Protein-Carbohydrate Interaction

IV. APPLICATION OF THE QUANTITATIVE PRECIPITIN METHOD TO POLYSACCHARIDE-CONCANAVALIN A INTERACTION*

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SUMMARY

Concanavalin A, a globulin found in the jack bean, reacts specifically to form a precipitate with a restricted group of branched polysaccharides. The various parameters optimal for this interaction were investigated by the quantitative precipitin method with a dextran as the precipitating polysaccharide. In this manner, an assay for concanavalin A activity was established. The analogy of this interaction with the antibody-antigen system is striking. A summary of findings follows.

1. Complete precipitation is achieved in 24 hours at 25°C.
2. The pH range optimum for the reaction lies between 6.1 and 7.2.
3. The concentration of sodium chloride has no effect on the reaction when the system is buffered at pH 7.0 with phosphate. KI and KCNS, however, are inhibitory.
4. The presence of foreign proteins does not affect the total amount of concanavalin A precipitated.
5. The precipitate formed between concanavalin A and dextran is slightly soluble (1.5 μg of nitrogen per ml) at 25°C.
6. More nitrogen is precipitated at 25°C than at 0°C, the relative amount depending upon the region of the equivalence curve examined.

More recently, the interaction between concanavalin A and polysaccharides was generalized to include all branched carbohydrate polymers which contain multiple α-D-glucopyranosyl (or its 2-acetamido-2-deoxy derivative), α-D-mannopyranosyl, or β-D-fructofuranosyl units at nonreducing chain ends (5-7).

The similarity of the interaction of concanavalin A with polysaccharide to the antibody-antigen system was first suggested by Sumner and Howell (1) in 1936 and later commented upon by Hehre (8) and the present investigators (5-7, 9). The parallel between the activity of phytohemagglutinins and immune antibodies was discussed by Boyd and Shapleigh (10) in their investigation of the lima bean hemagglutinin.

The polysaccharide-concanavalin A system has previously been investigated with the techniques of agar gel diffusion (7) and hapten inhibition (6). The present paper describes the application of the quantitative precipitin method to the concanavalin A system. This study was undertaken for the purpose of developing an assay system for monitoring the purification of the protein. It was also of interest to assess the concanavalin A system further as a model for antibody-antigen interaction and to apply the procedure to the study of polysaccharide structure.

EXPERIMENTAL PROCEDURE

Concanavalin A was isolated from jack bean meal in a highly purified form by the method of Agrawal and Goldstein (9). Solutions of concanavalin A in 0.1 M NaCl were stored at 4°C.

Dextran (NRRL B-1355-S) was dissolved in water and the concentration was determined by the phenol-sulfuric acid method (11).

Precipitation Reaction—Precipitation studies were carried out in duplicate in 3-ml centrifuge tubes calibrated at 1.0, 1.5, and 2.0 ml. The solutions were incubated at 25°C for at least 24 hours. The reaction mixture contained approximately 300 μg of protein and 200 μg of dextran in a total volume of 1.0 ml of 0.1 M NaCl containing 0.018 mmole of phosphate buffer, pH 7.2. These conditions were maintained throughout the course of the work except for the parameter under study. After a 24-hour precipitation period, the concanavalin A-dextran...
precipitate was centrifuged in an International Clinical centrifuge (3000 rpm) for 15 min at room temperature, the supernatant solution was carefully decanted, and the precipitates were allowed to drain with the tubes in an inverted position in a test tube rack. The precipitates were washed twice by suspending them in 50% ethanol and heated in a covered water bath maintained at 95°C for 20 min. The tubes were cooled and diluted to 10 ml with 50% ethanol.

Howe, and Kabat (13). The ninhydrin reagent (0.2 ml) was added to the 0.4 ml-aliquot, mixed with a Vortex mixer, and allowed to drain with the tubes in an inverted position in a test tube rack. The precipitates were washed twice by suspending them with an electrically heated sand bath at 110°C. When all of the water had evaporated, the temperature of the sand bath was raised to 180°C to complete the digestion. This usually required 1 hour more. The tubes were cooled, 30% H$_2$O$_2$ (0.1 ml) was added, and the tubes were heated at 110°C until the solution was water clear. The temperature was then raised to 180°C and maintained at that temperature for 1 hour to remove excess peroxide. The cleared digests were diluted to 2 ml with water and an aliquot (0.4 ml) was taken for determination of the (NH$_4$)$_2$SO$_4$ formed with the ninhydrin procedure. The method employed was essentially that of Rosen (12) as modified by Schiffman, Howe, and Kabat (13). The ninhydrin reagent (0.2 ml) was added to the 0.4 ml-aliquot, mixed with a Vortex mixer, and heated in a covered water bath maintained at 95°C for 20 min. The tubes were cooled and diluted to 10 ml with 50% ethanol and the absorbance at 570 nm was read against an alcohol blank by means of a Beckman DU spectrophotometer. A protein blank was run to time to correct for nonspecific precipitation. This amount was subtracted from the total nitrogen precipitated.

A standard curve employing (NH$_4$)$_2$SO$_4$ was established in the same fashion, and the amount of nitrogen in the precipitate was determined by reference to the standard curve.

For the concanavalin A-dextran precipitation curve, increasing amounts of polysaccharide were added to 300 µg of protein. The amount of H$_2$SO$_4$ used for digestion varied depending on the amount of nitrogen precipitated. For each 10 µg of nitrogen in the precipitate, 0.01 ml of 7 N H$_2$SO$_4$ was required. The proper dilutions were then made on the digests, 0.4 ml H$_2$O for every 0.01 ml of H$_2$SO$_4$ added, and the nitrogen was determined as described.

It has been established in this laboratory that concanavalin A is not a glycoprotein, and hence the analysis of carbohydrate by the phenol-H$_2$SO$_4$ method (11) may be conducted directly on the protein-polysaccharide precipitate. The amount of carbo-

hydrate in the precipitate was determined as follows. Washed precipitates of concanavalin A-dextran were dissolved in 2 ml of 0.1 M KCl-HCl buffer. An appropriate aliquot was removed for estimation of carbohydrate by the phenol-H$_2$SO$_4$ method. The total amount of dextran in the precipitation was determined by reference to a standard curve for glucose, making the necessary conversion to polysaccharide by multiplying the amount of glucose determined by 0.9.

Turbidimetric measurements were performed as follows. The reaction mixture contained 1.3 mg of concanavalin A and 290 µg of dextran in a total volume of 3.0 ml. The reaction was initiated by the addition of dextran to the concanavalin A solution. The mixture was stirred with a polyethylene rod and the tubes were incubated at 25°C for 12 min and read in a Bausch and Lomb spectronic 20 colorimeter at 420 µg against a blank containing protein. To correct for the turbidity of the dextran solution, a small amount of methyl α-d-mannopyranoside (approximately 1 mg), an inhibitor of the reaction (6, 7), was added to each tube to dissolve the precipitate. The absorbance of the resulting clear solution was subtracted from the reading obtained before addition of inhibitor.

**RESULTS**

Various parameters, such as pH, salt concentration, temperature, time, and volume, influence the precipitin reaction in most antibody-antigen systems. Although the concanavalin A-polysaccharide precipitation system appears to be analogous in almost all respects to the antibody-antigen precipitin reaction, it became necessary to investigate in detail the effect of all of the reaction variables.

**Effect of pH on Precipitate Formation**—Buffers (0.1 M) ranging from pH 2 to 11 prepared according to the procedure of Gomori (14) were employed in studying the effect of pH on concanavalin A-dextran interaction. Unless specified otherwise, these and subsequent studies were carried out in the region of maximum nitrogen precipitation. The amount of nitrogen in the precipitate as determined by ninhydrin analysis is plotted against the pH of the supernatant as illustrated in Fig. 1. The pH reaction curve shows an optimum and constant activity between pH 6.1 and 7.2. Below pH 4.7 and above pH 9.1, precipitation formation does not occur. Approximately 97% of the total protein is precipitated in the pH optimum range after correcting for solubility of the complex and loss due to washing (see below).

**Effect of Varying Concentrations of NaCl and of Various Inorganic Salts**—A study of the effect of NaCl on the precipitation reaction was carried out with salt concentrations ranging from 0.05 to 4.2 M. The amount of precipitable nitrogen increased as the ionic strength of the medium is increased, as shown in Fig. 2. The reaction medium in this particular instance contained NaCl as the only ionic species. The same experiment, when repeated in the presence of 0.018 mmole of phosphate buffer, pH 7.2, was independent of NaCl concentration (Fig. 2).

The effect of various other inorganic salts on the precipitation reaction was studied by turbidimetry. The data in Fig. 3, and 3B, indicate that the nature of the ionic species has a more pronounced effect on the reaction than the ionic strength. Sodium nitrate is without appreciable effect on the reaction whereas increasing the concentration of Na$_2$SO$_4$ results in an increased amount of nitrogen precipitated (Fig. 3A). The effect of salts of the halides is interesting in that increasing
concentrations of NaF and KCl enhance the amount of nitrogen precipitated, KBr decreases slightly, and KI markedly inhibits the amount of nitrogen precipitated (Fig. 3B). Potassium thiocyanate is similar to KI in this regard. At \( \Gamma/2 \) KI of 1.0 and \( \Gamma/2 \) KCNS of 2.75, the reaction was completely inhibited. With the exception of NaNO₃, an increase in salt causes an increase in the pH of the medium close to or at the optimum pH range. Increasing the ionic strength of phosphate buffer at pH 6.75 also caused inhibition of turbidity (Fig. 3A).

**Time and Temperature Effects on Precipitation**—The velocity of precipitate formation at \( \beta^\circ \) was studied at three points of the precipitation curve: (a) region of concanavalin A excess, (b) equivalence zone, and (c) region of dextran excess. Results of these studies at \( \beta^\circ \) are shown in Fig. 4. Completion of precipitation was most rapid in the equivalence zone, this being achieved in approximately 5 hours at \( \beta^\circ \). The rate of precipitate formation was slowest in the dextran excess region, 24 hours at \( \beta^\circ \) being required for complete precipitation. Approximately 22 hours were required for complete precipitation in the concanavalin A excess region.

These experiments were conducted over the course of 1 week and it was noted that the protein in the control tube aggregated nonspecifically on standing for long periods. The amount of nonspecific precipitation is also shown in Fig. 4 and this varies from preparation to preparation and with experimental conditions. More nonspecific precipitation occurred when freeze-dried preparations of concanavalin A were used (approximately 10% at \( \beta^\circ \) for 1 week, Fig. 4). With fresh solutions of concanavalin A aggregation was much less pronounced (approximately 5% at \( \beta^\circ \) for 1 week, Fig. 5). Inasmuch as precipitation is essentially complete in 24 hours, this does not pose any great difficulty.

We have observed that the presence of “substrate” or hapten inhibitor diminished the amount of aggregation. Thus d-glucose, which binds to the active sites of the concanavalin A molecule, protects it from aggregating, whereas d-galactose, a nonbinder, does not have this effect. However, addition of glucose to the protein control tube renders it unacceptable as a blank. In all of our experiments, therefore, a control tube containing the same amount of concanavalin A is routinely used as a blank to give a correction for nonspecific precipitation. For the duration of the normal incubation period, this amount is not appreciable (1 to 2%).
The solubility of the precipitate can be determined from the slope of the curve and amounts to 1.5 pg of nitrogen per ml.

Fig. 6. Effect of volume on concanavalin A-dextran precipitation. Concanavalin A, 40 µg of nitrogen; dextran, 180 µg.

Fig. 7. Quantitative precipitation curve of dextran B-1355-S with concanavalin A. The total amount of dextran in the precipitate is also illustrated. Concanavalin A, 42 µg of nitrogen.

The quantitative determination of the precipitin reaction was first attempted by Wu, Cheng, and Li (15) in 1927 and Wu, Sah, and Li (16) in 1928, when they determined the composition of washed, specific precipitates of antibody and antigen. However, the further refinement and perfection of this technique as an analytical tool for antibody determination is attributed to Heidelberger and Kendall (17, 18) and Heidelberger, Kendall, and Soo Hoo (19). It consists essentially of analysis of washed, specific precipitates of antibody and antigen. However, the further refinement and perfection of this technique as an analytical tool for antibody determination is attributed to Heidelberger and Kendall (17, 18) and Heidelberger, Kendall, and Soo Hoo (19). It consists essentially of analysis of washed, specific precipitates of antibody and antigen.

**Discussion**

The present study appears to substantiate the suggestion that the concanavalin A-poly saccharide interaction displays many of the characteristics of an antibody-antigen system. Previous work from this laboratory (5-7, 9, 20) demonstrated the specificity of the interaction, its dependence on the concentration of both protein and polysaccharide, and its ability to be inhibited specifically by low molecular weight substances (hapten). The present demonstration of an equivalence-type curve further strengthens this argument. In this system, concanavalin A is analogous to a specific antibody, the combining sites of which, complementary to the 2-deoxy-α-D-arabinohexopyranosyl ring
system, interact in a reversible manner with polysaccharide chain ends. In actual fact, any biopolymer which contains multiple residues of the "proper" carbohydrate determinant will form a precipitate with concanavalin A. This was shown recently with the aid of model substances (20) in which p-amino-phenyl glycosides of mono- and disaccharides were conjugated (by diazotization) to bovine serum albumin to afford multivalent antigens. The carbohydrate-protein conjugates containing the "proper" carbohydrate determinants interacted to form a precipitate with concanavalin A, whereas those with sugar residues which were not complementary to the concanavalin A binding sites failed to do so.

One of the most outstanding features of this system is the fact that this multivalent protein appears to contain only one type of combining site with respect to the carbohydrate determinant with which it interacts, a subject that will be dealt with in a future communication. This is in sharp contrast to immune antibodies to a given antigen which display many possible variations in their combining sites (21).

Approximately 95 to 98% of concanavalin A is specifically precipitated by dextran B-1355-S, an indication of the biological homogeneity of the protein. Detailed physicochemical studies carried out in this laboratory suggest that concanavalin A is physically homogeneous as well.² Foreign proteins, which do not carry the determinant carbohydrate units necessary for interaction with concanavalin A, are without effect on the amount of concanavalin A precipitated.

Since precipitate formation between concanavalin A and dextran involves a protein as one of the reacting species, it is to be expected that the interaction would be dependent upon the various factors which influence protein interaction, namely pH, ionic strength of the medium, temperature, etc. These have already been referred to above. There are however, several features of the concanavalin A polysaccharide interaction which bear comment, especially as they relate to immune antibody-antigen interactions.

Concanavalin A showed maximum and constant activity between pH 6.1 and 7.2. With immune antibodies, the optimum pH for antibody-antigen combination is also close to neutrality. In the egg albumin-anti-egg albumin system, for example, the optimal range for maximum precipitation lies between pH 6.26 and 8.45 (22).

Although most antibodies are known to be dissociated from their specific precipitates in hypertonic NaCl solutions, concanavalin A-polysaccharide precipitates buffered at the pH range of maximum activity are not effected by NaCl concentrations as high as 3.75 M. Mammalian antibodies give maximum precipitation at a salt concentration of 0.1 to 0.15 M (23), but there are certain instances in which more antibody nitrogen is precipitated at high NaCl concentration than in physiological 0.9% NaCl. Avian antisera in particular exhibit this type of behavior (24, 25).

The solubility of the concanavalin A-dextran complex was determined to be 1.5 µg of nitrogen per ml. With human antisemurn to blood group substances (26-28) and horse antipneumococcal antibody (26, 29), the solubility values of specific precipitates are very low, of the order of 1.5 µg of nitrogen per ml. However, with rabbit antisera, these same precipitates are considerably more soluble amounting to as much as 10 µg of nitrogen per ml (30, 31).

As with the antibody-antigen precipitin curve, the concanavalin A-dextran precipitation curve (Fig. 7) exhibits the presence of three zones, namely, a region of concanavalin A excess, an equivalence range, and a region of dextran excess. Up to and throughout the equivalence zone, virtually all of the dextran appeared in the precipitate. When more dextran is added, the reaction is inhibited, soluble complexes being formed.

Fig. 8 depicts a plot of the ratio of micromoles of concanavalin A to micromoles of dextran B-1355-S in the precipitate with respect to the amount of dextran added. A molecular weight of 100,000 is assumed³ for concanavalin A and the value of 44 X 10⁴ for dextran B-1355-S was obtained by light scattering.⁴ It is obvious that the composition of the concanavalin A-dextran precipitate varies continuously throughout the entire range of the precipitation curve. It was calculated that at the equivalence point there are 340 molecules of concanavalin A associated with each molecule of dextran. From these data, it is not possible to ascertain the valence of the protein. Equilibrium dialysis experiments are now in progress to establish this point.

Various anions were tested for their effect on the concanavalin A-dextran interaction. A striking similarity with a study reported by Kleinschmidt and Boyer (22) on the egg albumin-rabbit antibody system was noted in the effect of the various halogen salts and potassium thiocyanate on the concanavalin A system. Inhibition of precipitate formation in both systems parallel the familiar lyotropic series CNS⁻ > I⁻ > Br⁻ > Cl⁻ > F⁻. In the concanavalin A system, KI is somewhat more inhibitory than KCNS.

A rather unusual aspect of concanavalin A-polysaccharide interaction is the effect of temperature on the precipitation reaction. The amount of protein which is specifically precipitated is greater at 25⁰ than at 0⁰, the relative amount being a function of the equable point on the equivalence curve. The reasons for this behavior are currently under investigation.

³ Kindly determined by Dr. Kirsti Granath, Pharmacia, Uppsala, Sweden.

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Concanavalin A aggregates spontaneously upon standing. It appears to be more unstable at room temperature and neutral pH than at 0° and acid pH values. However, inasmuch as the quantitative precipitation reaction is complete within 24 hours, the error entailed is small. D-Glucose, which binds to the active sites of concanavalin A, assisted in protecting the protein from spontaneous aggregation. On the other hand, D-galactose, a noninhibitor of the concanavalin A-polysaccharide interaction, did not protect the protein from aggregation and in some instances actually appeared to increase the amount of aggregation over the control containing no carbohydrate.

The present investigation showed the feasibility of employing this quantitative precipitation procedure as a means for following the purification of concanavalin A. This has been done and the results of the purification procedure together with a discussion of the physical and chemical properties of concanavalin A will be published shortly.

The relative abundance of concanavalin A in jack bean meal (2.5 to 3% by weight) together with its ease of preparation in a highly purified state by specific binding to Sephadex (9) followed by elution with a competitive inhibitor such as D-glucose makes this protein-polysaccharide interaction an ideal system for intensive investigation. It is suggested that further study of the many parameters which effect concanavalin A-polysaccharide interaction will increase our knowledge of complex interacting systems in general, and the antibody-antigen reaction in particular.

REFERENCES
Protein-Carbohydrate Interaction: IV. APPLICATION OF THE QUANTITATIVE PRECIPITIN METHOD TO POLYSACCHARIDE-CONCANAVALIN A INTERACTION
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