ON THE SOLUBILITY OF GLIADINLIKE PROTEINS

II. Solubility in Aqueous Acid Media[1]

PETER MEREDITH

ABSTRACT

Gliadinlike proteins are completely miscible with dilute aqueous acid but form two-phase, liquid-liquid systems in aqueous acid salt solutions. The concentration-temperature diagram for incipient phase separation shows three maxima at characteristic protein concentrations; the temperatures of the maxima vary with acid and salt concentration. Precipitation and solubility of these proteins are dependent on the concentration of salt in the medium and not on combination of salt and protein. Evidence suggests that salt affects the inter- and/or the intramolecular bonding of the protein and that the amide groups may be concerned in this bonding system. It is concluded that further fractionation of the proteins is necessary before their solubility properties may be adequately explained, and that fractionation by precipitation or solubility is theoretically possible.

Many workers have considered the solubility of gluten and derived fractions from a qualitative standpoint, but with one exception (1) no quantitative studies of the type applied to protein purity problems have come to the author's attention. The anomalous behavior of "saturated" gliadin solutions was noted by Bungenberg de Jong and Klaar (2,3), but their observation of "separation" phenomena was not followed up. The effects of salt on the solubility and precipitation of gliadin have received considerable attention in the past, but no investigation of the mechanisms involved has been published.

During the earlier studies of gluten structure (4) it was found that part of lipid-free gluten was soluble in a methanol-chloroform mixture. In general properties, this part was similar to gliadin, which is also prepared by extraction into an organic solvent. It has been tentatively concluded that a relative lack of ionizable groups is responsible for the curious solubility of these proteins. The original preparation contained about 10% of nonnitrogenous material, probably carbohydrate (4).

Observations of the solubility of this type of protein fraction in dilute acid and salt solutions will be presented, and it will be shown that two-phase, liquid-liquid systems are formed. In these circumstances there is a possibility for fractionating the protein mixture.

[1]Manuscript received December 16, 1963. Contribution from the Wheat Research Institute, Dept. of Scientific and Industrial Research, Christchurch, New Zealand.

Materials and Methods

Several acid-soluble preparations from different bakers' flours have been used during the experiments, as indicated in part I of this series (5). For preparations used in plotting phase diagrams, particular care was taken in centrifuging the acid solutions so that all traces of gel-forming protein (6) were removed. Unless this precaution was taken, some parts of the curves could not be easily determined because of permanent turbidity in the solutions. For comparison, gliadin was prepared by extraction into 55% ethanol from flour previously extracted with 10% sodium chloride solution.

Phase diagrams were plotted by making successive small additions of solvent to the protein; the temperature of complete miscibility was determined for each concentration. Values are the means of readings taken with rising and falling temperature.

Glycine buffers were made according to Mills (7) but contained varying sodium chloride concentrations. For the solubility experiments, concentration of protein was determined by measurement of absorption of light at 280 mμ, using the original protein fraction for calibration. To the extent that there has been a fractionation of proteins differing in tyrosine and tryptophan content, these concentration data may be in error.

Results

Solubility of Protein in Acid Buffers. The protein preparations were miscible in all proportions with dilute acid solutions. That is, only one phase was present at all protein concentrations and the viscosity varied from that of the solvent through a thick gum to a glass. Thus the constant solvent solubility curve was a straight line of unit slope.

Addition of salts reduces the solubility in dilute acids. Curves of solubility against sodium chloride concentration were determined for two concentrations of protein in glycine-hydrochloric acid buffer with varying additions of sodium chloride. When data of solubility against concentration of sodium chloride in the buffer were plotted (Fig. 1, a), the inflections of the two curves agreed; whereas for the ratio of sodium chloride to protein (Fig. 1, b), they did not. This implies that it is the *concentration* of sodium chloride in the buffer which controls solubility of these proteins. As there are no sharp points of inflection, different components cannot be distinguished. Both phases were liquid at all stages.

Phase Diagrams. Since a two-phase, liquid-liquid system was present, diagrams of concentration against temperature (c,t) for incipient

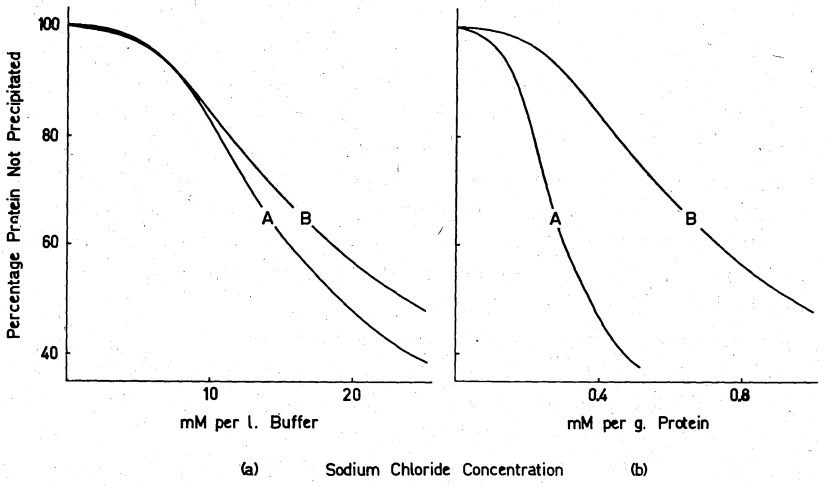

Fig. 1. Precipitation of gliadinlike protein from glycine buffer (hydrochloric acid 10 mM/liter, glycine 50 mM/liter) by increasing concentrations of sodium chloride. The same data are presented in a and b but with the sodium chloride concentration differently expressed. Initial protein concentration was 5.0% (curve A) and 2.5% (curve B).

phase separation in the acid-salt-water-protein systems were drawn. The fundamental type of curve is illustrated in Fig. 2, a. For conditions within the curve two liquid phases are present, whereas the system is homogeneous for conditions outside the curve. At all protein concentrations both phases could be obtained completely clear by prolonged centrifuging; because of the high viscosity of the lower phase they completely separate only with difficulty. Examination by microscope failed to show any solid phase formation. A faint opalescence was usually observed in the peak II region of concentrations at temperatures above the phase separation point.

As the concentrations of hydrochloric acid and sodium chloride were systematically varied, the family of curves shown in Fig. 3 was obtained. These have in common three maxima, as indicated by Roman numerals in Fig. 2, a. Similar maxima were shown in *c,t* diagrams for gliadin preparations under similar conditions (Fig. 2, b).

Partial *c,t* diagrams, for the peak I region, were plotted for three solvent systems: 1) HCl 10 mM/liter, NaCl 50 mM/liter; 2) HCl 10 mM/liter, NaCl 50 mM/liter, glycine 50 mM/liter; 3) formic acid 10 mM/liter, NaCl 50 mM/liter. The fact that there was no difference between the curves implies that in the presence of salt, the nature of the acid and the presence of glycine (as in the buffer) was not important.

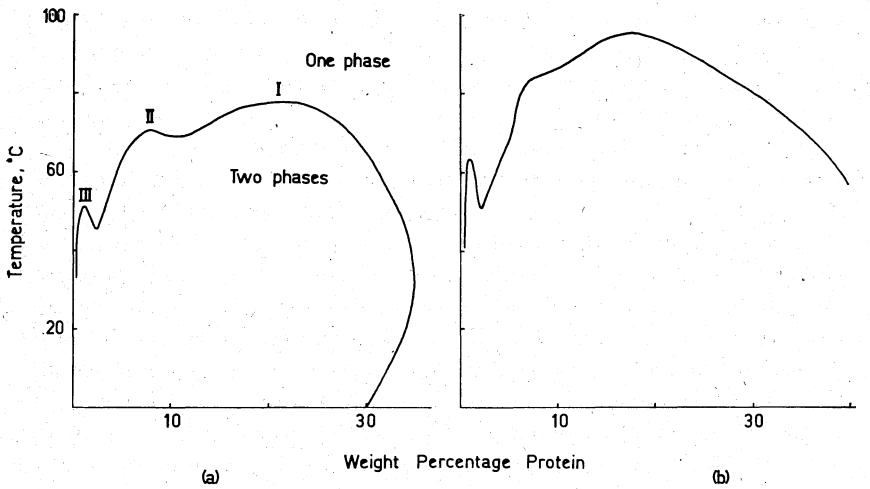

Fig. 2. Diagrams of protein concentration against temperature for incipient phase separation in the system hydrochloric acid 10 mM/liter, sodium chloride 30 mM/liter, water, protein: a, for an acid-soluble, methanol-chloroform-soluble preparation; b, for a gliadin preparation. I, II, and III are maxima referred to in the text.

Discussion

The solubility curves of Fig. 1, when extended to higher salt concentrations (not shown), are similar to the curves presented by Cunningham *et al.* (8) for the precipitation of gluten proteins from acid solutions by various simple salts. These workers assumed that salt precipitation was probably due to the formation of insoluble protein salts. The finding of Lusena (9) that salts at low concentration affect the solubility of gluten in acid has been substantiated in the present work. The ash concentration (0.50–0.75 g./liter of suspension) mentioned by Lusena as reducing substantially the solubility of gluten would, from the present data, reduce the solubility of this gliadinlike preparation in these circumstances to about 80% of maximum. Other components of gluten may be even more susceptible to variations in ionic atmosphere.

The solubility experiments described are incapable of providing information about the number of components in the protein preparation, since the separation of solid phases does not occur. But the graphs of solubility against sodium chloride concentration do provide evidence for the effect of sodium chloride in the protein-buffer system. It is clearly shown that separation into two phases is dependent on the concentration of salt in the buffer and not on combination of salt and protein. The effect is presumably due to changes in the electrical properties of the solvent.

Phase separations in solutions of high polymers have been extensively treated theoretically, and Schultz and Flory (10) demonstrate a *c,t* diagram having two inflections for a mixture of styrene polymers of differing molecular weight dissolved in cyclohexane. These authors and others (11,12) have considered that molecular weight of the polymers was the major determinant of the shape of the *c,t* diagram. Scott (11) concludes that whether phase separation will occur depends primarily on the molecular weights and interaction of the two (or more) polymers, the nature of the solvent being of only secondary importance; the function of the solvent is merely to diminish the interaction constant of the polymers. Present results suggest that salt alters the property of the solvent in this regard. Indeed, phase separation implies that the solvent is poor. On the other hand, Tanford (13) develops an equation for the critical temperature of such systems which includes two functions concerned with solvent-solute interaction. In such a poor solvent there is a preference for segment-segment contacts (of the polymer) rather than for having solvent molecules adjacent to polymer segments. Tanford also shows that poor solvents should become better solvents as the temperature is raised.

We thus conclude that the presence of salt in the acid medium makes the solvent poorer and hence increases the effect of the attractive forces within the protein molecules. Mass-action competition of the activities of hydrogen ions and sodium ions does not explain all the observed effects, since increasing hydrochloric acid concentration in the presence of 30 mM sodium chloride does not decrease the temperature of miscibility at lower protein concentrations (peak III region, Fig. 3). Hence we cannot simply suggest that the effectiveness or otherwise of these aqueous solvents is a function of their abilities to induce charges on the protein molecules. Peticolas (14) has recently discussed the critical salt concentration for biocolloids in terms of the long-range (repulsive) and short-range (attractive) coulombic forces acting between molecules. He suggests that helix formation (coalescence) occurs when the salt concentration in the solvent is sufficiently high to interfere with the long-range forces. Then the short-range forces between nonpolar parts of the molecule are responsible for the less soluble structure; that is, the protein aggregate is held together by "nonpolar" bonds.

The family of phase diagrams obtained when the salt and acid concentrations were varied has proved useful in predicting precipitation phenomena when salt, acid, or protein concentrations are varied, but the shapes of the diagrams have not yet been satisfactorily interpreted. The three maxima occurring at characteristic protein concen-

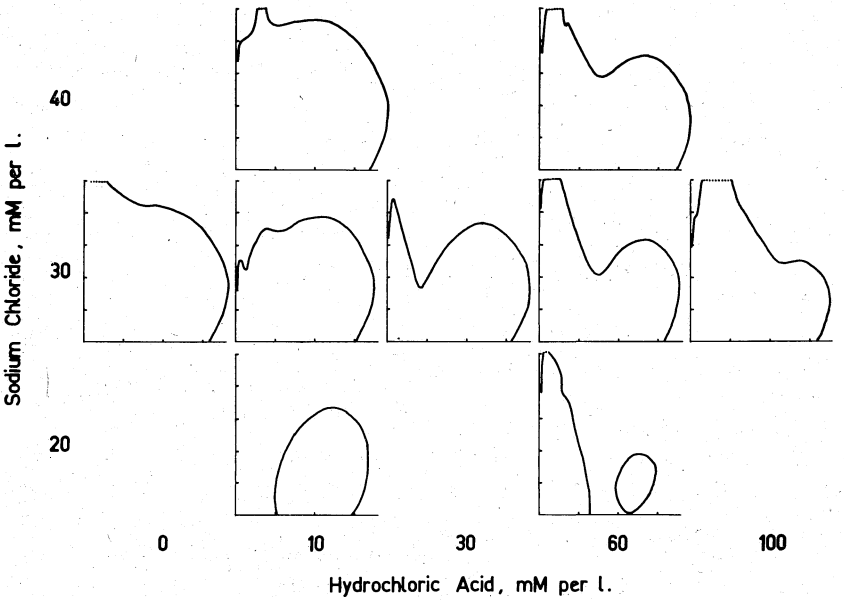

Fig. 3. A family of phase diagrams, each with coordinates similar to Fig. 2, a, showing the effects of varying hydrochloric acid and sodium chloride concentrations in the solvent.

trations could reflect one or more of several changing properties of the protein. Increasing acid concentration tended to increase peak III and decrease peaks I and II. Increasing salt concentration tended to increase peaks I and II and had little effect on peak III. The obvious inference from consideration of these changes is that the three ionic forms of the protein are represented, I being the switterion form and III the acid form. Alternatively, since there are several peaks in the electrophoretic pattern (to be demonstrated in part III of this series), these three maxima could reflect differing solubility properties of three protein components. It is also possible that the three maxima represent three aggregation states. Closely allied to this idea is the idea that merely three different types of solution are involved. Certainly it seems improbable that this type of phase diagram represents true solutions in all areas, and peak II might perhaps represent a liquid crystal structure.

The literature discussed above does not enable us to discriminate between the four different interpretations of the peaks in the *c,t* pattern. We can conclude, however, that differences in molecular weight or in chemical composition (i.e., distinct protein components) could cause this type of pattern without invoking electrical charge, aggregation, or kind of solution. To resolve between the four possibilities,

c,t diagrams for further fractionated material containing, if possible, only a single electrophoretic component will be examined. This is one of two reasons for attempting further fractionation of gliadin preparations, as will be described in part III of this series. The obtaining of single-component fractions would also be in itself desirable, since there is an obvious need to study such material in solution as a distinct type of macromolecule.

In part I of this series it was concluded that a molecule containing an active hydrogen atom plus a small, hydroxyl-containing molecule were requirements for one kind of solubility of these proteins. These requirements are met by dilute aqueous acid. It was further concluded that solubility was dependent on an interrelation between amide content of the protein and acid-salt concentration of the medium. It thus seems likely that the acid-salt concentration is affecting the inter- and/or intramolecular bonding of protein, thus limiting access of solvent to the protein molecules, and that either the amide groups of the proteins are a part of this bonding system, or free carboxyl groups on the proteins interfere with the bonding. Holme and Briggs (15) also concluded that the amide groups were concerned with gliadin solubility and that the imposition of a charge on gliadin molecules is effective in countering interactions between the protein molecules. They further concluded that the intermolecular attractions are of a hydrogen bond nature. Thus, as we can logically expect, all the kinds of bonds found in other proteins seem also to be of importance in the structure of these gliadinlike proteins.

Finally, it should perhaps be placed on record that these proteins, when freeze-dried from concentrated solution, exhibit a micalike structure, not as yet examined physically. This structure does not form from partially deamidated samples.

Literature Cited

1. Broekhuysen, J., and Broekhuysen, A. La solubilité du gluten de froment dans les solutions aqueuses alcalines. Bull. Soc. Chim. Biol. **39:** 883–903 (1957).
2. Bungenberg de Jong, H. L., and Klaar, W. J. Contribution to the knowledge of colloid-chemistry of the gluten. Cereal Chem. **6:** 373–378 (1929).
3. Bungenberg de Jong, H. L., and Klaar, W. J. Colloid chemistry of gliadin separation phenomena. Trans. Faraday Soc. **28:** 27–68 (1932).
4. Meredith, P., Sammons, H. G., and Frazer, A. C. Examination of wheat gluten by partial solubility methods. I. Partition by organic solvent. J. Sci. Food Agr. **11:** 320–328 (1960).
5. Meredith, P. On the solubility of gliadinlike proteins. I. Solubility in non-aqueous media. Cereal Chem. **42:** 54–63 (1965).
6. Meredith, P., Sammons, H. G., and Frazer, A. C. Examination of wheat gluten by partial solubility methods. II. Partition by dilute formic acid. J. Sci. Food Agr. **11:** 329–337 (1960).
7. Mills, G. L. Some observations on the electrophoresis of gliadin. Biochim. Biophys. Acta **14:** 274–281 (1954).

8. CUNNINGHAM, D. K., GEDDES, W. F., and ANDERSON, J. A. Precipitation by various salts of the proteins extracted by formic acid from wheat, barley, rye, and oat flours. Cereal Chem. **32:** 192–199 (1955).
9. LUSENA, C. V. Preparation of dried native wheat gluten. Cereal Chem. **27:** 167–178 (1950).
10. SCHULTZ, A. R., and FLORY, P. J. Phase equilibria in polymer-solvent systems. J. Am. Chem. Soc. **74:** 4760–4767 (1952).
11. SCOTT, R. L. The thermodynamics of high polymer solutions. V. Phase equilibria in the ternary system: polymer 1- polymer 2- solvent. J. Chem. Phys. **17:** 279–284 (1949).
12. TOMPA, H. On the precipitation threshold of solutions of heterogeneous polymers. Trans. Faraday Soc. **46:** 970–976 (1950).
13. TANFORD, C. Physical chemistry of macromolecules. Wiley: New York (1961).
14. PETICOLAS, W. L. Order-disorder transitions in biocolloids. J. Chem. Phys. **40:** 1463–1475 (1964).
15. HOLME, J., and BRIGGS, D. R. Studies on the physical nature of gliadin. Cereal Chem. **36:** 321–340 (1959).