The Significance of the Variable Solvent and Specific Property Solubility Tests in Protein Chemistry

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ABSTRACT An attempt has been made to review briefly the present status of the application of solubility measurements to the field of protein chemistry and in particular the quantitative significance of the measurable parameters obtained from the variable solvent solubility test has been evaluated.

Solubility methods have played a classic role (Northrop, Kunitz, and Herriott, 1948) in the work which led to the final recognition that enzymes were proteins, and they still constitute one of the solidly based methods for studying protein heterogeneity and for separating individual proteins.

Gibbs' deduction of the phase rule was completely general in that it applied to any number of phases, components, and degrees of freedom. In applying it to the study of protein solutions we are restricted to equilibria between solid and liquid phases. Detailed information can be obtained by measuring the protein in solution at equilibrium as a function of pH, temperature, and salt concentration. In practice, it is convenient to carry out more limited studies when the protein solubility is determined as a function of any one of the experimental variables while the others are held constant. Such experiments are referred to as solubility tests.

The work described in “Crystalline Enzymes” (1948) centered around the study of the solubility of highly purified proteins in a solvent of constant composition at constant pH and temperature. As solid phase is added, protein goes into solution until the solvent becomes saturated with it after which no more of that protein dissolves, and the curve relating protein in solution to protein added shows an abrupt discontinuity. The occurrence of more than one protein is shown by additional saturation points. This method has been referred to as the “constant solvent solubility test” (Falconer and Taylor,
1946), and its relationship to the variable solvent and specific property solubility tests defined (Falconer and Taylor, 1947).

The alternative method of experimental analysis in which total protein, temperature, and pH are constant and the precipitating salt concentration is varied has also been extensively used (Derrien, 1958), and has been termed the "variable solvent solubility test" (Falconer and Taylor, 1946). In order to define the limitations and uses of solubility methods the types of information they can yield may be summarized as follows:—

1. **The Determination of the Number of Proteins Present in Analytically Detectable Amounts**

   Usually only estimates of a lower limit to this number can be given. In complex mixtures such as cell extracts or blood plasma (Roche and Derrien, 1947) the majority of proteins will not be detectable as inflections by nitrogen analyses, but a few highly active enzymes may be estimated by individual methods; some information on the precipitation of these can be obtained by using the specific property test type of analysis.

2. **The Estimation of the Amounts of Each Protein**

   By plotting the first derivative of the variable solvent test with the sign changed, Roche and Derrien (1947) have attempted to form a relative idea of these values. The quantitative significance of these curves will be discussed later.

3. **The Quantitative Determination of the Steps Necessary for the Separation of Selected Individual Proteins**

   Methods are available for determining the experimental variables involved in salt fractionation when the protein to be separated has a specific measurable activity (Falconer and Taylor, 1946, 1947; Falconer et al., 1953). These methods have been applied to catalase by Brown (1952), to pancreatic trypsin inhibitor by Green and Work (1953), and to diphtheria toxin by Norlin (1955).

4. **Criteria of Protein Purity**

   These have been discussed in detail for the constant solvent test (Northrop et al., 1948), for the variable solvent test (Falconer and Taylor, 1946), and for the specific property test (Falconer and Taylor, 1946, 1947).
5. The Characterization of Separate Proteins

Solubility tests can provide values for the $\beta$ and $k$ constants in Cohn's equation and values for the activity per milligram in the case of active proteins.

6. The Variable Solvent Solubility Test

If a precipitating salt is added to a solution of pure protein, the concentration of the protein in solution will remain merely the content; i.e., indeterminate, until solid phase appears after which it becomes the solubility (Fig. 1). In variable solvent tests the presence of additional proteins is indicated by inflections corresponding to the beginning of each additional protein precipitate. In complex mixtures of proteins the number of such inflections can be quite large and unless there are many accurately determined points, the inflections do not show.

**Significance of Inflections**

The significance of the inflections found in variable solvent tests is important and the possibility that an inflection may not represent the beginning of pre-
The precipitation of another protein has been raised by Itano (1953, 1956). Smithies (1954) and Ogston and Tombs (1956) have provided experimental evidence that such inflections can occur when a change in structure of the solid phase occurs at a particular ionic strength. The few inflections that have been authenticated as arising from this cause have been in crystalline solid phases. There does not seem to be any detailed work available on the occurrence of inflections due to structural changes when the solid phase is amorphous.

The objections raised by Itano to attaching significance to inflections related to the possible heterogeneity of adult human hemoglobin. These objections have been discussed by Derrien (1958) and by Allison and Tombs (1957). Before an inflection can be regarded as indicating the presence of another protein, experimental verification is desirable and as Ogsten and Tombs have pointed out repetition of the variable solvent test at a different total protein concentration is a very useful way of doing this. If the total protein concentration is raised, inflections due to a solid phase alteration at a particular ionic strength will remain at the same salt strength whereas inflections due to the start of precipitation of a protein will appear at a lower value of the salt strength. There is some evidence that variable solvent solubility tests are best done under conditions where the solid phase is amorphous. Crystalline solid phases show greater tendencies to form solid solutions and equilibration times can be much longer (Smithies, 1954).

The evidence that the majority of inflections seen in variable solvent solubility tests, where the solid phase is amorphous, are caused by individual proteins has been summarized by Derrien (1958).

**Analysis of Variable Solvent Solubility Tests**

The relationship of protein solubility to salt strength is exponential and can be described by Cohn's equation

\[ \log S = \beta - kI \]

where \( \beta \) is the intercept on the solubility axis and \( k \) is the slope of the curve. Data from variable solvent tests on highly purified preparations containing only a few proteins can often be analyzed graphically and the component curves fitted to Cohn's equation.

(a) *Method of Derivative Curves*

This method of analysis was introduced by Derrien (1947) and Derrien, Laurent, and Reynaud (1951) and consists in plotting the slope of the line
joining two successive points against the precipitating salt strength. Derrien calls such curves derivative curves and they are an approximation to a first differential of the variable solvent curve with the sign changed. In order to develop a concept of what derivative curves may mean it is first necessary to consider the properties of the first differential of the variable solvent test.

Fig. 1, I represents a variable solubility test for a pure protein and II that for a mixture of two proteins. Curves III and IV are the first differentials of I and II with the sign changed to enable them to be plotted upright.

The problem to be solved concerns the calculation of the amount of each protein present from measurements available from the variable solvent test and its first differential. This can be investigated in cases of increasing complexity.

Cohn's equation for curve I can be written

\[ S = e^{(\theta - k)} \]

\[ \frac{dS}{d\theta} = ke^{(\theta - k)} \quad (3) \]

\[ \frac{dS}{d\theta} = kS \quad (4) \]

with the negative sign in equations (3) and (4) omitted because of the upright plot of the differential mentioned above. In Fig. 1

\[ \frac{dS}{d\theta} = AB = kS \quad (5) \]

\[ S = \frac{AB}{k} \quad (6) \]

and since \( S \) is the solubility at the inflection point it is also the content. Hence, if we know \( k \) the protein present can be estimated from the data in the first differential. In this case \( k \) is best obtained by plotting \( \log S \) against the salt strength and obtaining it from the slope of the straight line. In curves II and IV, although there are several possible procedures, that which can be applied to more complex cases will be used.

As before \( CD / k' \) gives the amount of the first protein present where \( k' \) is its slope constant.

From equation (5)

\[ CD = k'S' \]

\[ FG = k'S'' \quad (8) \]
where \( S'_1 \) and \( S'_2 \) are its solubility at salt strength \( I_1 \) and \( I_2 \).

\[
\therefore \quad k' = \frac{CD - FG}{S'_1 - S'_2} = \frac{CD - FG}{KI_1 - LI_2}
\]

hence \( k' \) can be estimated.

The abrupt start of the salting out of the second protein causes the curve \( CF \) to rise vertically from \( F \) to \( E \). Hence \( FE \) is the component of the slope of \( LN \) at \( L \) due to the appearance of the second protein.

\[
\frac{dS'_2}{dI} = k''S''_2
\]

or

\[
FE = k''S''_2
\]

and

\[
S''_2 = \frac{FE}{k''}
\]

again \( S''_2 \) is the amount of the second protein present and if we can find \( k'' \) it can be estimated.

As before

From (12)

\[
k'' = \frac{FE}{S''_2} \quad \text{or} \quad \frac{FE}{LI_2 - LM}
\]

but

\[
LM = S'_1 = \frac{FG}{k'}
\]

\[
\therefore \quad k'' = \frac{FE}{LI_2 - \frac{FG}{k'}}
\]

this gives \( k'' \) and the amount of the second protein present. It can be shown that by the successive determination of the slope constants of the proteins as they come out of solution, more complex curves can be analyzed. Essentially the increment \( FE \) in the first differential is determined, and divided by the slope constant of the protein to give its amount. However, the successive determination of the \( k \) constants leads to cumulative errors and it would seem advisable to have a method for the direct estimation of the slope constant of a protein without using values for prior constants. This can be approached from a different standpoint.

Let us examine the case of the \( ith \) protein in a complex mixture. Fig. 2, I is a portion of a variable solvent curve, \( M \) is the \( ith \) reflection, and II is the first differential of I. Here \( RQ \) is the sudden increment in the differential due
to the appearance of the $i$th protein and if $k_i$ is its slope constant, then its solubility at the inflection point and hence the amount of it present is given by

$$S = \frac{RQ}{k_i}$$  \hspace{1cm} (16)

The problem is to find $k_i$ from the data in Fig. 2.

As before, $RS$ is the slope of $SM$ at $M$ and $QR$ the slope of $MU$ at $M$. Let $P_b$ and $P_a$ be the protein in solution just before and after $M$. Then,

$$\frac{dP_b}{df} = RS$$  \hspace{1cm} (17)

and

$$\frac{dP_a}{df} = QR$$  \hspace{1cm} (18)
If we regard the proteins salting out just before $M$ as having a composite slope constant, $k_b$, and the $ith$ protein as having a slope constant, $k_i$, we can write

$$\frac{d^2P_b}{dT^2} = k_bRS \quad (19)$$

and

$$\frac{d^2P_i}{dT^2} = k_bRS + k_iQR \quad (20)$$

then

$$\frac{d^2P_o}{dT^2} - \frac{d^2P_b}{dT^2} = k_iQR \quad (21)$$

putting this value for $k_i$ into equation (16), we get

$$S_i = \frac{(QR)^2}{\frac{dP_o}{dT^2} - \frac{dP_b}{dT^2}} \quad (22)$$

The amount of the $ith$ protein therefore can be estimated by the square of $QR$ divided by the difference between the slopes of the tangents to the solid lines at $Q$ and $R$ respectively.

**THE SPECIFIC PROPERTY SOLUBILITY TEST**

The difficulty in purifying a protein by salt fractionation is largely due to the overlapping of the precipitation curves of the impurities onto that of the protein to be purified. The determination of the pH, temperature, total protein, and salt concentrations for minimal overlap of these impurities, gives the optimum conditions for separation.

The specific property solubility test as its name suggests is designed to analyze the information from variable solvent tests in which a protein or proteins have some accurately measurable specific and distinguishing characteristic such as metal content, prosthetic group, or the activity in the case of an enzyme. In a variable solvent solubility test, both protein in solution and the specific activities are determined and plotted. The inflection in the total protein curve which corresponds to the commencement of salting out of the active protein can then be identified. The use of the method, however, becomes clear when the values for total protein precipitation are plotted against precipitation of activity at the same salt concentrations (Falconer et al., 1953), and the method can be used to determine, quantitatively, the optimal conditions of pH, temperature, salt concentration, and total protein concentration for fractionation. Moreover, any proteins the precipitation of which can be determined from their specific activities can be used in the analysis of the
variable solvent test in that they provide some data on those proteins with overlapping precipitation ranges.

METHODOLOGY

From the foregoing analysis the need for many accurately determined points in the variable solvent test is obvious, and in fact this need constitutes one of the serious difficulties of the method. Since, after the mixing of protein and precipitating salt, several hours are needed for equilibration, it is unlikely that any technique, in which protein in solution is continuously determined as a function of gradually added precipitating agent, can be developed because of the very long time it would take to carry out the experiment.

The large number of points needed suggests mechanization, and here power-driven metering pumps might be used with advantage to deliver precise aliquots of protein solution to the equilibration flasks (Derrien, 1952). Moreover, the precipitating salt solutions might be similarly delivered from a reservoir in which the salt concentration was being slowly and accurately increased under precisely controlled conditions. High speed centrifugation at constant temperature of solutions after equilibration would seem to be preferable to filtration for removing the solid phase.

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REFERENCES


