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[CONTRIBUTION FROM THE NORTHERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION[1]]

An Ultracentrifugal Study on the Association–Dissociation of Glycinin in Acid Solution[2]

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Glycinin refers to phytate-free protein which precipitates from an aqueous extract of soybean meal at *p*H 5.1. Ultracentrifugal studies were conducted to discern the effect of *p*H, ionic strength and influence of various salts on the behavior of glycinin in acid systems. The results indicate that glycinin in acid solution is a freely reversible association–dissociation system containing three resolvable fractions having s_{20} values of approximately 2, 7 and 13S. An unresolvable fraction having an s_{20} value >13S is also present. The relative amounts of the unresolvable fraction as well as the resolvable components are dependent upon *p*H, ionic strength and type of salt present. Low *p*H and low ionic strength favor dissociation primarily into the 2 and 7S fractions. Mono- and divalent cations shift the equilibrium toward dissociation. Experiments with calcium chloride, sodium chloride and sodium sulfate indicate that sulfate ions cause a marked shift in association toward formation of large amounts of unresolved, high molecular weight material.

Osborne and Campbell[3] proposed the name glycinin for the "salt-solution soluble globulin which precipitates on dialysis." In spite of the low yield obtained, they considered glycinin to be the principal protein of the soybean.

Several other workers[4–8] have used the name glycinin for soybean protein fractions isolated by procedures different from that described by Osborne and Campbell but apparently they isolated essentially the same protein. However, none of the glycinin preparations or their fractions have been shown to be a homogeneous protein.

In this Laboratory[9,10] the major part of the protein of the soybean has been isolated by extracting defatted meal with water and precipitation of the protein in the *p*H region of 4.0–4.6. The *p*H of maximum precipitation of protein varies with removal of non-protein components from the system. Smith and Rackis[11] found that if the water extract of soybean meal is dialyzed at *p*H 7.0, followed by treatment with Dowex 1-X10, an anionic exchange resin, to remove the phytate, the *p*H of maximum precipitation occurs at 5.1. This procedure isolates approximately 80–85% of the meal protein and eliminates some of the complicating factors encountered in the isolation and characterization of the acid-precipitated soybean protein. The data presented in this investigation and a survey of the literature led us to conclude that the glycinin, as previously described, and the acid-precipitated protein contain the same components but differ mainly in protein yield and in the amount of phytate present. Thus, in this report we have used the name glycinin for the phytate-free acid-precipitated protein described in detail by Smith and Rackis.[11]

This glycinin is readily and completely soluble in the *p*H region of 1–4, as well as above *p*H 7.0; whereas, the protein prepared without resin treatment contains substantial amounts of insoluble protein in the *p*H range of 1–4.

The present ultracentrifugal investigation is concerned with the association–dissociation of glycinin in acid solution as affected by *p*H and by type and concentration of salt. Ultracentrifugal investigations of soybean protein in alkaline solutions have been reported by Naismith,[12] Wolf and Briggs[13] and Wolf.[14] Naismith in ultracentrifugal experiments found four resolvable fractions corresponding to s_{20} value of 2, 7, 11 and 15S. Wolf and Briggs and Wolf found the same ultracentrifugal components as Naismith plus considerable amounts of unresolved material >15S, and also showed that the decrease in extractability of soybean protein from the meal at intermediate concentrations of salt was due primarily to the decreased solubility of the 11S, 15S and unresolved fractions.

Experimental

Protein Isolation and Preparation of Samples for Ultracentrifugal Analysis.—Adams soybeans, 1955 crop, were used throughout this study. The water extracts from hexane-extracted meal were obtained by extracting with a 1:10 ratio of meal to water followed by extraction of the residue using a 1:5 ratio. The phytate-free glycinin was obtained by precipitation at *p*H 5.1, according to the method of Smith and Rackis.[11] The protein was washed three times with small portions of water in order to remove whey proteins. The glycinin contained 16.35% nitrogen and 0.33% phosphorus, which has been assayed as 95% nucleic acid phosphorus. For the ultracentrifugal analysis, enough of the washed glycinin was slurried in water to give an approximate concentration of 1% and brought to the desired *p*H with HCl. Aliquots of the protein solution were dialyzed 18 to 24 hours against the appropriate buffer and then diluted to the desired protein concentration before ultracentrifugation. The factor 6.12 was used in converting nitrogen content to protein concentration.

Ultracentrifugal Analysis.—Sedimentation studies were carried out at room temperature with a Spinco ultracentrifuge, Model E, at 35,600 r.p.m. The bar angles and time

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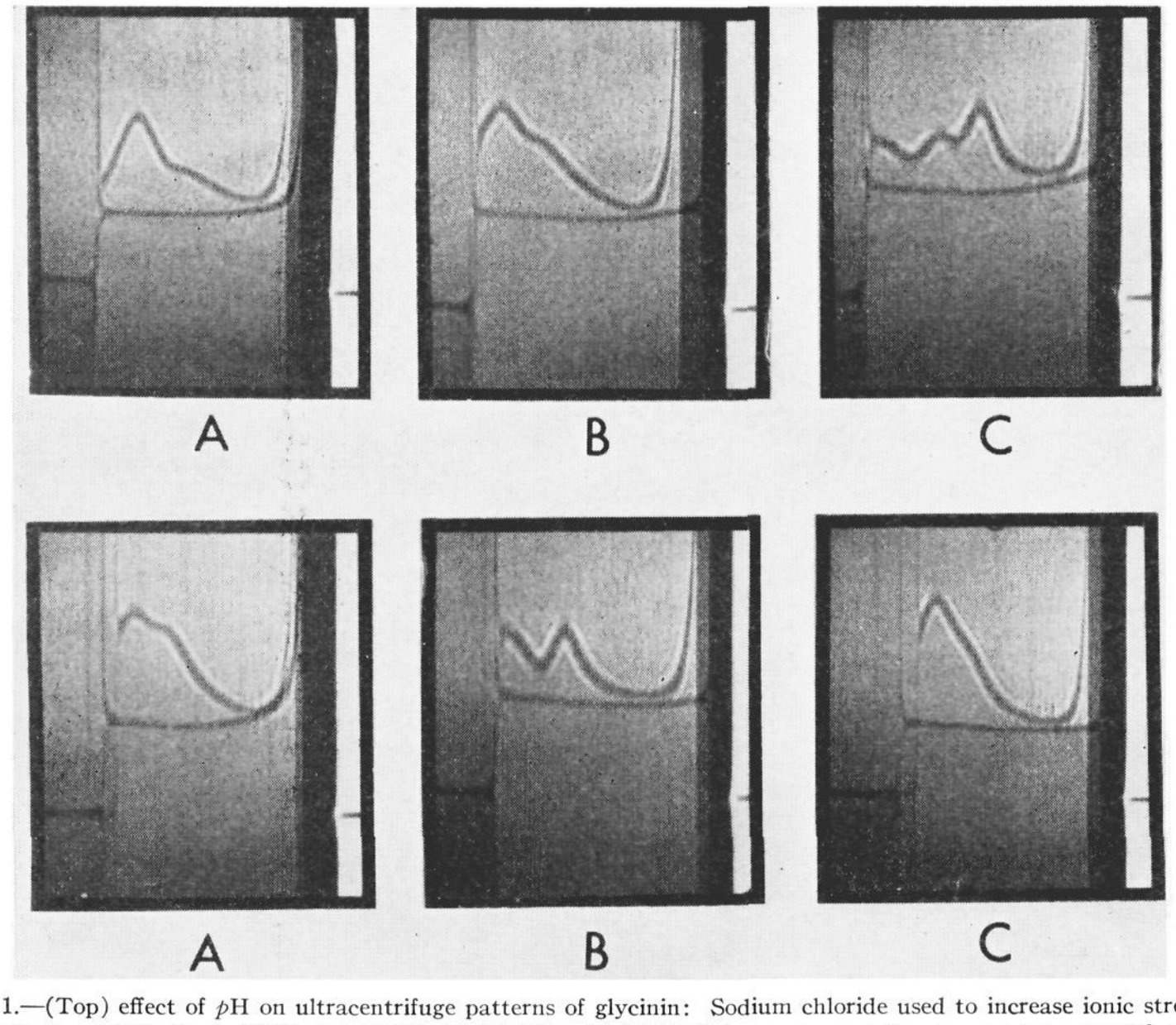

Fig. 1.—(Top) effect of *p*H on ultracentrifuge patterns of glycinin: Sodium chloride used to increase ionic strength of 0.02 *M* glycine–HCl buffer to 0.058; (a) *p*H 2.0, (b) *p*H 3.0 and (c) *p*H 3.8, bar angles and time in minutes were 50°-157 min., 45°-93 min., and 40°-77 min., respectively.

Fig. 2.—(Bottom) effect of type of salt on ultracentrifuge patterns of glycinin: Ionic strength of 0.02 *M* glycine–HCl buffer *p*H 3.0 raised to 0.058 with the following salts: (a) NaCl, (b) Na_2SO_4 and (c) $CaCl_2$. Time for each 77 min. and bar angles 45, 40 and 50°, respectively.

of centrifugation varied for some photographs and these values are given in their respective captions. A double sector plastic centerpiece in a 30-mm. cell was used. The protein solution was placed in one sector and the buffer solution in the other. Sedimentation constants were calculated according to the method of Svedberg and Pedersen.[15] The areas were calculated by the method of Pickels[16] assuming a value of 0.00186 for the specific refractive increment of the protein.

Results

Effect of *p*H on Association of Glycinin.—Figures 1a, b and c show ultracentrifugal patterns at *p*H values of 2.0, 3.0 and 3.8, respectively, and constant ionic strength. Patterns a and b have only two components with s_{20} values of 2.0–2.6S and 5.7–7.8S; whereas, in pattern c, there is an additional component with an s_{20} value of 12.6S. These fractions are designated as the 2, 7 and 13S fractions, respectively. However, these components do not account for all of the protein in solution. On comparing the area under the peaks of the ultracentrifugal patterns with the calculated area corresponding to the total protein concentration, there is a large amount of unresolved material sedimenting either faster than the 13S fraction or slower than the 2S fraction. Since extended centrifugation showed that very little material was present as low molecular weight, the unaccounted for protein has been designated as the >13S fraction. Because of the type of cell employed, a top practical limit of only 35,600 r.p.m. can be used which gave very good delineation at the high end of the protein spectrum. This limit in turn prevents the low end of the protein spectrum from being completely pulled away from the meniscus. As a result, some doubts exist as to the nature of the material lower than the 2S peak.

Realizing that a very complex system of proteins exists in soybeans, this initial study with the ultracentrifuge is intended to serve as a guide in determining the approximate limits within which the protein system can be investigated in detail and to describe the initial conditions which would best represent the entire protein system. With this in mind, the conclusions reached as far as compositional analyses are concerned are subject to error especially since the low end of the spectrum is not completely resolved. Nevertheless, the percentages given for the 2S fractions in terms of total protein do not increase more than 5% when a com-

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TABLE I

EFFECT OF *p*H, IONIC STRENGTH, TYPE OF SALT, AND PROTEIN CONCENTRATION ON DISSOCIATION OF GLYCININ

Treatment	Conditions *p*H	Conditions Ionic strength	Type of salt	Protein concn.	Area % of calcd. area[a]	Ultracentrifuge data: Area % of total area under curve[b] 2S	7S	13S	Total protein %[c] 2S	7S	13S	>13S
*p*H	2.0	0.058	NaCl[d]	0.65	65	61(2.6)	39(5.7)	.....	40	25	..	35
	3.0	.058	NaCl	.65	67	50(2.3)	50(6.6)	.....	34	34	..	32
	3.8	.058	NaCl	.65	47	23(2.0)	27(7.8)	50(12.6)	11	13	23	53
Ionic strength	3.0	.011	Gly.–HCl[e]	.65	67	49(2.8)	51(5.0)	.....	33	34	..	33
	3.0	.061	Gly.–HCl	.65	65	38(2.0)	62(6.4)	.....	25	40	..	35
	3.0	.13	Gly.–HCl	.65	56	25(2.1)	43(6.8)	32(12.2)	14	24	16	44
	3 0	.058	NaCl[d]	.65	63	47(2.0)	53(5.7)	.....	30	33	..	37
	3.0	.15	NaCl	.65	60	30(1.9)	42(7.1)	28(12.1)	18	25	17	40
	3.0	.30	NaCl	.65	31	44(1.7)	56(6.7)	.....	14	17	..	69
	3.0	.51	NaCl	.65	24	41(1.9)	60(6.9)	.....	10	14	..	76
Salt	3 0	.058	NaCl[d]	.65	66	41(2.3)	59(6.9)	.....	27	39	..	34
	3.0	.058	Na_2SO_4	65	29	43(1.7)	57(7.8)	.....	12	17	..	71
	3.0	.058	$CaCl_2$	.65	54	59(2.2)	41(6.4)	.....	32	22	..	46
Protein concn.	3.0	.058	Na_2SO_4[d]	.25	27	35(1.7)	65(7.8)	.....	10	18	..	72
	3.0	.058	Na_2SO_4	.65	29	43(2.0)	67(6.8)	.....	12	17	..	71
	3.0	.058	Na_2SO_4	1.0	25	45(1.8)	55(7.6)	.....	11	14	..	75
	3.0	.058	Na_2SO_4	2.0	10	42(1.9)	58(7.2)	.....	4	6	..	90
	3.0	.058	NaCl	0.25	60	40(2.0)	60(6.8)	.....	24	36	..	40
	3.0	.058	NaCl	0.65	66	41(2.3)	59(6.9)	.....	27	39	..	34

[a] Total area of the ultracentrifuge pattern in respect to the calculated area corresponding to the original protein concentration of 0.65%. [b] s_{20} values are given in parentheses. [c] Based on the original protein concentration of 0.65%. [d] Ionic strength of a 0.02 *M* glycine–HCl buffer was increased to desired value with the indicated salt. [e] Glycine–HCl buffer, ionic strength in respect to amount of HCl required to acidify a 0.02, 0.2 and 0.4 *M* glycine solution to *p*H 3.0.

parable protein solution was allowed to centrifuge for as much as 160 minutes instead of the usual 77 minutes. This increase in the area of the resolvable proteins will concomitantly decrease the amount of material actually present as the >13S fraction.

As shown in Table I, about 65% of the total protein can be accounted for as the 2 and 7S fractions at *p*H 2.0 and 3.0; whereas, at *p*H 3.8, only 47% of the total protein was present in the ultracentrifugal pattern. At *p*H 3.0, the 2S fraction is the major protein component accounting for 40% of all the protein. With an increase in *p*H to 3.8, the 2 and 7S fractions decrease with a concomitant increase in the 13 and >13S fractions.

These results demonstrate the *p*H dependency of the association of glycinin. It appears that the 2 and 7S fractions associate into the 13S component which is then capable of aggregating into high molecular weight material.

To determine the stability of glycinin in acid solution, the protein was allowed to stand 5 days at *p*H 2.0. The sedimentation values and relative areas were the same as for a freshly prepared solution.

Effect of Ionic Strength on Association of Glycinin.—Table I shows the association of glycinin as a function of ionic strength at *p*H 3.0 where the variation in ionic strength was effected by increasing the molarity of the glycine–HCl buffer. The data show two components with s_{20} values corresponding to the 2 and 7S fractions in solutions of 0.011 and 0.061 ionic strength and an additional peak is present at 0.13 ionic strength. Also, Table I shows that with an increase in ionic strength to 0.13 both the 2 and 7S fractions decrease in amount with a concomitant increase in the amount of 13S and > 13S material resulting from the association of the 2 and 7S peaks into higher molecular weight material. Photographs were taken during the early part of the centrifugation runs in these patterns as well as in the following experiments, and the presence of high molecular weight protein having a very broad distribution was observed.

Experiments also were conducted with the buffer composition kept constant at 0.02 *M* glycine and the ionic strength varied with the addition of NaCl. The results of these experiments, in Table I, show that with ionic strengths of 0.058, 0.30 and 0.51, respectively, there are two resolvable peaks having s_{20} values corresponding to the 2 and 7S fractions; whereas, at 0.15 ionic strength, there is an additional peak corresponding to 13S.

It can be seen from the data that there is a large decrease in the amount of the 2S and 7S fractions in terms of per cent. of total protein, when the ionic strength was increased to 0.51 with sodium chloride. This is the result of a progressive increase in the association of glycinin into material having an s_{20} value >13S. At this salt concentration, 76% of the protein is in the rapidly sedimenting form. Above 0.3 ionic strength, the protein solutions become very milky and protein precipitates on standing. In these experiments the low end of the spectrum was not entirely pulled away from the meniscus. The time of centrifugation was extended and again the increase in the relative amount of the 2S fraction in terms of the total protein concentration was about 5%, indicating very little material is present having a sedimentation value less than 2S.

From a comparison of the data on ionic strength variation, it can be seen that at approximately the same ionic strengths the 2S, 7S and 13S fractions were present in about the same relative amount.

In order to determine the reversibility of the as-

sociative processes occurring with changes in ionic strength, a protein solution in 0.15 ionic strength buffer was dialyzed to an ionic strength of 0.011 and then re-equilibrated to ionic strength of 0.15. In all three cases, the ultracentrifugal patterns were characteristic of the ionic strengths employed, thus showing that the associative changes were complete as to sedimentation rate and relative distribution of the various components indicating that the system was reversible.

Effect of Salts on Association of Glycinin.—In order to determine whether the association of glycinin was dependent upon the type of salt employed in the buffer medium, the ionic strength of 0.058 of the buffer was made up either with sodium chloride, sodium sulfate or calcium chloride. The results are illustrated in Fig. 2, patterns a, b and c. Two resolvable peaks with s_{20} values of 2S and 7S are present irrespective of the type of salt. With a sedimentation time of 112 minutes (not shown in figures), the 2S and 7S fractions are completely resolved.

On comparing the area under the peaks of the ultracentrifugation patterns with the calculated area corresponding to the total protein concentration, not all of the area was accounted for by the 2S and 7S fractions. As shown in Table I, the two peaks in the buffer containing sodium chloride, sodium sulfate and calcium chloride, accounted for 66, 29 and 54%, respectively, of the calculated area. Thus varying amounts of >13S material were sedimented ahead of the 7S fraction, the amount being dependent upon the type of salt in the buffer medium. In the presence of sodium sulfate, 71% of the protein was present as the >13S fraction. At 0.2 ionic strength sodium sulfate, large amounts of protein were precipitated.

Patterns taken during the first few minutes of ultracentrifugation in buffers containing sodium sulfate showed the presence of high molecular protein which had a sedimentation value of approximately 75S. It has a small maximum on a very broad distribution curve indicating that the material was quite heterogeneous.

The type of salt not only determines the amount of >13S fraction, but also determines the relative distribution of both the 2S and 7S fractions. For example, it can be seen from the data in Table I that in the presence of sodium chloride the 7S fraction is the major peak, whereas in calcium chloride the 2S fraction predominates. Thus, it appears that the equilibrium distribution of the 2S, 7S and >13S fractions is dependent upon the type of salt present in the buffer medium.

Effect of Protein Concentration on Association.—The effect of protein concentration on the association of glycinin was determined in buffers containing sodium sulfate. The results in Table I show that an increase in glycinin concentration from 0.25 to 1.0% had very little effect on the ultracentrifugal pattern. However, at the 2% level, 90% of the protein sedimented in the >13S form.

Discussion

There are marked differences in the ultracentrifugal patterns of glycinin in solutions acid and alkaline to the isoelectric point. Naismith[11] and Wolf and Briggs,[12] working in alkaline solutions found four distinct sedimenting species having s_{20} values approximating 2, 7, 11 and 15S as well as appreciable amounts of unresolved material >15S. According to Wolf,[14] the >15S material may be partly the result of disulfide polymer formation.

In acid solution, we found only three resolved components having s_{20} values of 2, 7 and 13S, with varying amounts of unresolved material >13S. In the acid systems, the 13S fraction was present in only two instances, *p*H 3.0 and 0.15 ionic strength, and *p*H 3.8 and 0.058 ionic strength. The reason for this is not apparent at this time. It would appear that of all the resolvable components, the 13S fraction is the most sensitive to small changes in *p*H and ionic strength. The 11S and 15S components found in alkaline solutions by other workers were not present in acid solution; however, the 13S component found in acid systems may be the same as the 11S component. Naismith also showed that glycinin in alkaline solutions was very susceptible to change with ionic strength but not to *p*H variation, and that its maximum association was near 0.1 ionic strength, whereas in acid systems glycinin was very susceptible to change with both *p*H and ionic strength. Part of the >13S material probably contains some disulfide polymers formed during the isolation of glycinin. However, the large increase in the amount of this material in acid solution must be caused by associative forces which are electrostatic in nature.

We made some ultracentrifugal runs at *p*H 7.6 and 0.5 ionic strength with glycinin prepared according to the method of Smith and Rackis and found components 2S, 7S, 11S and 17S. The 17S fraction was diffuse and probably corresponds to the 15S of Wolf and Briggs and the 16-19S fractions of Naismith. Additional information is required to reconcile the ultracentrifugal results obtained on the acid and alkaline sides of the isoelectric point of glycinin.

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