^{-2} g/g)–D(6.4×10^{-2} g/g) system on ionic strength at pH 5.14. The intensity of scattered light is expressed in conditional units.

Fig. 5. Dependence of the light scattering of the H_2O–G–D system in the region of a thermodynamically stable, single-phase state ($C_{HCl} = 5.5 \times 10^{-3}$ mole/l) on the ratio between the weight concentrations of gelatin and dextran. The intensity of scattered light is expressed in conditional units. The scale of the D/G concentration ratio is non-linear.

The above data reveal some typical features inherent in the nature of the thermodynamic incompatibility of gelatin and DGL in isoionic conditions. The thermodynamic incompatibility of these polymers is apparently determined by self-association of gelatin macro-ions, due to interaction between the charge fluctuations.

It has been proved theoretically[14,15] and experimentally[16–19] that, in isoionic solutions, long-range attractive forces act between polyampholyte macro-ions which on the whole are electroneutral. The forces are determined by fluctuations of the polyampholyte macro-ion charge. The interaction between the charge fluctuations is characterized by a negative contribution to the chemical potential of the polyampholyte.

Dilution of the isoionic polyampholyte solution is attended by an increase in the average distance between macro-ions and leads, in the thermodynamic sense, to greater electrostatic energy of the solution. If the polyampholyte solution is diluted with a solvent or a solution of a low-molecular non-electrolyte, this increase in energy is compensated by the higher entropy of the system, associated with an increase in volume. In this case, dilution is thermodynamically advantageous. However, when the isoionic polyampholyte solution is diluted with a solution of an uncharged polymer (*e.g.* DGL), the entropy of the system increases but apparently not to a great extent, since a considerable part of the added volume is inaccessible to polyampholyte macro-ions by virtue of the size of the uncharged polymer macromolecules (excluded volume effect). In this case, the effect of increase in electrostatic energy predominates, and for this reason concentration of polyampholyte and of uncharged polymer in different phases is thermodynamically substantiated.

The interaction between charge fluctuations is suppressed when the pH is shifted to one or another region in relation to the pI of the polyampholyte, as well as when the ionic strength is sufficiently increased (generally up to 0.1 mole/l)[16]. Simultaneously, a sharp increase in co-solubility of the polyampholyte with uncharged polymers should be observed, which is corroborated experimentally. It is precisely under such conditions that the H_2O–G–DGL system undergoes a reversible phase transition from a two-phase to single-phase state.

The interaction between the charge fluctuations is intrinsically linked with the nature of proteins as typical polyampholytes. There is sufficient ground for assuming that the incompatibility of proteins with DGL under isoionic conditions is a general phenomenon. For instance, according to our data[20], human serum albumin is incompatible with amylopectin under isoionic conditions. However, the co-solubility of these polymers sharply increases when the pH is shifted to a more-acid region.

Woodside *et al.*[3–8] have discussed the possibility of formation of G–DGL complexes in aqueous medium. Proof of the existence of these complexes is provided by the considerable solubility of DGL in acidic ethanol in the presence of gelatin. Woodside *et al.* suggested that G–DGL complexes are formed in distilled, deionized water, *i.e.* under isoionic conditions. This conclusion conflicts with our findings. The predominant tendency of polymers towards separation, accompanied by phase separation of the H_2O–G–DGL system, is incompatible with the formation of complexes of higher solubility.

From the data of Woodside *et al.*[4–6], it is evident that addition of a sufficient quantity of trichloroacetic or some other acid to the diluted H_2O–G–DGL system always preceded the identification of complexes, by ethanol precipitation of uncombined DGL. Consequently, it cannot be assumed that soluble G–DGL complexes exist in the H_2O–G–DGL system before the addition of acid.

We find that sufficiently concentrated H_2O–G–DGL systems, given a certain acidity of the medium, undergo a phase transition from the hetereogeneous to the homogeneous state, which evidently corresponds to a drastic change in the nature of interaction between the polymeric components of this system. The single-phase state of the system, which is thermodynamically stable under such conditions, no longer contradicts the assumption that soluble G–DGL complexes exist in it. Thus, the formation of G–DGL complexes established by Woodside *et al.* should be related to quite definite conditions of acidity of the medium in which the single phase state of the H_2O–G–DGL system is thermodynamically stable. It is precisely in such conditions that DGL is not precipitated by ethanol.

The formation of soluble G–DGL complexes at pH<pI of gelatin is also confirmed by our nephelometric and viscosimetric data[21,22]. At pH 3–4, there is a sharp increase in the light scattering and viscosity of the H_2O–G–DGL system without a change of the single phase state. In this case, the light scattering of the system depends in a peculiar way on the ratio between the concentrations of polymers. Fig. 5 shows the dependence of the light scattering of a thermodynamically stable, single phase H_2O–G–D system on the ratio between the weight concentrations of

gelatin and dextran. The sharp increase in light scattering with D/G = 0.6–3.5 is noteworthy. Maximum scattering is observed at D/G = 3.5, which consequently conforms to the optimal composition of the G–D complexes. These results are in satisfactory agreement with the data of Woodside *et al.*[5] on the precipitation of dextran by ethanol from its acid mixtures with gelatin.

In attempting to clarify the influence of phenol on G–DGL complex formation, Woodside *et al.*[5] treated the isoionic H_2O–G–DGL system with a 45% solution of phenol. The fact that gelatin was extracted by phenol was considered to indicate destruction of the G–DGL complexes. However, adequate separation of gelatin from DGL could have been achieved upon phase separation of a sufficiently concentrated H_2O–G–DGL system in the course of centrifugation. Experiments[5] to ascertain the influence of complex formation on the gel formation of gelatin, studied in the case of a clearly two-phase, isoionic, concentrated H_2O–G(4%)–DGL(4%) system, are also inconclusive.

EXPERIMENTAL

Materials. — Commercial, alkali-precursor, photographic gelatin, when purified[23] and deionized[24] by literature procedures, had pI 4.90. The diffusion coefficient and the intrinsic viscosity of the purified preparation were determined in 2M potassium thiocyanate at 25°. On the basis of these data, the molecular weight of the gelatin was calculated[25] to be 3.0×10^5 g/mole. Such a high value indicates the presence of multichain collagen destruction products. The physico-chemical parameters of the gelatin preparation are: diffusion coefficient at infinite dilution, $D_0 \times 10^7 = 2.25 \pm 0.08$ cm^2/sec; intrinsic viscosity $[\eta] = 0.57$ dl/g; molecular weight, $3.0 \pm 0.5 \times 10^5$.

Amylopectin was isolated from waxy-corn starch and subjected to prolonged Soxhlet extraction with water–*p*-dioxane to remove the fatty acids, soluble salts, and protein admixtures[26]. The defatted starch was dissolved under nitrogen in 0.5M potassium hydroxide with continuous stirring. After neutralization, the starch solution was centrifuged to remove insoluble material, subjected to exhaustive electrodialysis, and lyophilized. The purity of the isolated amylopectin was established by colorimetric determination[27] of the "blue value", using B.D.H. amylopectin as standard. The "blue value" was similar to that of the standard.

Chemically pure, rabbit-liver glycogen "Biomed" (PO_4^{3-}, undetectable; N, 0.63%) and dextran "Serva" (PO_4^{3-}, N, undetectable) were used without additional purification. The molecular weight of the dextran, determined by the light-scattering method, was 8.4×10^4 g/mole.

Stock solutions. — The gelatin solutions were prepared by weight from a concentrated (10–15%) gel. At a lowered temperature (~4°) and 100% humidity, the gel could be kept without change in concentration for 7–14 days. To prevent bacterial infection, traces of bis(tri-*n*-butyl) stannoxide (<0.005%) were added to the gel.

Stock polysaccharide solutions (8–16%) were prepared by weight at room, or

slightly elevated (50–60°), temperature, followed by centrifugation to remove the insoluble material. The concentration of the stock solutions was determined from the weight of dry residue. Ordinary distilled water was used for diluting the stock solutions.

Methods. — The behaviour of the H_2O–G–DGL systems during centrifugation (30 min; 1000 *g*) served as a qualitative criterion of their stability; unstable systems separated into two liquid phases.

The DGL concentration in the co-existing phases was determined spectrophotometrically at 322.5 nm by the sulphuric acid method. This method, initially suggested[28] for sugars of low molecular weight, proved highly effective for determining DGL in the presence of gelatin.

The concentration of gelatin in the co-existing phases was determined spectrophotometrically at 230 nm. Before analysis, the solutions were heated for 1 h at 40° to disaggregate the gelatin. Beer's law was followed up to an absorbance of 0.9; the accuracy of the determinations was within ±3%. The extinction coefficient of gelatin was $E^{1\%}_{1cm} = 19$, irrespective of the DGL concentration. A "Specord UV-VIS" spectrophotometer (Carl Zeiss, Jena) was used for spectrophotometry; the accuracy, when using a recording device and a bridge measuring-scheme, did not exceed ±2%. The extinction coefficients of gelatin and DGL were regularly checked by appropriate standard solutions.

Determination of the phase diagram of the H_2O–G–D system. — On centrifugation, the phases separated quite rapidly. The volumes of the phases reached a constant value within 1 h. This time, however, was insufficient for the system to attain an equilibrium between the phases in all the components. Moreover, during rapid separation, as a result of coalescence of the dispersion phase drops, the phase interface contact-area sharply diminished, and the conditions for mass exchange between phases deteriorated. Therefore, when determining an equilibrium phase diagram, centrifugation was not used.

The aliquots of the stock dextran solution and gelatin gel were added by weight into 5-ml test-tubes having well-ground stoppers. To ward off evaporation of water, the microsections of the test-tubes were thoroughly greased with a vacuum lubricant. The test-tubes were then placed in an ultrathermostat (temp. 42.5±0.1°) for 7 days. The contents of the test-tubes were thoroughly stirred by inversion with shaking.

Samples of the phases for analysis were withdrawn by syringe, the part of the solution adjoining the interface being rejected. The samples (1–2 g) were diluted to a concentration ≥ 0.3 g/dl. Subsequently, all dilutions required for analyses were carried out by volume. The compositions of the co-existing phases were calculated on a weight scale (g/g). In every case, in conformity with the law of mass conservation in a closed system, the points of the phase diagram, representing the compositions of the co-existing phases, lay on the same straight line as the point representing the initial composition of the system, thereby confirming the satisfactory quality of the analyses.

Nephelometric titration. — These titrations were carried out at 436 nm on a

"Spekol" spectrocolorimeter (Carl Zeiss, Jena) equipped with a special titration attachment "Ti". The device was also furnished with an appliance for regulating the temperature of the cell by a thermostat. The intensity of light scattered at an angle of 90° (I_{90}) was recorded through a multi-purpose amplifier "ZV" and a voltage divider by the recording device "EZ-2" (Lp, Prague). The recording device was supplied with a polarographic filter for smoothing the fluctuations of the signal caused by variations of optical heterogeneity of the stirred solution.

Before titration, equal volumes (2.5 ml) of the stock gelatin (0.8%) and DGL (12.6%) solutions at 40° were put into a 5-ml cylindrical cell. The cell was then placed in a spectrocolorimeter and kept there for 1 h, with careful magnetic stirring. During this time, light scattering reached a constant level. The titrant (12.5mM HCl or KOH) was added to the cell in small portions (0.05 ml) by means of an Autoburette ABU-1b "Radiometer". After the addition of each portion of the titrant, especially before the inflection point of the nephelometric curve, the light scattering sharply increased and then relaxed during 5–7 min to the constant value. These "equilibrium" values were used to plot curves of nephelometric titration*. The "equilibrium" curves** of nephelometric titration, in the coordinates I_{90}–volume of the titrant, were typically S-shaped. The accuracy of determining their inflection point was within ±7%. These curves were replotted into ξ(pH) curves, proceeding from the data on potentiometric titration.

The method of titration with sodium chloride was essentially similar. However, in this case the curves of nephelometric titration had no inflection point, which may be linked with the relatively high (1.499M) concentration of the titrant.

The stability of the systems at the particular points of the nephelometric titration curves was determined during centrifugation, as described above.

Potentiometric titration. — These titrations were effected with 12.5mM HCl or KOH, using a TTT-1c "Radiometer" Autotitrator, the pH meter being preliminarily calibrated by two standard buffer solutions (pH 6.5 and 4.01). The titration curves of the H_2O–G(1.6×10^{-2} g/g)–D(6.4×10^{-2} g/g) system were plotted at intervals of 0.1 pH unit. In the pH range 5.14–5.80, the titration curve approximated to one straight line, and in the range 5.14–4.40 to two straight lines so as to facilitate interpolation. The equations of the straight lines, obtained by the method of least squares, were used for transforming the experimental curves of nephelometric titration into curves ξ(pH).

All operations (mixing, centrifuging, and nephelometric and potentiometric titrations) were conducted at 40°.

*The curves obtained upon continuous addition of the titrant at a constant rate had an anomalous appearance in the precritical region, which is linked with their non-equilibrium[21].

**Strictly speaking, these curves should be termed quasi-equilibrium, since it is not certain that an equilibrium distribution of the components between the co-existing phases corresponds to each point of these curves.

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REFERENCES

1 T. C. LAURENT, *The Chemistry and Physiology of Mucopolysaccharides, Proc. Symp., Milan, 1965*, (Publ. 1968), p. 153.
2 E. L. ROZENFELD AND E. G. PLYSHEVSKAYA, *Usp. Sovrem. Biol., 46* (1958) 130.
3 E. E. WOODSIDE AND W. KOCHOLATY, *Blood*, 16 (1960) 1173.
4 E. E. WOODSIDE, C. A. FRICK, AND W. KOCHOLATY, *Proc. Soc. Exptl. Biol. Med.*, 119 (1965) 327.
5 E. E. WOODSIDE, G. F. TROTT, R. J. DOYLE, AND C. W. FISHEL, *Arch. Biochem. Biophys.*, 117 (1966) 123.
6 R. J. DOYLE, E. E. WOODSIDE, AND C. W. FISHEL, *Carbohyd. Res.*, 5 (1967) 274.
7 E. E. WOODSIDE, G. F. TROTT, R. J. DOYLE, AND C. W. FISHEL, *Carbohyd. Res.*, 6 (1968) 449.
8 R. J. DOYLE, E. E. WOODSIDE, AND C. W. FISHEL, *Life Sci.*, 8 (1969) 121.
9 E. E. SMITH, Z. H. GUNJA SMITH, AND I. J. GOLDSTEIN, *Biochem. J.*, 107 (1968) 715.
10 M. W. BEIJERINCK, *Zentr. Bakteriol. Parasitenk., Abt. II*, 2 (1896) 627, 698; *Kolloid Z.*, 7 (1910) 16.
11 O. BÜTSCHLI, *Untersuch. Strukt.*, (1898) 251.
12 W. OSTWALD AND R. H. HERTEL, *Kolloid. Z.*, 47 (1929) 258, 357.
13 K. DOI, *Biochim. Biophys. Acta*, 94 (1965) 257.
14 S. A. RISE AND M. NAGASAWA, *Polyelectrolyte Solutions*, Academic Press., New York, 1961, p. 178.
15 J. G. KIRKWOOD AND J. B. SCHUMAKER, *Proc. Natl. Acad. Sci. U.S.*, 38 (1952) 855, 863.
16 G. EHRLICH AND P. DOTY, *J. Amer. Chem. Soc.*, 76 (1954) 3764.
17 S. N. TIMASHEFF, H. M. DINTZIS, J. G. KIRKWOOD, AND B. D. COLEMAN, *J. Amer. Chem. Soc.*, 79 (1957) 782.
18 H. BOEDTKER AND P. DOTY, *J. Phys. Chem.*, 58 (1954) 968.
19 A. VEIS, *J. Polym. Sci.*, 26 (1957) 113.
20 V. B. TOLSTOGUZOV AND V. YA. GRINBERG, *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1970) 1423.
21 V. YA. GRINBERG, V. B. TOLSTOGUZOV, AND G. L. SLONIMSKY, *Vysokomolekul. Soedin.*, A12 (1970) 1593.
22 V. B. TOLSTOGUZOV, V. YA. GRINBERG, AND L. I. FEDOTOVA, *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1969) 2839.
23 J. LEOB, *Proteins and the Theory of Colloid Phenomena*, Gizlegprom Publishing House, Moscow, 1932, p. 52.
24 J. W. JANUS, A. W. KENCHINGTON, AND A. G. WARD, *Research (London)*, 4 (1951) 247.
25 V. N. TSVETKOV AND S. I. KLENIN, *J. Polym. Sci.*, 30 (1958) 187.
26 T. J. SCHOCH, *J. Amer. Chem. Soc.*, 64 (1942) 2954.
27 G. A. GILBERT AND S. P. SPRAGG, *Methods Carbohyd. Chem.*, 4 (1964) 168.
28 J. H. BATH, *Analyst*, 83 (1958) 451.