ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS **124**, 218–229 (1968)

Protein–Carbohydrate Interaction

VII. Physical and Chemical Studies on Concanavalin A, the Hemagglutinin of the Jack Bean

B. B. L. AGRAWAL[1] AND I. J. GOLDSTEIN[2]

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan

Received June 8, 1967; accepted August 15, 1967

Concanavalin A yielded a monodiperse pattern in the analytical ultracentrifuge in the pH range 2–5. The sedimentation coefficient, $s^0_{20,w}$ in pH 5 acetate buffer containing 0.1 M NaCl (total $\Gamma/2 = 0.20$) was 4S. At pH 7 and above, a two-peak schlieren pattern was observed, the faster component (about 7S) probably representing a dimer. On the basis of an $s_{20,w}$ of 3.9S; a $D_{20,w}$ of 5.43×10^{-7} cm²/second, and a $\bar{V}$ of 0.73 ml/gm, a molecular weight of 68,000 was calculated (0.10 M acetate, NaCl, $\Gamma/2 = 0.45$) for the homogeneous component at pH 5. From free-boundary electrophoresis data the isoelectric point of concanavalin A was determined to be 7.1 ± 0.1. Starch gel electrophoresis patterns of concanavalin A in borate buffer (pH 8.6) were characterized by an anodically migrating streak. Incorporation of D-glucose into the gel gave a single sharp band which migrated slowly toward the cathode, the glucose probably inhibiting protein-starch interaction. Gel filtration experiments with Biogel P-100 suggested that at pH 5 the molecular weight of concanavalin A is in the vicinity of 50,000; at pH 7.5 concanavalin A is completely excluded, an indication that a high molecular weight species probably is formed by the association of subunits. Concanavalin A has an extinction coefficient, $E^{1\%}_{1cm}$, of 11.4 ± 0.1, the molar ratio $M_{tyrosine}/M_{tryptophan}$ being 1.78. Unlike other phytohemagglutinins, concanavalin A does not contain cysteine (or cystine) or carbohydrate. Aspartic acid and serine constitute the most abundant residues, and the protein has a high amide content (68.6 groups/10^5 gm protein). Amino terminal studies showed the presence of 1.5 moles DNP-alanine/68,000 gm protein together with very small amounts of DNP-serine and DNP-glycine. The manganese content of concanavalin A was found to be 0.029 and 0.036%, by activation analysis and emission spectroscopy, respectively.

In an earlier report (1) we described a method for the isolation and purification of concanavalin A, a jack-bean globulin, which interacts to form a precipitate with certain specific types of branched polysaccharides (2). Our method for isolation of concanavalin A depends upon its specific adsorption on cross-linked dextran gels (Sephadexes) followed by its displacement by D-glucose, an inhibitor of dextran-concanavalin A interaction. The protein obtained by this method is approximately 98% active as determined by a specific quantitative precipitation assay (3) developed in this laboratory. No detailed information on the physicochemical properties of concanavalin A is available except for brief reports on the content of cystine, tryptophan, and tyrosine (4) and on molecular weight based on limited

[1] This work represents a portion of a dissertation submitted by B. B. L. Agrawal to the Graduate School of the State University of New York at Buffalo in partial fulfillment of the requirements for the Degree of Doctor of Philosophy. Present address: Abbott Laboratories, Scientific Division, North Chicago, Illinois 60064.

[2] Established investigator of the American Heart Association; to whom inquiries regarding this paper should be sent.

sedimentation-diffusion data (5, 6). With the availability of concanavalin A in a highly purified state it was possible for us to undertake physicochemical studies, which is the subject of this report. Such information is basic to an understanding of the mechanism of interaction of concanavalin A with carbohydrates, serum glycoproteins, and erythrocytes. The added significance of this interaction is due to its striking similarity to the antibody-antigen system. It may be emphasized that such interactions are more widespread than hitherto realized. Loyter and Schramm's demonstration (7) of the binding of α-amylase to its polymer substrate (glycogen) is a further example of a reaction which appears to be analogous to the binding of an immune antibody to its antigen.

MATERIALS AND METHODS

Concanavalin A was prepared by our Sephadex adsorption method (1).

Ultracentrifugal studies. A 1% solution of concanavalin A was examined in a Spinco model E analytical ultracentrifuge at 59,780 rpm in various buffers of the following pH values: 2, 4, 5, 7, 8, and 11. The ionic strength was maintained at 0.45 with NaCl.

Sedimentation-diffusion studies. The sedimentation coefficient of concanavalin A at pH 5 was determined in 0.10 M acetate buffer containing NaCl ($\Gamma/2 = 0.45$) at the following protein concentrations: 2.45, 4.80, 6.90, 8.30, 9, and 11.50 mg/ml. The diffusion-coefficient (protein conc. 6.9 mg/ml) was determined with a Spinco model H electrophoresis-diffusion instrument in the same medium in a standard 11-ml cell. Calculations were made by the height-area method. The sedimentation-diffusion data were obtained following the procedure described by Shulman *et al.* (8).

Sedimentation studies at pH 5 and 7. The sedimentation behavior of concanavalin A was studied in 0.10 M acetate buffer, pH 5, containing 0.10 M NaCl at the following protein concentrations: 2.06, 4.12, 8.25, and 16.50 mg/ml. Similar studies were made in pH 7 phosphate buffer containing 0.10 M NaCl at 8.50 and 17 mg protein/ml. The total ionic strength of both buffer systems was 0.20. The sedimentation was carried out at 59,780 rpm.

The observed values of sedimentation and diffusion coefficients were corrected to the standard form $s_{20,w}$ and $D_{20,w}$ in the usual way (9).

Moving-boundary electrophoresis. Concanavalin A (10 mg/ml) was examined in a Spinco model H electrophoresis-diffusion instrument and a standard 11-ml cell. The buffers ($\Gamma/2 = 0.05$) were prepared according to Long (10). The mobilities were calculated from the descending boundary.

Cellulose acetate electrophoresis. An approximately 1% solution of the protein was studied in the following buffers ($\Gamma/2 = 0.05$): acetate, pH 5; phosphate, pH 7; and barbital, pH 8.6. Electrophoresis was conducted at constant voltage (250 V) for 1 hour. The strips were stained with Ponceau S and washed with acetic acid (5%, v/v).

Paper electrophoresis. Electrophoresis on paper was carried out at constant current (8 mA) for 8 hours in the following buffers: acetate (pH 4, 6.8), phosphate (pH 4.6, 6.5, 7.8), phthalate (pH 5.9), barbital (pH 8.6), borate (pH 9), and glycine (pH 9.6). The paper strips were stained with amido black and washed with methanol-acetic acid-water (5:1:5, v/v).

Starch gel electrophoresis. Starch gel electrophoresis was conducted in borate buffer, pH 8.6, in the presence and absence of 0.10 M glucose. The gels were stained with amido black and washed with methanol-acetic acid-water (5:1:5, v/v).

Molecular weight estimation by gel filtration. A Sephadex G-100 column (2 × 60 cm) and a Biogel P-100 column (2 × 75 cm) were prepared. The flow rate was maintained at 30 ml/hour. The gel filtration of concanavalin A was studied in the following media: pH 7.5 Tris-HCl containing 0.10 M KCl, $\Gamma/2 = 0.15$, and pH 5 acetate with 0.10 M NaCl, $\Gamma/2 = 0.20$. The void volume was determined with blue dextran (M.W. 2×10^6). The sample (10 mg protein in appropriate buffer) was applied to the column, and fractions (2 ml) were collected and read at 280 mμ. Bovine serum albumin, α-chymotrypsin, cytochrome *c*, ovalbumin, and pepsin were employed as standards.

Determination of extinction coefficient, and nitrogen content of concanavalin A. Triplicate samples (3 ml) of an approximately 1% solution of the protein in 0.10 M NaCl were lyophilized in tared weighing bottles and finally dried *in vacuo* at 110° for 48 hours to obtain the dry weight of concanavalin A.

Suitable aliquots of the above concanavalin A solution were used for the determination of its extinction coefficient at 280 mμ, and the nitrogen content was determined by a micro-Kjeldahl method (11). The results were expressed on a dry weight basis. Elemental analyses of concanavalin A and jack-bean meal for C, H, N, and ash were carried out by Galbraith Laboratories, Knoxville, Tennessee.

Absorption spectra. Absorption spectra of the protein in 1 M NaCl, and 0.10 N NaOH were obtained with a Cary 15 automatic recording spectrophotometer. From the absorption data in 0.10 N NaOH the amounts of tyrosine and tryptophan

were determined as described by Beaven and Holiday (12).

Determination of amide groups. The amide content of concanavalin A was determined by the procedure of Hamilton (13).

Analysis for carbohydrate. Concanavalin A (10–20 mg) was analyzed for carbohydrate by colorimetric methods based on the orcinol (14), phenol-H_2SO_4 (15), and anthrone reactions (16). D-Glucose was used as standard.

Concanavalin A was also examined for the presence of hexosamine by the methods of Winzler (14) and Boas (17), and for sialic acid by Aminoff's procedure (18).

Paper chromatographic analysis of protein hydrolyzate for free sugars. Samples of concanavalin A (40 mg) were dissolved in 2 N H_2SO_4 (3 ml) and hydrolyzed at 100° in sealed tubes for 4–7 hours. The hydrolyzates were neutralized ($BaCO_3$) and filtered. The filtrates were evaporated, and the residues were dissolved in water and chromatographed on Whatman No. 1 paper (glucose, fructose, and sucrose were used as standards) for 37 hours in *n*-butanol–ethanol–H_2O (4:1:5, v/v) solvent system. The chromatograms were sprayed with the $AgNO_3$-NaOH reagent (19).

Amino acid analysis. Essentially the procedure described by Moore and Stein (20) was followed. The protein samples were hydrolyzed in constant boiling-HCl for 22, 48, 72, and 96 hours. The analyses were carried out in a Spinco amino acid analyzer, model 120 B.

A sample of performic acid-oxidized concanavalin A prepared by the method of Hirs (21) was hydrolyzed for 22 hours and examined in the usual manner.

Amino terminal analyses. These studies were carried out with 20-mg samples of protein by the fluorodinitrobenzene and phenylisothiocyanate procedures as described by Fraenkel-Conrat *et al.* (22).

Activation analysis. Concanavalin A (50 mg) was subjected to neutron activation in a nuclear reactor. The nature of the metal was confirmed by comparison of the energy spectrum of the activated sample with standard spectra (23, 24). The manganese content of the protein was determined by comparison with a standard sample of $MnCl_2$, run simultaneously with the protein sample.

Emission spectroscopy. The manganese content of concanavalin A was also determined by emission spectroscopy by using an a.c. arc and the method of internal standard (platinum 3043 Å). The manganese analytical line was 2949 Å. A calibration curve for manganese was prepared by using aqueous solutions containing 0.5, 1, and 3, ppm/gm sample.

TABLE I

SEDIMENTATION-DIFFUSION DATA ON CONCANAVALIN A (pH 5, $\Gamma/2$ = 0.45)

Protein conc. (mg/ml)	$s_{20,w} \times 10^{13}$	Sedimentation-diffusion data
2.45	3.67	$s_{20,w}$ 3.92 × 10^{-13} sec
4.80	3.73	$D_{20,w}$ 5.43 × 10^{-7} cm^2 sec^{-1}
6.90	4.11	$\bar{V}$ 0.73 ml/gm
8.30	3.88	
9.00	4.17	$\rho_{20,sol}$ 1.0170 gm/ml
11.50	3.96	M.W. 68,000
	Avg. 3.92	

RESULTS

Ultracentrifugal studies. In the pH range 2–5 concanavalin A moved as a single symmetrical boundary. At pH 7 and 8 two components were observed. The ultracentrifuge pattern at pH 11 showed gross polydispersity.

Our results indicate that concanavalin A exists as a single homogeneous species in the pH range 2–5; however, at pH values of 7 and above the protein exhibits a pronounced tendency to form molecular aggregates.

Molecular weight from sedimentation diffusion data. The data are summarized in Table I. The partial specific volume, $\bar{V}$, of concanavalin A was calculated from its amino acid composition by the method described by Cohn and Edsall (25), and the calculated value of 0.73 was identical to that obtained by Sumner *et al.* (5, 6). The sedimentation coefficient under the listed conditions (pH 5, $\Gamma/2$ = 9.45) appeared to be independent of the protein concentration, and thus an average value was taken. A molecular weight of 68,000 was calculated from these data.

Sedimentation behavior at pH 5 and 7. The data are given in Table II. The sedimentation patterns (Figs. 1 and 2) show that the slower moving, minor component at pH 7 migrates at a rate similar to the homogeneous species at pH 5. The faster

TABLE II
SEDIMENTATION DATA ON CONCANAVALIN A

Experimental conditions	Protein conc. (mg/ml)	$s_{20,w}$
Acetate	16.50	3.54
0.10 M NaCl	8.25	3.47
pH 5, Γ/2 = 0.20	4.12	4.34
	2.06	3.98
Phosphate	17.0	3.61
0.10 M NaCl		5.81[a]
pH 7, Γ/2 = 0.20		4.09
	8.5	6.44[a]

[a] Refers to faster sedimenting component.

component at pH 7 could represent a dimer. The concentration dependence of $s_{20,w}$ at pH 5 is slight and an $s^0_{20,w}$ of 4S was obtained by extrapolation to infinite dilution. Such data, however, were not available at pH 7 since the protein was studied at only two different concentrations.

Moving-boundary electrophoresis. From the mobilities listed in Table III an isoelectric point of 7.1 ± 0.1 was determined. In the pH range 3.4–6.8 the electrophoretic pattern was essentially that of a single component. Two peaks were observed at pH 2.4 and

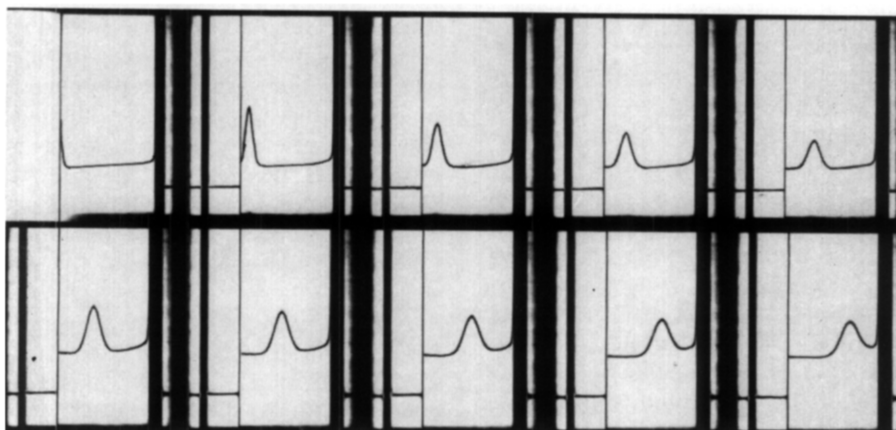

FIG. 1. Schlieren patterns of concanavalin A (8.25 mg/ml) in pH 5 acetate buffer containing 0.10 M NaCl, Γ/2 = 0.20. Photographs were taken at 16-minute intervals; speed, 59,780 rpm. Sedimentation proceeds from left to right. Bar angle, upper frame 65°; lower frame 50°.

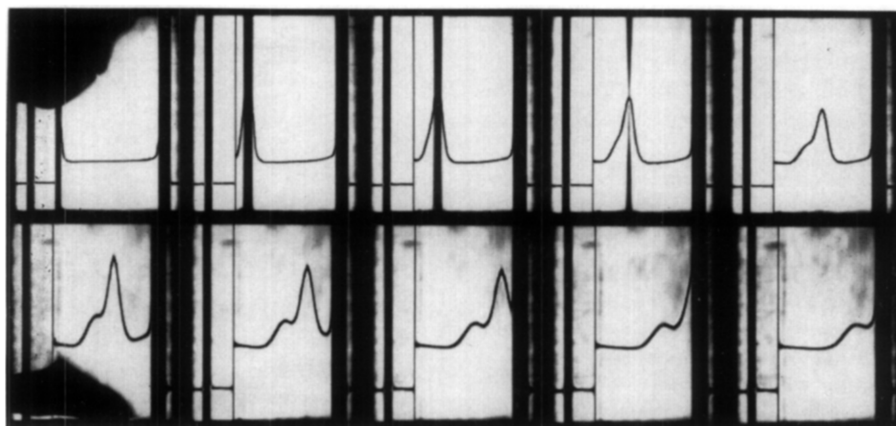

FIG. 2. Schlieren patterns of concanavalin A (17 mg/ml) in pH 7 phosphate buffer containing 0.10 M NaCl, Γ/2 = 0.20. Photographs were taken at 16-minute intervals. Speed, 59,780 rpm. Sedimentation proceeds from left to right. Bar angle, upper frame 65°; lower frame 50°.

TABLE III
ELECTROPHORETIC MOBILITY DATA OF CONCANAVALIN A

Buffer	pH	Mobility × 10^5 (cm^2/volt-sec)
Glycine-HCl	2.4	7.8
Formate	3.4	7.3
Acetate	4.4	4.1
Histidine	6.0	1.2
Phosphate	6.4	1.5
Phosphate	6.8	0.6

three peaks at pH 7.4 (Fig. 3); the significance of these data is discussed below.

Cellulose-acetate electrophoresis. At pH 5 concanavalin A migrated as a single band toward the cathode. In barbital buffer, pH 8.6, the protein migrated anodically; the slight streaking following the major band probably indicates the aggregation of the protein at this pH. At pH 7 the protein did not migrate to either electrode, and thus supported the results of free-boundary electrophoresis studies.

Paper electrophoresis. Figure 4 shows paper electrophoretic results at various pH values. Only one band is observed between pH 4 and 6.8. At pH 7.8 the protein still migrated toward the cathode, but two zones were discernible. In barbital buffer, pH 8.6, the major band moved a short distance toward the cathode; an anodically migrating streak was also present. In glycine buffer, pH 9.6, the major component remained at the origin and a streak moved toward the anode. The anomalous migration of concanavalin A in phosphate buffer, pH 4.4, could possibly be attributed to the binding of phosphate anions to the protein.

Starch gel electrophoresis. The electrophoretic pattern of concanavalin A in borate buffer, pH 8.6, was characterized by an anodically migrating streak indicating polydispersity. Incorporation of D-glucose in the gel gave a single sharp band which migrated a short distance toward the cathode.

Gel filtration. Biogel P-100: At pH 7.5 concanavalin A emerged with the void volume (R_F = 1) indicating its molecular weight to be 100,000. At this pH ovalbumin (M.W. 46,000) had an R_F of 0.85. In acetate buffer, pH 5, the R_F of ovalbumin was practically unchanged (0.81), but concanavalin A was definitely retarded with an R_F of 0.80 very similar to that of ovalbumin. These results together with ultracentrifugal data suggest association of subunits around pH 7. The data are summarized in Table IV.

When a solution consisting of a mixture (1:1 by weight) of concanavalin A and ovalbumin was applied at pH 7.5, the two proteins emerged as a single peak and suggested a complex formation. A similar though somewhat diminished tendency was apparent at pH 5. *Sephadex G-100:* At pH 5 and 7.5 a small quantity of concanavalin A (about 5%) appeared with the void volume. The amount of this component was slightly higher if the freeze-dried protein was used. The remaining protein appeared in a broad peak (R_F 0.63). This suggested the interaction of concanavalin A with cross-linked dextran gel despite the fact that the column was equilibrated with 0.10 M glucose. Thus it was not possible to estimate the molecular weight by using Sephadex.

Extinction coefficient and nitrogen content. On a dry weight basis an extinction coefficient, $E_{1cm}^{1\%}$, of 11.4 ± 0.1 was obtained for concanavalin A. The nitrogen content was

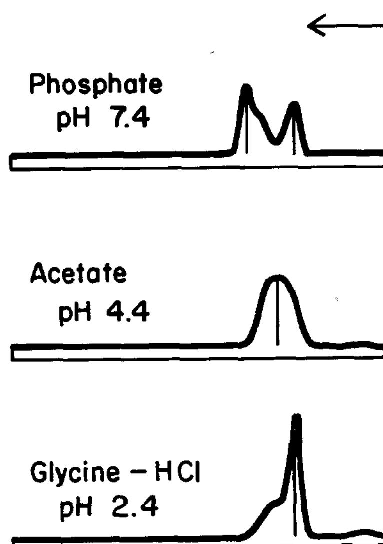

FIG. 3. Moving-boundary electrophoresis patterns of concanavalin A. Arrow indicates the direction of migration (descending boundary).

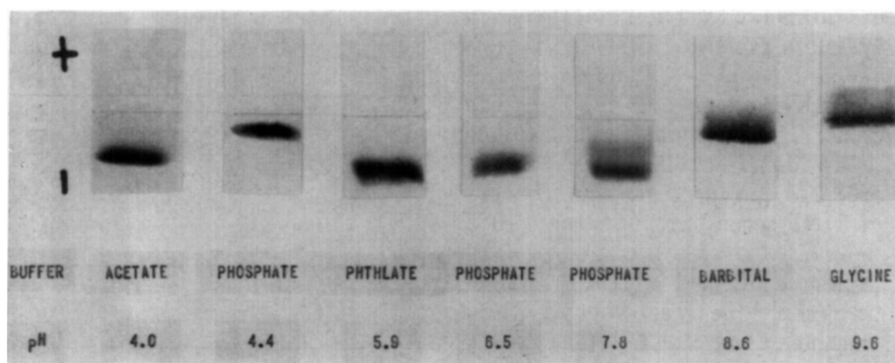

FIG. 4. Patterns of concanavalin A on paper electrophoresis at various pH values. Constant current 8 mA, 8 hours. Strips were stained with amido black.

TABLE IV
BIOGEL P-100 GEL FILTRATION DATA

Sample	M.W.	Tris-HCl, (0.10 M KCl, pH 7.5, Γ/2 = 0.15)			Acetate (0.10 M NaCl, pH 5, Γ/2 = 0.20)		
		V_0 (ml)	V_e (ml)	R_F	V_0 (ml)	V_e (ml)	R_F
Blue dextran	2×10^6	59	59	1.0	59	59	1.0
Bovine serum albumin	70,000					63	0.94
α-Chymotrypsin	25,000					88	0.67
Concanavalin A			59	1.0		74	0.80
Cytochrome *c*	12,400					104	0.57
Ovalbumin	46,000		69	0.85		73	0.81
Ovalbumin-concanavalin A			59	1.00		68	0.87
Pepsin	35,000					85	0.69

found to be 15.2%, in excellent agreement with the elemental analysis of the protein (C, 50.2%; H, 7.3%; N, 15.2%; ash, none). The analysis of the jack-bean meal was as follows: N, 4.6%; S, 0.2%, Ash, 3.29%.

Ultraviolet absorption spectra. The spectrum of concanavalin A in 1 M NaCl was typical of a protein with an E_{280}/E_{260} ratio of 1.8, which indicated the absence of nucleotides. The maximum corresponded to 278.6 mμ, the absorption at 280 mμ being approximately 0.66% less than at the λ_{max}.

From the absorption data in 0.10 N NaOH the molar ratio M_{tyr}/M_{trp} was determined to be 1.78 corresponding to 28.3 and 15.9 residues, respectively, of tyrosine and tryptophan per 10^5 gm protein. The extinction coefficient, $E_{1cm}^{1\%}$, of the protein in 0.10 N NaOH was 12.7 ± 0.1. Figure 5 shows the spectra of concanavalin A.

Amide content. The amide nitrogen constituted 6.3% of the total nitrogen. A value of 68.8 amide residues/10^5 gm protein was calculated.

Analysis for carbohydrates. All the colorimetric reactions for neutral and basic sugars yielded negative results. No reducing sugar was detected on paper chromatographic analysis of the protein hydrolyzate. If the sensitivity of these methods is considered, it is inferred that concanavalin A does not contain any detectable quantity of carbohydrate.

Amino acid analysis. These analyses revealed the complete absence of cystine (cysteine) in concanavalin A. This was supported by the absence of cysteic acid in the hydrolyzate of performic acid-oxidized protein. Under these conditions more than 90% of the methionine was oxidized to methionine sulfone. Concanavalin A contains very large amounts of aspartic acid and serine residues. Methionine (7.5 mole/

10^5 gm protein) is the least abundant residue. The analyses are presented in Table V.

Amino terminal. DNP-Alanine with small amounts of DNP-serine and DNP-glycine were present. The amount of alanine corresponded to 0.023 μmole/mg protein or 1.5 mole/68,000 gm protein. The concentration of DNP-serine was less than 10% that of alanine.

Alanine was confirmed as the principal amino-terminal residue by the phenylisothiocyanate method. The isolated phenylthiohydantoin, on hydrolysis, yielded alanine which was identified by paper chromatography in n-butanol–acetic acid–water (4:1:5, v/v). Traces of serine and glycine were also detected. The absorption spectrum of the isolated phenylthiohydantoin had the expected shape with a maximum at 267.6 mμ; the ratio $E_{260}/E_{\max}$ was 0.81.

Metal content. The manganese content of concanavalin A as determined by activation analysis was found to be 0.029%, which corresponds to 1 gm-atom Mn/about 190,000 gm protein. In addition to the 0.85 and 1.80 MeV peaks of Mn, 1.27 MeV Na^{24} and 1.64 MeV Cl^{38} peaks were also present. These were not quantitated.

From emission spectroscopy, the Mn^{2+} content was determined to be 0.036%, which corresponds to 1 gm-atom Mn/about 130,000 gm protein.

TABLE V

AMINO ACID COMPOSITION OF CONCANAVALIN A

Amino acid[a]	Moles/10^5 gm protein	Residue (gm/100 gm protein)
Lysine	46.6	5.96
Histidine	25.9	3.55
Ammonia	68.7	
Arginine	25.4	3.97
Aspartic	125.0	14.38
Threonine	75.3	7.61
Serine	121.1	10.55
Glutamic	46.1	5.96
Proline	41.9	4.06
Glycine	62.4	3.56
Alanine	70.8	5.02
Cysteine	0.0	0.0
Valine	64.9	6.43
Methionine	7.5	0.98
Isoleucine	56.3	6.37
Leucine	71.1	8.04
Tyrosine	28.8	4.69
Phenylalanine	39.2	5.76
Tryptophan	16.2	3.01

[a] Values for serine and threonine were corrected for losses, 5.5 and 3.9%, respectively, during the 24-hour hydrolysis period. The values for isoleucine and valine are derived from 72- and 96-hour hydrolyses. Other values are averages of the four hydrolyses. Amide groups were determined according to Hamilton (13). The spectrophotometric method described by Beaven and Holiday (12) was used for the determination of tryptophan.

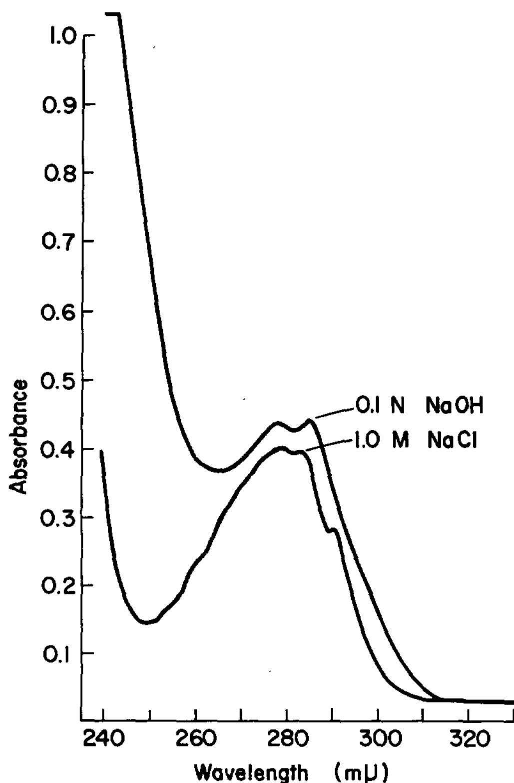

FIG. 5. Ultraviolet absorption spectra of concanavalin A in 0.10 N NaOH (upper curve), and 1 M NaCl (lower curve).

DISCUSSION

The determination of the homogeneity of proteins is a complex problem (26). Studies reported here were carried out on concanavalin A, which was shown to be approximately 98% pure by an assay conducted under rigidly defined conditions, thus indicating that the protein was homogenous by the criterion of its activity, i.e., polysaccharide-

precipitating ability. We would like to refer to this property as biological homogeneity. The physical heterogeneity observed under various conditions may not be due to the presence of any impurity; rather it may be an expression of the complex behavior of the protein itself. The tendency of the protein to associate near and above its isoelectric point, the presence of nonstoichiometric amounts of manganese (27, 28), its ability to interact with specific carbohydrates in addition to its interactions with buffer ions, etc., contribute to the complexity of the problem.

We have undertaken these studies of concanavalin A in the hope that the information obtained would be useful in understanding the mechanism of its interaction with specific carbohydrates, and its tendency for association. Such information should also contribute to a better understanding of the properties of other complex macromolecular interactions.

Between pH 2 and 5 concanavalin A migrated as a single symmetrical boundary in the analytical ultracentrifuge. Of the two species observed at pH 7 and above, the slower component is probably identical with the monodisperse species seen in the acidic pH medium, but the faster migrating component probably represents a dimer. The sedimentation-diffusion data indicated the $s_{20,w}$ of concanavalin A (in 0.10 M acetate pH 5, NaCl $\Gamma/2$ 0.45) was 3.9 compared with a value of 6 reported by Sumner *et al.* (5, 6). The determinations of the latter investigators were carried out in unbuffered 5% NaCl with the Svedberg ultracentrifuge. The difference in the value of the sedimentation coefficient in the two studies is too large to be due to experimental error.

The presence of a faster sedimenting component in our ultracentrifugal studies is definite evidence of association at pH 7. The undetected presence of such a component in an unbuffered medium could also lead to a higher value for the sedimentation coefficient.

On the basis of a calculated value of 0.73 for the partial specific volume, we obtained a molecular weight of 68,000, in contrast to the value of 96,000 reported by earlier workers. This difference is due largely to the higher value for the sedimentation coefficient reported by Sumner and co-workers (5, 6).

Unlike the single symmetrical boundary obtained at pH 5 and below, the sedimentation pattern at pH 7 exhibited the behavior typical of an interacting protein system involving a single component: $n\,\mathrm{A} \leftrightarrows \mathrm{B}_n$ (29). In rapidly equilibrating systems bimodal boundaries are anticipated if the equilibria involve aggregates larger than dimers (30, 31).

In a study of the polymerization of carboxypeptidase A in NaCl, Bethune (32) observed a single symmetrical boundary in 1–2 M NaCl. Studies of the concentration dependence of sedimentation coefficients led to the postulation of a rapid equilibrium involving the monomer and the dimer. In 2.5 M NaCl, however, a bimodal pattern was obtained, and the concentration of the faster component increased with temperature (33). On the basis of this temperature-dependence, the role of hydrophobic groups was indicated in the carboxypeptidase polymerization. The association and aggregation of concanavalin A is much more pronounced at temperatures higher than at 0°. The tendency of concanavalin A to form faster sedimenting components at pH 7 may be related to the dissociation of some charged groups in this region, the imidazole group being the most likely candidate. Dissociation of this group (pK about 7) probably results in conformational changes that favor protein-protein interaction. Our studies show concanavalin A to be an associating system near and above pH 7. However, more information is necessary to determine the nature of the equilibrium involved.

Results of our ultracentrifugal studies at pH 7 are at variance with those of Olson and Liener (34), who obtained a value of 3.18S for the sedimentation coefficient of concanavalin A. In our studies at pH 7 a distinct shoulder on the fast migrating peak of the schlieren pattern was invariably observed approximately 48 minutes after attaining maximum speed (59,780 rpm) in the ultracentrifuge. Such information is not

available from the data presented by Olson and Liener.

From the studies of mobility in moving-boundary electrophoresis, the isoelectric point of concanavalin A was determined by us to be 7.1 ± 0.1, in contrast to a value of 5.5 obtained by the minimum solubility method (35) and by cataphoresis (36). The earlier low values for the isoelectric point of concanavalin A could be attributed to the impurity of the protein and the technique employed.

On cellulose acetate electrophoresis we observed a zero net migration of concanavalin A at pH 7. However, on paper electrophoresis concanavalin A migrated to the cathode, even at pH 8.6. Similar observations were made by Nakamura and Suzuno (37). This retrograde migration probably reflects electroendosmosis and adsorption effects on paper (38).

At present no simple explanation can be offered to account for the electrophoretic heterogeneity of concanavalin A observed in free-boundary electrophoresis at pH 2.4 and 7.4. Heterogeneity of bovine serum albumin (39) at low pH values has been ascribed to the phenomenon of "isomerization." In the case of ovalbumin and plakalbumin, multiple peaks have been reported to be due to the presence of nonstoichiometric amounts of phosphorus (40–42). An analogous situation exists in the case of concanavalin A which contains 0.029% manganese (1 gm-atom Mn/about 190,000 gm protein) (28).

Association of molecules to form higher molecular weight units could also result in a change in the distribution of charges (43). Since this tendency is observed in the case of concanavalin A at pH 7 and above, these effects possibly contribute to the observed electrophoretic behavior. Protein-buffer interaction may also play some role. A more intensive examination of the electrophoretic behavior of this protein could be very valuable in increasing our understanding of protein-protein interaction.

A single sharp band on starch gel electrophoresis in the presence of glucose probably reflects the inhibition of concanavalin A-starch interaction by glucose. Alternately, binding of glucose to concanavalin A could result in conformational changes which prevent protein-protein interaction responsible for polydispersity.

Exclusion chromatography yields unreliable estimates of molecular weight for solutes which interact with the medium. Proteins with high carbohydrate contents also have been observed to deviate from the normal behavior on gel filtration with cross-linked dextran gels (44). Similar experience with concanavalin A using Sephadex G-100 can be rationalized on the basis of Sephadex-concanavalin A interaction.

Gel filtration experiments with Biogel P-100 lend support to the results of ultracentrifugal studies in that at pH 5 the molecular weight of concanavalin A is close to 50,000; association of subunits to a higher molecular weight species occurred at pH 7.

The nature of the complex formed between concanavalin A and ovalbumin in Biogel P-100 experiments is not known. Ovalbumin (45) contains covalently linked carbohydrate side chains composed of D-mannose and 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine), both of which possess the necessary determinants (if joined by α-glycosidic linkages) to interact with concanavalin A. The inability to form an insoluble complex with concanavalin A could be analogous to the situation with linear polysaccharides which cannot form a matrix due to the presence of only a single reactive end. Alternately, the complex could be a result of protein-protein interaction, the nature of which may be electrostatic or otherwise.

Ultraviolet absorption studies of concanavalin A revealed a high content of aromatic amino acids and exclude the presence of nucleotides in the protein.

We have been unable to detect the presence of carbohydrate in concanavalin A. Since our isolation procedure is very mild, avoiding extremes of pH, it appears that concanavalin A does not contain any carbohydrate moiety. Similar observations have been made by Olson and Liener (34). The complete absence of carbohydrate distinguishes concanavalin A from soy and wax bean hemagglutinins (46, 47) and immunoglobins (48), all of which are glycoproteins. The role of the carbohydrate moiety in

these proteins is not known. However, in the case of immunoglobulins, carbohydrate-free papain fragments have been shown to retain their ability to interact with specific antigens (49, 50). Similarly, concanavalin A, which has been shown to be devoid of carbohydrate, displays the ability to interact with specific polysaccharides and to agglutinate erythrocytes (34).

Amino acid analysis of concanavalin A revealed the complete absence of cysteine and cystine. A similar situation is observed with hemagglutinins from soy (46) and wax beans (47). In contrast, the cystine content of immunoglobulins is very high. Except for the content of cysteine and cystine, there is a remarkable similarity in the amino acid compositions of phytohemagglutinins (46, 47, 51) and γ-globulins (52, 53). For instance, all of these proteins have a very low methionine content (about 7 moles/10^5 gm protein), very large amounts of acidic, and substantial quantities of basic amino acid residues. In addition they also contain high proportions of amino acids with hydroxyl groups. Generally speaking, vegetable proteins contain more aspartic acid (asparagine) than glutamic acid (glutamine). A large number of acidic residues occur as their amides in concanavalin A and other phytohemagglutinins and in γ-globulins.

The tyrosine content of concanavalin A as obtained by Beaven and Holiday's method (12) (28.3 moles/10^5 gm protein) is in remarkable agreement with that obtained from amino acid analysis of the protein hydrolyzate (28.8 moles/10^5 gm). A similar value for tyrosine was noted by Sumner and Graham (4). These workers also reported a cystine content of 0.4% for this protein, which probably reflects some contamination with proteins rich in cysteine or cystine. Preliminary analysis of the proteins emerging in the void volume (Peak 1) in the Sephadex G-50 adsorption method for the isolation of concanavalin A (1) revealed the presence of small quantities of cystine. This may account for Sumner and Graham's findings. A very small amount of hexosamine was also detected in this fraction.

Results of our amino acid analyses are in excellent agreement with those of Olson and Liener (34), except for slight differences in the content of aspartic acid, histidine, serine, threonine, and amide nitrogen.

Amino-terminal studies show only alanine to be present in stoichiometrically significant amounts (about 1.5 mole/68,000 gm protein). This could indicate a multichain structure for the protein. The presence of small amounts of serine and traces of glycine could be interpreted as indicative of heterogeneity or premature release of these amino acids. Inasmuch as dinitrophenyl and phenylthiohydantoin derivatives of serine and glycine undergo extensive destruction, the present data are insufficient to rule out the contribution of these amino acids to the amino terminal end of concanavalin A.

We have confirmed earlier reports (27) of the presence of manganese in concanavalin A. From the results of activation analysis, a manganese (Mn^{2+}) content of 0.029%, corresponding to 1 gm-atom/about 190,000 gm protein was obtained. If a value of 68,000 is used for the molecular weight of a subunit, it is suggested that concanavalin A is composed of three (possibly four) subunits. Since metal is essential for activity, this indicates that the activity at pH 5 would be due solely to subunits containing manganese. The maximum activity at neutral pH values is most likely due to the formation of a quaternary structure composed of three or four subunits. At present, however, it is very difficult to indicate the exact number of subunits due to the variation in the manganese content determined by the two different techniques.

Formation of a stable structure by association of independent subunits has been observed with several proteins. Such structures may be stabilized by metals, e.g., the tetrameric form of alcohol dehydrogenase is stabilized by zinc (54). In proteins which lack sulfhydryl or disulfide groups, bivalent metals could assume the role of disulfide groups in maintaining a particular three-dimensional structure. In the case of concanavalin A the metal is tightly bound to the protein; consequently concanavalin A satisfies the definition of a metalloprotein (55). The role of the bivalent cation in concanavalin A-polysaccharide interaction will be discussed in a later paper (56).

ACKNOWLEDGMENTS

We thank Dr. E. A. Boettner, Dr. L. F. Haas, Dr. N. R. Harvie, Dr. Margaret Hunter, Dr. G. W. Jourdian, Dr. I. E. Liener, Dr. D. V. Myrhe, Mr. H. G. Olson, Dr. J. Sri Ram, Dr. Sidney Shulman, and Dr. C. H. Williams, Jr. for liberal use of their facilities. We express our sincere appreciation to Mr. Paul Bronson, Mr. F. I. Gunder, Mr. Mike Mulich, Mr. John Van Atta, and Mr. Marshall Wakat for their assistance in various phases of this work. We are indebted to Professor J. L. Oncley for his interest in these studies. This work was supported by grants GM-12922, A1-06795, and AM-10171 from the National Institutes of Health.

REFERENCES

1. Agrawal, B. B. L., and Goldstein, I. J., *Biochim. Biophys. Acta* **147,** 262 (1967).
2. Goldstein, I. J., Hollerman, C. E., and Merrick, J. M., *Biochim. Biophys. Acta* **97,** 68 (1965).
3. So, L. L., and Goldstein, I. J., *J. Biol. Chem.* **242,** 1617 (1967).
4. Sumner, J. B., and Graham, V. A., *J. Biol. Chem.* **64,** 2571 (1925).
5. Sumner, J. B., Gralen, N., and Eriksson-Quensel, I. B., *Science* **87,** 395 (1938).
6. Sumner, J. B., Gralen, N., and Eriksson-Quensel, I. B., *J. Biol. Chem.* **125,** 45 (1938).
7. Loyter, A., and Schramm, M., *J. Biol. Chem.* **241,** 2611 (1966).
8. Shulman, S., Landaburu, R. H., and Seegers, W. H., *Thromb. Diath. Haemorrhag.* **4,** 336 (1960).
9. Schachman, H. K., *Methods Enzymol.* **4,** 32 (1957).
10. Long, C., *in* "Biochemists Handbook," p. 29. Van Nostrand, Co., Princeton, New Jersey (1961).
11. Kabat, E. A., and Mayer, M. M., *in* "Experimental Immunochemistry," 2nd edition, p. 476. Thomas, Springfield, Illinois (1961).
12. Beaven, G. H., and Holiday, E. R., *Advan. Protein Chem.* **7,** 320 (1952).
13. Hamilton, L. D. G., *in* "Analytical Methods of Protein Chemistry" (P. Alexander and R. J. Block, eds.), Vol. 2, p. 71. Pergamon Press, New York (1960).
14. Winzler, R., *Methods Biochem. Anal.* **2,** 279 (1955).
15. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F., *Anal. Chem.* **28,** 350 (1956).
16. Scott, T. A., and Melvin, E. H., *Anal. Chem.* **25,** 1656 (1953).
17. Boas, N. B., *J. Biol. Chem.* **204,** 553 (1953).
18. Aminoff, D., *Biochem. J.* **81,** 384 (1961).
19. Trevelyan, W. E., Proctor, D. P., and Harrison, J. S., *Nature* **166,** 444 (1950).
20. Moore, S., and Stein, W. H., *Methods Enzymol.* **6,** 819 (1963).
21. Hirs, C. H. W., *J. Biol. Chem.* **219,** 611 (1956).
22. Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., *Methods Biochem. Anal.* **2,** 360 (1955).
23. Crouthamel, C. E., *in* "Applied Gamma Ray Spectrometry," p. 183. Pergamon Press, New York (1960).
24. Heath, R. L., *in* "Scintillation Spectrometry." Phillips Petroleum Co., Idaho Falls, Idaho (1957).
25. Cohn, E. J., and Edsall, J. T., (Eds.), "*Proteins, Amino Acids and Peptides.*" Reinhold, New York (1943).
26. Sober, H. A., Hartley, Jr., R. W., Carroll, W. R., and Peterson, E. A., *in* "The Proteins" (H. Neurath, ed.), 2nd edition, Vol. III, p. 2. Academic Press, New York (1965).
27. Sumner, J. B., and Howell, S. F., *J. Biol. Chem.* **115,** 583 (1936).
28. Agrawal, B. B. L., and Goldstein, I. J., *Biochim. Biophys. Acta* **133,** 376 (1967).
29. Nichol, L. W., Bethune, J. L., Kegeles, G., and Hess, E. L., *in* "The Proteins" (H. Neurath, ed.), 2nd edition, Vol. II, p. 305. Academic Press, New York (1964).
30. Gilbert, G. A., *Discussions Faraday Soc.* **20,** 68 (1955).
31. Gilbert, G. A., *Proc. Roy. Soc. (London), Ser. A* **250,** 377 (1959).
32. Bethune, J. L., *Biochemistry* **4,** 2691 (1965).
33. Bethune, J. L., *Biochemistry* **4,** 2698 (1965).
34. Olson, M., and Liener, I. E., *Biochemistry* **6,** 105 (1967).
35. Czonka, F. A., Murphy, J. C., and Jones, D. B., *J. Am. Chem. Soc.* **48,** 763 (1926).
36. Sumner, J. B., and Howell, S. F., *J. Bacteriol.* **32,** 227 (1936).
37. Nakamura, S., and Suzuno, R., *Arch. Biochem. Biophys.* **111,** 499 (1965).
38. Waldmann, Meyer, H., *Methods Biochem. Anal.* **13,** 47 (1965).
39. Aoki, K., and Foster, J. F., *J. Am. Chem. Soc.* **79,** 3385 (1957).
40. Linderstrom-Lang, K., and Ottesen, M., *Compt. Rend. Trav. Lab Carlsberg* **26,** 16 (1949).
41. Perlman, G. E., *Nature* **164,** 961 (1949).
42. Perlman, G. E., *Nature* **166,** 870 (1949).
43. Ansevin, A. T., Stevens, C. L., and Lauffer, M. A., *Biochemistry* **3,** 1512 (1964).
44. Andrews, P., *Biochem. J.* **96,** 595 (1965).

45. NEUBERGER, A., AND MARSHALL, R. D., *in* "Glycoproteins" (A. Gottschalk, ed.), p. 299. Elsevier, Amsterdam (1966).
46. WADA, S., PALLANSCH, M. J., AND LIENER, I. E., *J. Biol. Chem.* **233,** 395 (1958).
47. TAKAHASHI, P., RAMACHANDRAMURTHY, P., AND LIENER, I. E., *Biochim. Biophys. Acta* **133,** 123 (1967).
48. PRESS, E. M., AND PORTER, R. R., *in* "Glycoproteins" (A. Gottschalk, ed.), p. 395. Elsevier, Amsterdam (1966).
49. PORTER, R. R., *Biochem. J.* **73,** 119 (1959).
50. FLEISCHMAN, J. B., PORTER, R. R., AND PRESS, E. M., *Biochem. J.* **88,** 220 (1963).
51. LIS, H., SHARON, N., AND KATCHALSKI, E., *J. Biol. Chem.* **241,** 684 (1966).
52. PHELPS, R. A., AND PUTNAM, F. W., *in* "The Plasma Proteins" (F. Putnam, ed.), p. 143. Academic Press, New York (1960).
53. SMITH, E. L., MCFADDEN, M. L., STOCKELL, A., AND BOETTNER-JANUSCH, V., *J. Biol. Chem.* **214,** 197 (1965).
54. KAGI, J. H. R., AND VALLEE, B. L., *J. Biol. Chem.* **235,** 3188 (1960).
55. VALLEE, B. L., *Advan. Protein Chem.* **10,** 317 (1955).
56. AGRAWAL, B. B. L., AND GOLDSTEIN, I. J., in preparation.