SOLUBILITY METHODS IN THE STUDY OF PROTEINS[1]

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Gibbs' phase rule permits one to make certain general predictions about the number of components in a preparation from its solubility curve. If the solubility does not vary with the amount of solid, i.e., it has a constant solubility, the material will in general be a single pure component. If the solubility varies with the amount of solid, two possible cases exist. The material may be a mixture or a solid solution of two or more components.

The curves, methods of distinguishing, and techniques involved in these three cases are discussed in detail.

Use of solubility methods in the purification of proteins is described and illustrated with the cases of pepsin and diphtheria antitoxin.

I. INTRODUCTION

The historical and theoretical development of protein solubility has been thoroughly discussed by Northrop and Kunitz (7, 12) and more recently by Butler (1), so that it is necessary to mention only the more important aspects.

Sørensen (15) seems to have been the first to apply the phase rule to studies of protein solubility. However, when his protein preparations, even after repeated fractionation, did not have a constant solubility as the amount of solid was varied, he suggested (16) that perhaps proteins are dissociable molecules and were for this reason behaving in his solubility experiments as "multiple component systems."

Kunitz and Northrop (7) were the first to obtain highly purified protein preparations of constant solubility. Their work not only demonstrated that proteins could be obtained which had constant solubility and therefore behaved like other molecules of organic or inorganic nature but also proved that not all proteins conformed to the dissociating theory of Sørensen (16). At the present time a number of proteins have been prepared which show a constant solubility, among which are chymotrypsinogen (Kunitz and Northrop (7)), trypsin (Kunitz (5)), swine pepsin (Herriott, Desreux, and Northrop (3)), salmon pepsin (Norris and Elam (10)), ribonuclease (Kunitz (6)), the luteinizing hormone of swine, metakentrin (Shedlovsky, Rothen, Greep, Van Dyke, and Chow (14)), the lactogenic hormones of beef and sheep (Li, Lyons, and Evans (9)), and the oxytocic, pressor, and antidiuretic hormone from beef pituitary (Van Dyke, Greep, Rothen, and Chow (17)).

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II. THE PHASE RULE AND SOLUBILITY CURVES

Crystalline proteins do not have melting points, so that this classical criterion of purity, so useful in organic chemistry, cannot be applied in the study of proteins. However, the somewhat analogous determination of solubility furnishes much the same information.

In most phase-rule studies, changes in certain properties are examined as the temperature or pressure of the system is varied. In the solubility determination the temperature and pressure are held constant, and the variation in the concentration of solute is noted as the amount of solid phase is varied.

The Gibbs' phase rule, which applies only to systems at equilibrium, states that the number of phases plus the number of degrees of freedom equals the number of components plus two, or $P + F = C + 2$. Since the temperature and pressure are held constant during the solubility determination the equation simplifies to $P + F' = C$, in which $F' = F - 2$. In view of the fact that much depends upon the meaning of the terms, the following definitions are taken directly from Findlay's book on the phase rule (2). Thus, "homogeneous physically distinct and mechanically separate portions are called phases." By components are meant "only those constituents which can undergo independent variation in the different phases." For the number of components in a system "there are to be chosen the smallest number of independently variable constituents by means of which the composition of each phase participating in the state of equilibrium can be expressed in the form of a chemical equation." The number of degrees of freedom is "the number of variable factors, temperature, pressure, and concentration of the components, which must be arbitrarily fixed in order that the condition of the system may be perfectly defined."

In order to apply the phase rule to solubility data it is necessary to measure the solubility of the substance in the presence of varying quantities of the solid phase. This is done by stirring varying quantities of the precipitate with a constant volume of solvent until equilibrium is obtained. The concentration of dissolved material is then determined and plotted against the total protein concentration. The various types of curves possible are shown in figure 1.

In the first part of the curve, as small amounts of the unknown solid are added to pure solvent all will dissolve, giving a clear solution, and the points will fall on the 45° line. There is only one phase, the solution, and two or more components one of which is the solvent; therefore there is at least one degree of freedom. As soon as the solid phase appears, there are two phases present. Three types of curves are now possible.

(*1*) A curve with a slope of zero, such as curve A in figure 1 in which the composition of both phases remains constant and independent of the quantity of the phases. This result shows that the system is fixed and has no degrees of freedom. The number of phases and of components is therefore equal. The simplest case is two components, solvent and solute, and two phases, one liquid and one solid. The solid phase consists, therefore, of only one component. It is possible that two solid phases would appear at precisely the same point and the system would then consist of three phases and three components. Such a

result could occur if the solid consisted of two proteins present in proportion to their solubility. It corresponds to the case in which a mixture of two substances has the same melting point as one pure substance and is an improbable occurrence. This possibility can be ruled out, however, by repeating the determination in another solvent, since the relative solubility of two substances is, in general, different in different solvents. A racemic mixture of *d*- and *l*-isomers, however, could not be recognized by this method, although if either isomer was present in excess its presence would be detected.

A curve such as curve A in figure 1 with a slope of zero would also be obtained if the solid were a solid solution of two or more proteins having exactly the same solubility. This possibility can also be ruled out by repeating the determination in another solvent.

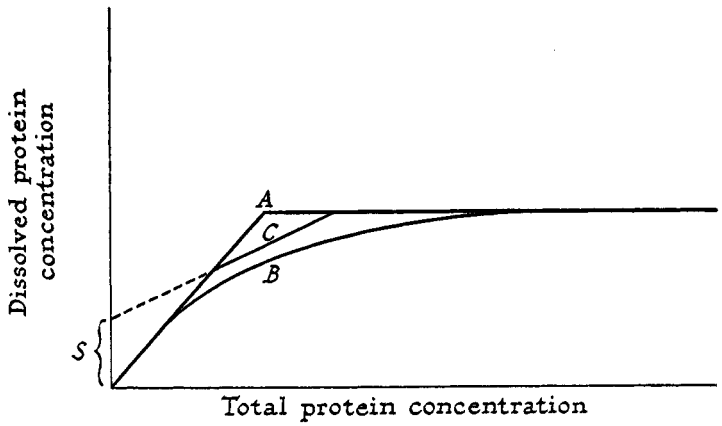

FIG. 1. General types of solubility curves. Curve A, results from a single protein component; curve B, results from a solid solution of two or more protein components; curve C, results from a mixture of two protein components.

(*2*) A rounded curve like curve B of figure 1, in which there is only one solid phase but of varying composition (solid solution of two or more components). This system has at least one degree of freedom.

(*3*) A curve like curve C of figure 1 (a mixture), made up of straight lines in which each break in the curve represents the appearance of a new solid phase.

It may be seen from examination of these three curves that in each case the solubility becomes constant when the excess of solid is great enough. Therefore, it is not sufficient to determine the solubility in the presence of two or more different amounts of solid if these amounts are high relative to the concentration of the dissolved material. One must examine most carefully that region of the curve where the solid phase first appears. If the solubility is the same at this point as in the presence of a large excess of solid, then the preparation has a *constant solubility*.

When it is necessary to distinguish between a mixture and a solid solution with greater certainty than from merely the shape of the curve, this can be

done by testing the prediction that the first solid phase that appears along the solubility curve is a pure component if the components are present as a mixture. Thus, if the solid that first appears along the curve is separated off and found to have a constant solubility, the original preparation is a mixture of two or more components. If the solid that is separated off produces a rounded curve similar to the original curve, then the original material is a solid solution. In either case the maximum solubility will be less than that of the original, but the main point is that, in the case of a mixture, the first solid to appear is a single pure component, while in a solid solution it is not.

In the event that one is dealing with a mixture of proteins, certain additional information is obtainable from the solubility diagram (assuming ideal solutions). Thus, if the line obtained when the first solid appears, indicated in figure 1 as C, is extrapolated back, the value at the intersection of this extrapolation with the ordinate is the solubility of the pure component that is appearing as a solid phase. One minus the slope of that line represents the fraction of the pure component in the original suspension. This follows from certain graphic and algebraic considerations which are derived and discussed in the previous papers of Northrop and Kunitz (7, 12) and are not a part of the phase rule.[2]

It may be seen from the preceding discussion that if one obtains a curve of constant solubility similar to curve A in figure 1, the material under examination is either a single component or one of the rare cases of two or more components that has a constant solubility. By determining the solubility in as widely different solvents as the material will permit,—in the case of proteins variations in pH being of particular importance,—one may decide between the possibilities. If, in the different solvents and after all types of fractionation, the material persists as a preparation of constant solubility over the entire range of suspension concentrations, one is forced to conclude that the material is a single pure component or a theoretically possible, though very unlikely, case of a combination of proteins which have the same relative solubility in all solvents used.

III. USE OF THE SOLUBILITY CURVE IN THE PREPARATION OF PROTEINS

Theoretical

One of the most powerful uses that can be made of the solubility curve lies in the development of methods of separating out single protein components. As has already been discussed, in the case of a mixture the first solid to appear is a pure component, so that one has at hand a simple scheme for its preparation.

When the solubility curve is that of a solid solution (curve B of figure 1), the predictions with regard to purifying the components with the same solvent are as follows, assuming that Raoult's law governs at least approximately: (*1*) The greater the difference between the point at which the solid first appears along the curve and the point where the solubility does not change with amount

[2] Kunitz and Northrop (8) have demonstrated with known crystalline proteins how the determined solubility and composition of mixtures and solid solutions agree with the predicted values. They have a case of two proteins which behave as a mixture at one pH and at only 1.5 pH units away form a solid solution.

of solid, the greater the difference in the solubility of the pure components and the more suitable the solvent is for separation of the components. (*2*) The solid phase will be richer than the starting material in the less soluble components, especially when most of the total material is dissolved.[3] (*3*) The solution phase will be richer in the more soluble components, particularly when most of the protein is present as the solid phase. (*4*) The maximum separation of components possible for a single treatment will be C times the composition of the starting material, where C is the ratio of the solubilities of the pure components and the composition is expressed in mole fractions. Treating the first fraction a second time will yield a maximum separation of C^2 times the composition of the original preparation, etc. If enough material is available and the difference in solubility is appreciable in the solvent used, relatively pure fractions should be obtainable by repeating the procedure several times. In several instances this has been an experimental fact, as is adequately demonstrated below.

Purification of pepsin and diphtheria antitoxin

Crystalline pepsin prepared by the method originally described usually has a solubility curve characteristic of a solid solution, as may be seen in figure 2. This preparation had been twice fractionally crystallized and had been found to be strictly homogeneous in the Tiselius electrophoresis cell (4). Similar preparations were homogeneous in the ultracentrifuge (Philpot (13)). Recently a procedure was designed (Herriott, Desreux, and Northrop (3)) for the purification of pepsin on the basis of its being a solid solution of proteins. Starting with the crude commercial pepsin protein, the soluble fraction in the presence of excess solid was saved. The results of the important steps are shown as solubility diagrams in figure 3. The solubility curve of the original crude pepsin is indicated in curve B of figure 3. The solid phase began appearing very early in the curve, as may be seen, and the solubility did not become constant until the suspension concentration was many times greater than that shown on the graph. This is typical of a solid solution curve. The material was then extracted with a volume of solvent in which only a third of the total protein dissolved but, as may be seen from the curve marked "soluble fraction" in figure 3, that portion that was soluble in the extracting solvent contained very little of the less soluble material. The residue from the extraction, on the other hand, was rich in the less soluble component, as is indicated by the solubility curve marked "insoluble fraction." When the soluble fraction was precipitated and reëxtracted in the same solvent as used before, the amount dissolved was considerably greater, as was to be expected. When the soluble portion after this second extraction was crystallized it had a constant solubility, as seen from the solubility curve A of figure 3 and it remained constant on further fractionation.

The case of diphtheria antitoxin (Northrop (11)) is similar to that of pepsin

[3] In the paper by Northrop and Kunitz (12) line 8 of the text on page 787 should read, "the first precipitate that appears will be $\frac{C}{F}$," $\left(\text{not } \frac{1}{FC}\right)$.

but is worth discussing in order to emphasize that purification may be accomplished and examined by the solubility technique.

Figure 4 contains the solubility curves of the fractions of diphtheria antitoxin protein at various stages in the purification. The entire protein fraction, after precipitation of the antitoxin with toxin and then removal of the toxin, had a

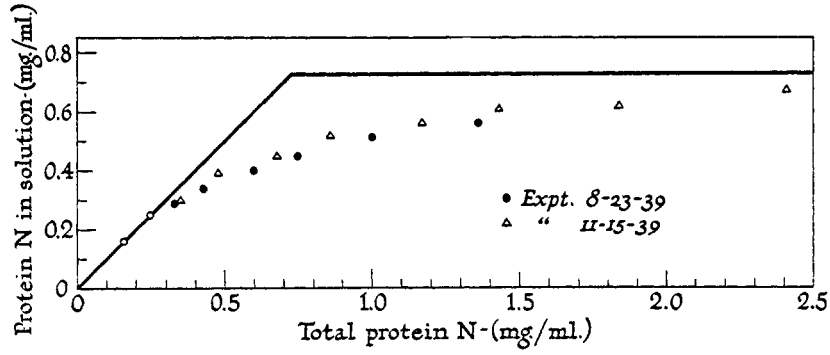

FIG. 2. Solubility curve of twice-crystallized pepsin

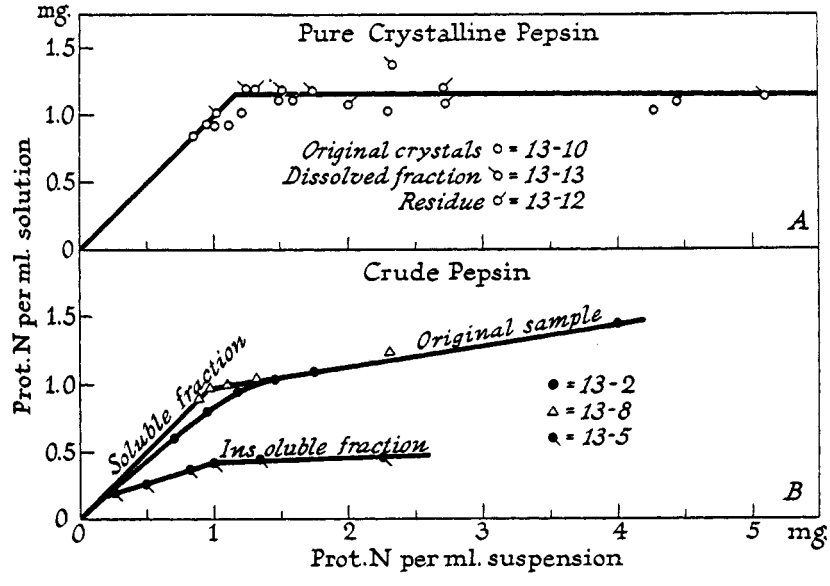

FIG. 3. Solubility curves of pepsin at various stages in the purification

solubility curve similar to the top curve, A, of figure 4. The fraction of this preparation which was insoluble between 0.1–0.35 saturated ammonium sulfate is shown in curve A. It was a very insoluble material, for the coördinates are on a 10 to 1 scale. The fraction precipitated between 0.35–0.65 saturated ammonium sulfate is shown in curve B. Here the coördinates have the same scale,

and it is quite clear that this fraction was more soluble but far from homogeneous. When this last fraction was refractionated by saving only that part which was soluble in 0.50 ammonium sulfate but insoluble in 0.65, the curve C was obtained. Upon repeating this fractionation between 0.5–0.65 saturation a material of constant solubility was obtained, as seen by curve D.

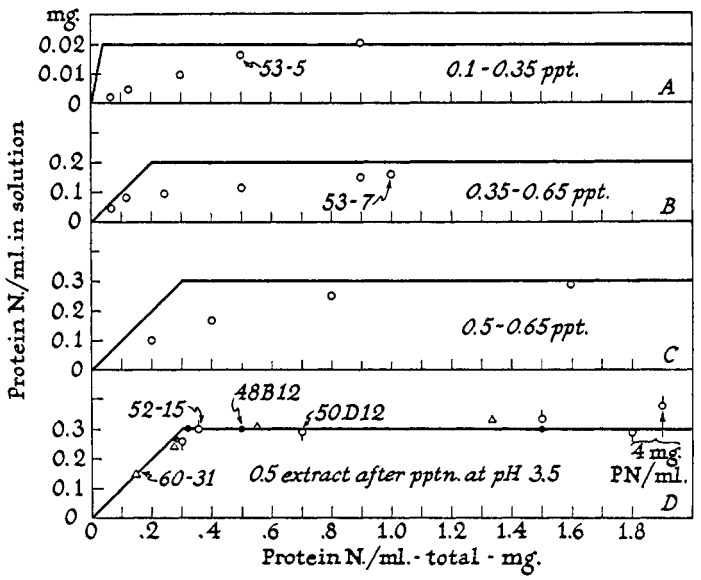

FIG. 4. Solubility curves of diphtheria antitoxin (Northrop) at various stages in the purification.

IV. CONDITIONS AND CERTAIN DETAILS OF SOLUBILITY TECHNIQUES

Solvent.—Although there are few definite rules that apply to all proteins, certain general aspects of the problem must be kept in mind. Thus, it is important when choosing the conditions to arrange them so as to obtain as near the maximum stability of the protein as possible. A solvent of such a nature (usually a salt solution) should be chosen that the amount of dissolved protein will be such as to be estimated precisely and yet not such that too large a proportion of the protein in a concentrated suspension is in solution.

Amorphous and crystalline forms.—With some proteins it is possible to perform the solubility experiment on either the crystalline or the amorphous form. There are certain advantages to each but, other things being equal, a solubility experiment on the crystals is open to less objection. On the other hand, solubility curves of amorphous forms are often extremely convenient and useful, especially for proteins that do not crystallize.

Equilibrium from both sides.—In addition to the choice between solubility curves for amorphous and for crystalline forms, one may choose to approach the equilibrium point from either the supersaturated or the undersaturated side.

Both approaches should be made when the amount of material and other considerations permit, and it is rather important that at least one or two points be approached from both sides to insure one that a true equilibrium value is actually being measured. Approach from the supersaturated side of the equilibrium can be conveniently carried out with amorphous preparations, using a split solvent. This is done by dissolving the solid phase or suspension in the water or buffer part of the solvent and precipitating with the concentrated salt part of the solvent. With crystals, supersaturation is easily obtained by varying the temperature so that some of the crystals dissolve and, on returning to the original temperature, crystallization takes place, but slowly.

Approaching from the undersaturated side of the equilibrium is usually accomplished for both amorphous and crystalline forms by stirring the solid phase with the complete solvent. The time required to attain equilibrium will depend upon a number of factors, paramount among which are the quantity of solid, the size of the solid particles, and the rate of stirring. The time will therefore vary with conditions and must in each case be determined experimentally.

Preliminary equilibration.—In bringing the solid into equilibrium with the solvent before carrying out the solubility-curve experiment, much time can be saved by first washing the solid on a Büchner funnel with small aliquots of the complete solvent or by dialysis in cellophane bags against the solvent. The solid is then stirred or precipitated with successive aliquots of the solvent until several successive aliquots contain the same amount of dissolved protein. If one does not obtain a constant amount dissolved after a few washings it may be due to a change in the protein composition, owing to removal of a relatively large amount of the more soluble components in the washing, and not because salt equilibrium had not been obtained. When several successive aliquots do have the same amount of dissolved protein, it may be presumed that equilibrium exists between the solid and the solvent.

Distribution.—Solvent equilibrium having been obtained, the suspension is then distributed in varying amounts in a number of test tubes, followed by the addition of the solvent. The amount of suspension in each tube is adjusted, so that at equilibrium a range from complete solution of the protein to a large excess of solid exists in the series of tubes. For reasons already discussed in the earlier sections of this paper, it is more important to have most of the tubes in the region of a small excess of solid than of a great excess of solid.

Final equilibration.—In stirring the protein solutions or suspensions, foaming should be avoided as this may bring about considerable denaturation. It has been found that glass beads or marbles in test tubes that are completely filled, stoppered, and then rotated, suffice to stir the suspensions. The time of stirring necessary to attain equilibrium must be determined experimentally.

Separation of solid and solution phases.—After equilibrium has been attained, the solid is separated from the solution by centrifugation or filtration. Care must be taken in the case of "amorphous" solubility curves that are approached from the supersaturated side that equilibrium is attained before separation of the solid phase. It has usually been assumed that one could not have a pro-

tein solution supersaturated with respect to the amorphous form, but certain experiments with pepsin seem to indicate that supersaturation is possible. The filtrates should remain clear for several hours unless crystallization is taking place, which can be determined by microscopic examination. In the event the preparation tends to crystallize in the complete solvent, one may depress this tendency by allowing the solution of protein to stand in the dissolving half of the split solvent for several hours before adding the precipitating half of the solvent. This allows the last traces of crystals to dissolve, which would otherwise act as seeding crystals. The supernatant or filtrate must, therefore, be crystal-clear. It has been found that filtration through Whatman's No. 42 filter paper gives uniformly the best results. If the amount of dissolved protein is under 0.5 mg. per milliliter the filter paper takes up some of the protein, so that the first portion of filtrate may be low in dissolved protein and is therefore usually discarded.

Estimation of dissolved protein.—The amount of dissolved protein may be determined by any of a number of methods, such as Kjeldahl nitrogen, turbidity, biological activity, tyrosine color value, or any other easy and precise method. In the event that one is examining an unidentified substance having a specific property such as a biological activity, one can learn a great deal about the class of substances in which the unknown belongs by making as many different tests on the solubility solutions as possible. For example, one will find that the protein, carbohydrate, nucleic acid, or other properties do or do not run parallel to the specific property that is being studied.

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