The Association and Dissociation of Concanavalin A, the Phytohemagglutinin of the Jack Bean*

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ABSTRACT: Gel filtration of concanavalin A on Sephadex G-200 in the presence of 8 M urea at pH 7 separates the protein into three components having molecular weights of approximately 16,500, 42,000, and >200,000, respectively. Sedimentation velocity measurements under the same conditions also revealed three components having respective $s_{20,w}$ values of 0.71, 4.76, and 7.56 S. Dissociation and aggregation could also be demonstrated by gel filtration on Bio-Gel P-100 under acid and alkaline conditions, respectively; at pH 2.2, 7.0, and 10.2, the molecular weight of concanavalin A was estimated to be 40,000, 71,000, and >100,000, respectively. Amino acid analyses and peptide maps of the three components isolated by chromatography on Sephadex G-100 in the presence of 8 M urea revealed very little difference in composition and peptide distribution. Quantitative N-terminal amino acid analysis by the cyanate method gave 3.3 moles of alanine/71,000 g of protein.

Concanavalin A thus appears to be composed of four identical subunits having a molecular weight of about 17,500.

Concanavalin A, first isolated from the jack bean (*Canavalia ensiformis*) by Sumner (1919), possesses hemagglutinating activity (Sumner and Howell, 1936) as well as the ability to precipitate various polysaccharides such as glycogen, yeast mannan, amylopectin, and dextrans (Sumner and O'Kane, 1948; Cifonelli and Smith, 1955; Cifonelli *et al.*, 1956; Manners and Wright, 1962; Goldstein *et al.*, 1965a,b) as well as blood serum glycoproteins (Nakamura and Suzuno, 1965). It has recently been demonstrated that con A,[1] like the phytohemagglutinin from *Phaseolus vulgaris* (Nowell, 1960), also displays the phenomenon of being able to stimulate the mitosis of human leukocytes (T. Punnett and S. Surrey, personal communication; W. G. Jaffe, personal communication).

Essential to an understanding of the mechanism whereby con A can produce such diverse effects is a detailed knowledge of the structure and properties of this protein. Such studies have been facilitated by the finding that con A can be readily prepared in a high state of purity from a crude extract of the jack bean by taking advantage of its specific binding to cross-linked dextran from which it may be eluted with dilute acid (Olson and Liener, 1967) or glucose (Agrawal and Goldstein, 1965). Some of the more important physical and chemical properties of con A have been recently described (Olson and Liener, 1967; Agrawal and Goldstein, 1967). Such studies have revealed a high degree of uncertainty regarding the size and molecular weight of con A. Sumner *et al.* (1938) originally reported a sedimentation coefficient ($s_{20,w}$) of 6.0 S and a molecular weight of 96,000, but the more recent studies cited above have shown that con A displays $s_{20,w}$ values of 3.8–3.9 S at pH 2.0–5.5 and 6.0–7.0 S at pH 7. Olson and Liener (1967) noted that the apparent molecular weight of con A in 8 M urea was about 42,000 compared to a value of 71,000 in the absence of urea. These observations, coupled with the fact that there was more than one N-terminal amino group (Olson and Liener, 1967), strongly suggested that con A was a molecule comprised of one or more dissociable subunits. This paper describes the results of further studies designed to elucidate the nature of this dissociation with particular regard to the size and number of subunits which make up the con A molecule.

Experimental Section

Materials. Con A was prepared by adsorption and elution from Sephadex G-100 as described previously (Olson and Liener, 1967). Sephadex G-100 and G-200 were purchased from Pharmacia, Piscataway, N. J. Bio-Gel P-2 and P-100 were products of the Bio-Rad Laboratories, Richmond, Calif. Trypsin and pepsin were twice-crystallized preparations obtained from Worthington Biochemical Corp., Freehold, N. J. All urea solutions used in this study were deionized by passage through a column of Amberlite MB-3, a product of Mallinckrodt Chemical Works, St. Louis, Mo. The following proteins of known molecular weight were used for the purpose of calibrating the Sephadex and Bio-Gel columns: cytochrome *c* (Nutritional Biochemical Corp., Cleveland, Ohio), chymotrypsinogen

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[1] Abbreviation used: con A, concanavalin A.

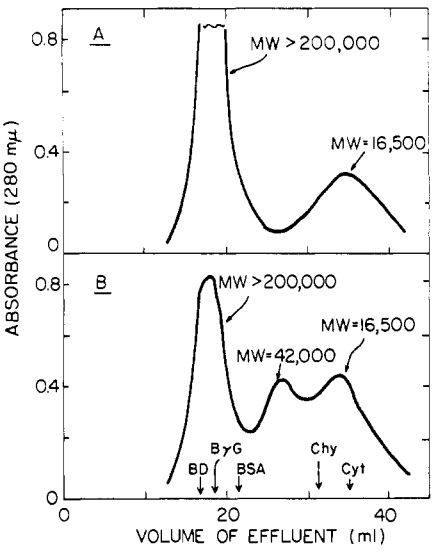

FIGURE 1: Chromatography of con A on Sephadex G-200 in the presence of 6 (A) or 8 M urea (B). The elution volumes of Blue Dextran and proteins of known molecular weight are indicated by the following abbreviations on the ordinate of B: BD, Blue Dextran; BγG, bovine γ-globulin; BSA, bovine serum albumin; Chy, chymotrypsinogen; Cyt, cytochrome *c*. Molecular weights of components from con A were obtained from curve relating elution volume to logarithm of molecular weights of these proteins (Figure 7). See text for further details.

(a gift from Dr. Rufus Lumry), bovine serum albumin (Pentex Inc., Kankakee, Ill.), and bovine α-globulin (Sigma Chemical Co., St. Louis, Mo.).

Gel Filtration Experiments. A column of Sephadex (1.2 × 58 cm) was equilibrated with buffers containing 6 or 8 M urea in 0.1 M phosphate buffer made up to include 0.1 M NaCl and 0.001 M EDTA, final pH 7.0. The relationship between elution volume and the logarithm of the molecular weights of various proteins was established on this column according to the procedure of Andrews (1965). The following proteins were used for this purpose (molecular weights shown in parentheses): cytochrome *c* (12,400), chymotrypsinogen (25,000), bovine serum albumin (69,000), and bovine α-globulin (150,000). Blue Dextran (Pharmacia) was used for determining the void volume of the column. Gel filtration experiments were conducted on a similar column in the absence of urea using Bio-Gel P-100 which had been equilibrated either with (a) 0.1 M glycine buffer, adjusted to pH 2.2 with 1 M HCl, (b) 0.1 M phosphate buffer (pH 7.0), or (c) 0.1 M glycine buffer, adjusted to pH 10.2 with 1 M NaOH. The Bio-Gel column was calibrated in the same manner as the Sephadex column using the pH 7 phosphate buffer for equilibration of the column and as the solvent for the various proteins.

A 1% solution of con A was dialyzed overnight against the appropriate buffer, and 1 ml was then applied to either Sephadex G-200 or Bio-Gel P-100 which had been equilibrated against the same buffer. Fractions (3 ml) were collected at a constant flow rate of 12 ml/hr maintained with a Sigma motor pump. The effluent was monitored at 280 mμ in a continuous fashion by passage through a flow cell placed in a Gilford Model 2000 multiple-sample absorbance spectrophotometer equipped with a recorder. All runs were made at room temperature.

Gel filtration experiments on Sephadex G-200 were conducted on a preparative scale (100 mg of con A) in the presence of 8 M urea under the same conditions as described above except that the size of the column was increased to 1.9 × 155 cm and the flow rate to 15 ml/hr.

Sedimentation Studies. Sedimentation velocity experiments were performed on a Spinco Model E ultracentrifuge employing a 2-mm, single-sector aluminum cell at a rotor speed of 59,780 rpm and a temperature of 20°. Runs were made on 1% solutions of con A in buffers containing 6 or 8 M urea prepared as described for the gel filtration experiments with Sephadex G-200. Sedimentation coefficients were calculated according to the Svedberg equation (Svedberg and Pederson, 1940).

Disc gel electrophoresis was conducted at pH 4.5 using the 7.5% acrylamide system described by Reisfeld *et al.* (1962). In studying the effects of urea on con A, the system was modified by substituting 8 M urea for the water used in all of the gel solutions and buffer. The gels were strained with Amido Black and photographed with a Polaroid camera using the technique described by Burns and Pollak (1963).

Amino acid analyses were made with a Spinco Model 120 analyzer according to Spackman *et al.* (1958). Samples of protein were hydrolyzed with 6 N HCl in sealed, evacuated tubes at 110° for 22 hr. The nitrogen content of the acid hydrolysate was determined by direct nesslerization (Lanni *et al.*, 1950), and this value multiplied by 6.25 was used to calculate the amount of protein subjected to amino acid analysis.

Peptide Maps. Samples of protein (4 mg) were dissolved in 0.8 ml of 0.1 M ammonium carbonate containing 2 M urea and the pH was adjusted to 8 with CO_2 (Kimmel *et al.*, 1965). Three additions of trypsin of 0.1 mg each were made over a period of 40 hr at room temperature. Urea and salts were removed from the digest by passage through a column (1.2 × 50 cm) of Bio-Gel P-2. The absorbance of the effluent was measured at 230 mμ for the detection of peptides, and a colorimetric assay (Levine *et al.*, 1961) was used to locate those tubes containing urea. The peptide-containing tubes were pooled and evaporated to dryness. The residue was dissolved in 0.05 ml of water and applied to a sheet (46 × 57 cm) of Whatman 3MM paper. Chromatography was carried out in one direction of 13 hr using 1-butanol–acetic acid–water (4:1:5), and high-voltage electrophoresis (2000 v, 90 min) was performed in the second dimension at pH 3.7 (Katz *et al.*, 1959).

Peptic digests were prepared by adding two 0.08-mg portions of pepsin over a 40-hr period to 4 mg of

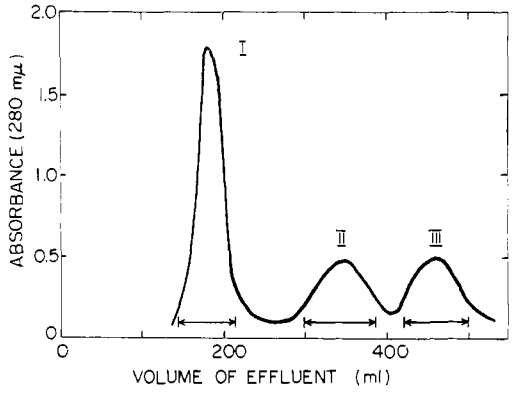

FIGURE 2: Large-scale chromatography of con A on Sephadex G-200 in the presence of 8 M urea. Double-headed arrows denote fractions which were pooled and subjected to further study.

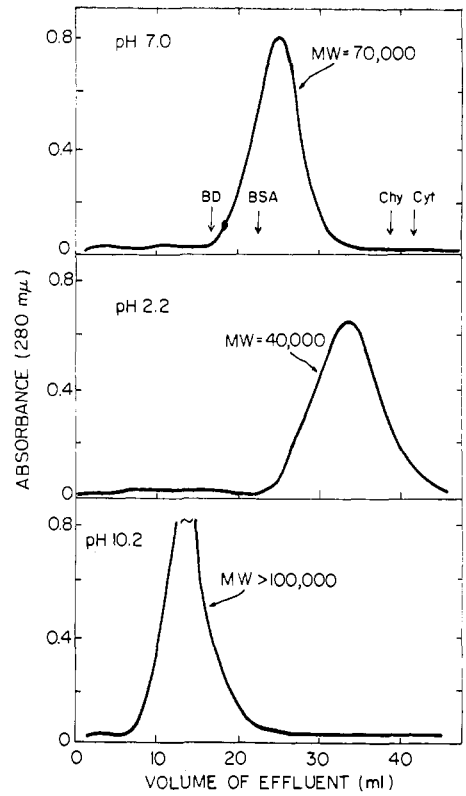

FIGURE 3: Effect of pH on the chromatographic behavior of con A on Bio-Gel P-100. The elution volumes of Blue Dextran and proteins of known molecular weight were determined at pH 7 and are indicated by vertical arrows. See legend to Figure 1 for abbreviations used. Molecular weights were estimated from curves relating elution volume to the logarithm of the molecular weights of these proteins determined at pH 7.0.

protein dissolved in 1 ml of 5% formic acid, the solution being kept at 37° during this time. Peptide maps were then made in the same manner as described for the trypsin digests.

Amino-Terminal Groups. The number of N-terminal acid residues was determined by the cyanate method of Stark and Smyth (1963). The hydantoin derivatives of the N-terminal amino acids were identified and quantitated after acid hydrolysis (6 M HCl, 110°, 96 hr) or alkaline hydrolysis (0.2 M NaOH, 110°, 20 hr). Recovery of the amino acids from their hydantoin derivatives was corrected by using the values given in Tables III and VIII in the paper by Stark and Smyth (1963). Negligible amounts of amino acids were found in a blank run in which the uncarbamylated con A was put through the same procedure. The lysine and homocitrulline content of the native and carbamylated proteins was also determined after acid hydrolysis (6 M HCl, 110°, 22 hr) in order to determine the extent of carbamylation. Lysine was recovered to the extent of 18% in the carbamylated protein. Since lysine is formed in 24% yield when free homocitrulline is hydrolyzed under these conditions, lysine recoveries below this value indicate complete carbamylation of the protein including the α-amino groups (Stark and Smyth, 1963).

Results

Gel Filtration Experiments. The results of gel filtration experiments of con A on Sephadex G-200 in 6 and 8 M urea are shown in Figure 1. Although con A is tightly bound to cross-linked dextran in the absence of urea (Agrawal and Goldstein, 1965; Olson and Liener, 1967), essentially quantitative recovery of the protein is obtained from Sephadex G-200 in either 6 or 8 M urea. In the case of 6 M urea (Figure 1A), two components were observed, one (80%) which presumably had a molecular weight in excess of 200,000 since it was completely excluded from the column, and the other (20%) which was estimated to have a molecular weight of approximately 16,5000. In the presence of 8 M urea, three components were clearly evident (Figure 1B). The fraction which was eluted in the void volume was followed by two components having molecular weights of 42,000 and 16,500. When a solution of con A was dialyzed against 8 M urea for 70 hr instead of overnight, as in the case of Figure 1B, the number and distribution of the various protein fractions was essentially unchanged.

Gel filtration with Sephadex G-200 in 8 M urea was repeated on a preparative scale in order to facilitate the isolation of sufficient quantities of each component for further characterization. As shown in Figure 2, the resolution of the three components of con A was actually superior to that which had been obtained with the smaller column. The recovery of protein, however, in this instance was somewhat less than quantitative (84%); of the recovered protein, 57, 23, and 20% were distributed as component I (molecular weight, >200,000), component II (molecular weight, 42,000), and component III (molecular weight, 16,500), respectively.

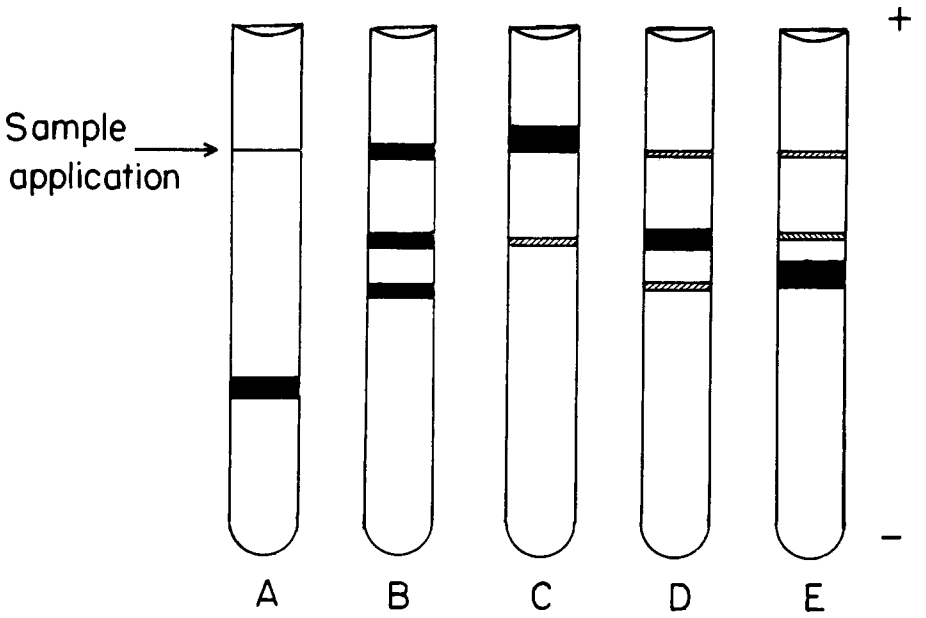

FIGURE 4: Disc gel electrophoresis of con A and the various components which are produced in the presence of 8 M urea. (A) Con A in the absence of urea; (B) con A in the presence of 8 M urea; (C) component I; (D) component II; (E) component III. Components I–III were fractions isolated by chromatography on Sephadex G-200 in the presence of 8 M urea (Figure 2). Patterns B–E were obtained by electrophoresis in urea-containing gels. Protein (0.1 mg) was applied in all cases.

Since the gel filtration of con A on cross-linked dextran cannot be performed in the absence of urea, the polyacrylamide gel, Bio-Gel P-100 (exclusion limit, 100,000), was used in studying the possible effects of pH on the dissociation and association of this protein. The results presented in Figure 3 show the marked influence which the pH exerts on the apparent molecular size of con A. At each of the three different pH values studied here, only one molecular species could be discerned, but each of these differed very significantly from the other. The estimated molecular weights at pH 2.2, 7.0, and 10.2 were 40,000, 70,000, and >100,000, respectively.

Disc Electrophoresis. When con A was examined by disc gel electrophoresis at pH 4.5 in the absence of urea, only one component was observed (Figure 4A), whereas in the presence of 8 M urea at the same pH three zones were produced (Figure 4B). As shown in Figure 4C–E, these three zones could be identified as components I–III on the basis of a comparison with runs made with fractions isolated by gel filtration. A minor degree of cross-contamination was evident in these various fractions which suggests that gel filtration was not completely effective in resolving the original mixture of these components.

Sedimentation Studies. Figure 5A shows the sedimentation pattern of con A in 6 M urea (pH 7). Under these conditions one major, rapidly sedimenting component having an $s_{20,w}$ value of 22 S was observed. A small amount of a slower moving component could be discerned after 148 min of centrifugation. The 22S component most likely corresponds to the aggregated form of con A which had a molecular weight in excess of 200,000 previously noted in Figure 1A. The small amount of the lighter component seen late in the sedimentation run would appear to be equivalent to the one which had a molecular weight of approximately 16,500.

In 8 M urea, shown in Figure 5B, three components are clearly apparent. In order of decreasing size, their sedimentation coefficients were calculated to be 7.6, 4.8, and 0.7 S. These values would be compatible with the molecular weights of components I (>200,000), II (42,000), and III (16,500), respectively, observed by gel filtration under the same conditions (Figure 1B).

Amino Acid Composition. The amino acid composition of components I–III, isolated by gel chromatography in the presence of 8 M urea, is presented in Table I. Also included in this table are data pertaining to the composition of the original sample of con A which had not been treated with urea. Individual amino acid values, expressed on a per unit weight basis, were remarkably consistent among the various components of con A as well as con A itself.

Peptide Maps. Shown in Figure 6 are peptide maps contained with trytpic and peptic digests of con A and components I–III produced as a result of treatment with 8 M urea. Within each type of digest the general appearance of these maps does not show any significant differences in the distribution of peptides from these various proteins. On the basis of the total number of lysine and arginine residues in con A (based on a molecular weight of 71,000; see Table I), one would

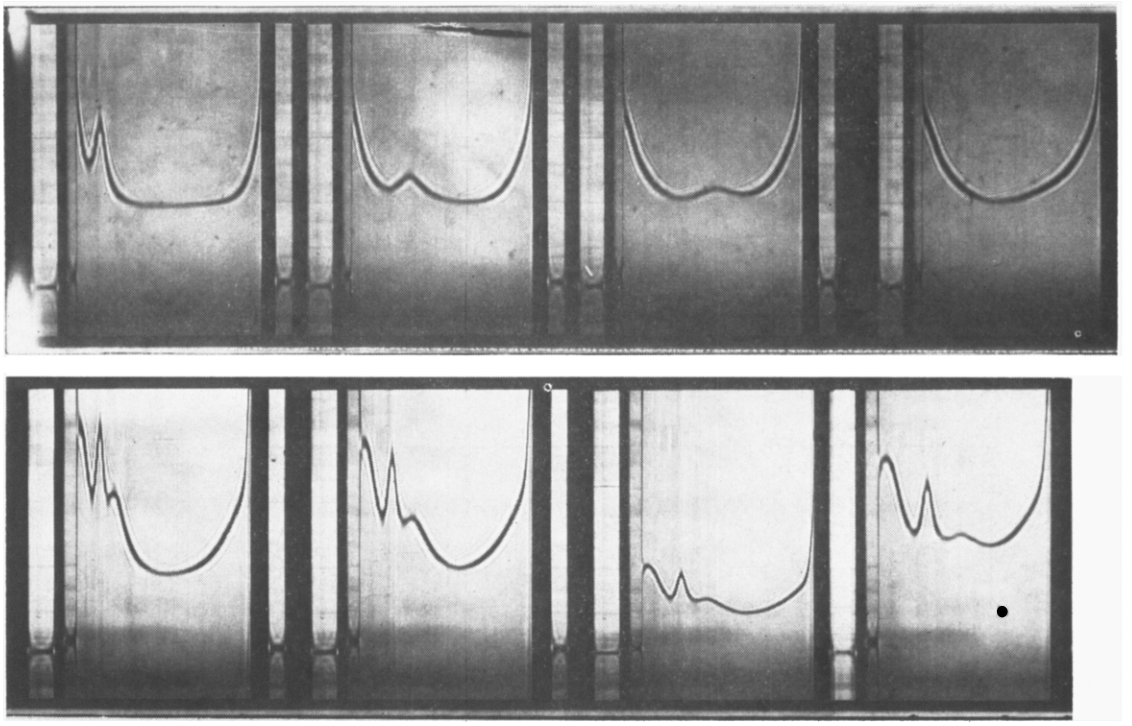

FIGURE 5: Sedimentation patterns of con A in 6 M (A) and 8 M (B) urea. Concentration of protein, 1%. Photographs were taken from left to right at 16-min intervals in 6 M urea. The intervals between pictures in the 8 M urea system were 28, 20, and 28 min, respectively.

expect a total of at least 48 peptides in its tryptic digest. Since no more than 20 different peptides were produced, it must be concluded that con A must be composed of subunits which are identical in their sequence of amino acids. Because of the ever present possibility of some nonspecific cleavage with trypsin, the significance of the number of peptides obtained in these tryptic maps must be interpreted with caution. If 20 is accepted as a maximum value, there should be at least two identical subunits comprising the con A molecule.

Amino-Terminal Residues. In confirmation of previous reports that alanine constitutes the N-terminal amino acid of con A (Olson and Liener, 1967; Agrawal and Goldstein, 1967), the cyanate method of Stark and Smyth (1963) likewise proved alanine to be the predominant N-terminal amino acid accompanied by traces of glycine, serine, aspartic, and glutamic acids. Essentially the same picture was obtained regardless of whether acid or alkaline hydrolysis of the hydantoins was employed for the quantitative determination of the N-terminal groups. Since serine and threonine are completely destroyed by acid hydrolysis but only partially destroyed by alkaline hydrolysis (Stark and Smyth, 1963), these particular amino acids were apparently not N-terminal residues of con A. Of particular significance was the finding of 3.3 moles of alanine/71,000 g of protein. This value, which is undoubtedly a minimal one, would indicate that con

TABLE I: Amino Acid Composition of Components Derived from Con A in the Presence of 8 M Urea.

	Components in 8 M Urea[a]			
Amino Acid	Con A[a]	I	II	III
Lysine	0.44	0.42	0.45	0.047
Histidine	0.29	0.28	0.25	0.26
Arginine	0.24	0.21	0.22	0.23
Aspartic acid	1.33	1.10	1.20	1.47
Threonine	0.69	0.65	0.62	0.65
Serine	1.05	1.04	1.02	1.02
Glutamic acid	0.43	0.45	0.43	0.37
Proline	0.38	0.45	0.37	0.39
Glycine	0.60	0.63	0.58	0.56
Alanine	0.67	0.68	0.69	0.71
Half-cystine	0	0	0	0
Valine	0.61	0.61	0.59	0.62
Methionine	0.07	0.06	0.08	0.06
Isoleucine	0.54	0.51	0.49	0.56
Leucine	0.67	0.63	0.60	0.62
Tyrosine	0.24	0.22	0.23	0.28
Phenylalanine	0.40	0.49	0.40	0.39
Tryptophan[b]				

[a] Based on a nitrogen content of 15.6% multiplied by 6.25. In millimoles per gram of protein. [b] Not determined.

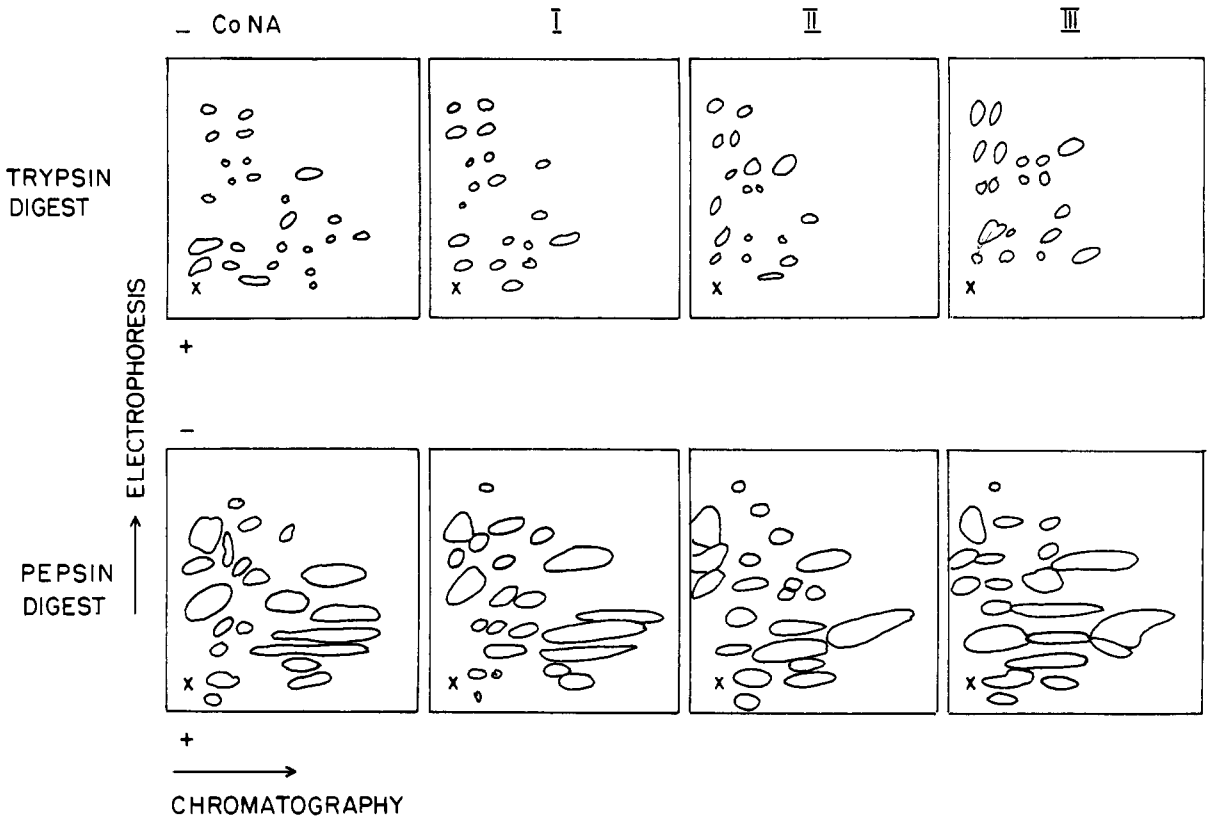

FIGURE 6: Peptide maps of tryptic and peptic digests of con A compared with the various components obtained by treatment with 8 M urea. Crosses in lower left corner of each map denote point of application of each digest.

A probably has a multichain structure of four peptides, each with an N-terminal alanine residue.

Discussion

The dissociation and association of proteins under special conditions has come to be regarded as a rather common phenomenon of proteins (Reithel, 1963; Klotz, 1967). Previous work on con A from this laboratory (Olson and Liener, 1967) and elsewhere (Agrawal and Goldstein, 1967) has shown that the molecular weight of this protein is highly dependent on environmental conditions. In the present study urea and pH were found to be highly effective means for demonstrating that con A can in fact undergo dissociation as well as association.

The results of these different techniques (gel filtration, disc gel electrophoresis, and ultracentrifugation) point to the fact that con A can exist in the form of three different molecular species in the presence of 8 M urea. The molecular weights of each of these components, based on their elution from Sephadex G-200, were estimated to be >200,000, 42,000, and 16,500 for components designated as I, II, and III, respectively.

These estimates of molecular weights based on gel filtration data should be regarded only as rough approximations. It is known that the chromatographic behavior of proteins on Sephadex can be appreciably altered in the presence of urea (de la Llosa *et al.*, 1966). Although the molecular weights of the con A units were estimated from a calibration curve established in the presence of 8 M urea, the different proteins employed for this purpose might be expected to unfold to different extents. In particular the number of disulfide bonds would be expected to influence the degree of unfolding produced by urea. Confidence in the validity of these estimates for the molecular weight of con A units is somewhat enhanced, however, by the observation that, in spite of these uncertainties, a good linear relationship was observed between elution volume and the logarithm of the molecular weights of the several known proteins that were examined in the presence of 8 M urea (Figure 7).

The three components observed during the sedimentation of con A in 8 M urea had sedimentation coefficients of 7.56, 4.76, and 0.71 S which may be presumed to correspond to components I, II, and III, respectively. The amino acid composition and peptide maps of tryptic and peptic digests of con A and components I–III indicated little if any difference among these various protein fractions. These results permit the conclusion that con A is in fact composed of identical subunits.

In order to determine the number of subunits comprising the con A molecule, it was assumed that the "preparative" weight, as defined by Reithel (1963),

of con A is approximately 71,000 at pH 7 in the absence of urea (Olson and Liener, 1967). This value may also be considered the molecular weight of "active" con A since this protein is most active in precipitating dextran in the pH range 6.1–7.2 (So and Goldstein, 1967). Since end-group analysis had indicated that con A was most likely comprised of four chains, it may be concluded that con A is a tetramer of component III which had a molecular weight (16,500) approximately equal to one-fourth that of con A.

Reducing the urea concentration to 6 M seemed to favor aggregation as evidenced by the fact that approximately 80% of the protein was excluded from Sephadex G-200 under these conditions. On the basis of sedimentation data, this aggregate has an $s_{20,w}$ value of 22 S which is much higher than the value of 7.6 S observed for the heaviest component of con A in 8 M urea. In addition to this highly polymerized form of con A, a small amount of the monomeric species having a molecular weight of 16,500 was produced. A dimer such as that observed in 8 M urea was not detected in the 6 M urea system. It is not clear why 6 M urea should have an effect on the dissociation and association of con A which is different in these respects from the effect of 8 M urea.

The extent to which con A can dissociate or aggregate is markedly influenced by the pH of its environment. Under acid conditions (pH 2.2) only one component having an estimated molecular weight of 40,000 was noted;[2] this species is presumably a dimer or a half-molecule of con A. Alkaline conditions (pH 10.2) favored the exclusive formation of a molecular species having a molecular weight somewhere in excess of 100,000. At pH 7, where con A is known to be most active (So and Goldstein, 1967), a single component with a molecular weight of approximately 70,000 was the only molecular species noted. Inasmuch as the sedimentation coefficient of con A under neutral conditions is 6–7 S (Sumner *et al.*, 1938; Olson and Liener, 1967; Agrawal and Goldstein, 1967), the value of 3.8–3.9 S, which has been reported in the pH range 2–5.5 (Olson and Liener, 1967; Agrawal and Goldstein, 1967), is no doubt a reflection of the formation of half-molecules under these conditions.

Rigas *et al.* (1966) have recently reported that a phytohemagglutinin from *Phaseolus vulgaris* could be dissociated into at least eight subunits in 8 M urea. Chromatography on IRC 50 resin in the absence of urea resulted in dissociation and reassociation of the subunits in various ratios to give components having varying degrees of hemagglutinating and mitogenic activity. They concluded that one type of subunit is hemagglutinating and another type is mitogenic. In the present study with con A, it was not possible to examine the activity of component III since the removal of urea led to an insoluble product most of which could not be resolubilized in the absence of urea. The small amount which could be made to go back into solution (1% NaCl) gave a disc electrophoresis pattern indistinguishable from that of con A (unpublished observations), suggesting that a reassociation of subunits takes place once urea is removed.

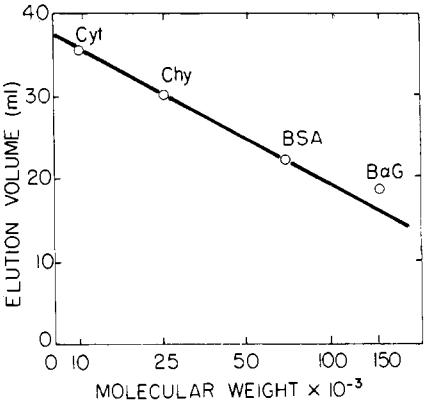

FIGURE 7: Calibration curve relating elution volume to logarithm of molecular weight obtained with proteins of known molecular weight chromatographed on Sephadex G-200 in the presence of 8 M urea buffer. See legend to Figure 1 for abbreviations.

Disulfide bonds have been frequently implicated in the dissociation and association reactions of seed proteins (Reithel, 1963). Since con A is devoid of disulfide linkages (Olson and Liener, 1967), noncovalent bonds must be responsible for the quaternary structure of this protein. Since the isoelectric point of con A is 7.1 (Agrawal and Goldstein, 1967), the absence of a net charge would seem to favor the existence of con A in the form of a tetramer. Dissociation into monomeric units under acid conditions would suggest that the bonds responsible for holding these subunits together are not sufficiently strong to resist the electrostatic repulsion brought about by an increased net cationic charge. Under alkaline conditions where the protein would have a net anionic charge, electrostatic repulsion is apparently not of sufficient strength to overcome whatever forces are involved in the aggregation of con A molecules. The mechanism whereby urea leads to a simultaneous process of dissociation as well as aggregation remains unexplained not only for con A but for most proteins in general (Reithel, 1963).

[2] It should be pointed out that if aggregation and disaggregation were a rapid process, the apparent molecular weights obtained from the Bio-Gel experiments might be average values rather than properties of discrete entities.

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