STUDIES ON PROTEINS BY MEANS OF SALTING-OUT CURVES

I. METHOD OF ESTABLISHMENT OF SALTING-OUT CURVES OF PROTEINS

by

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INTRODUCTION

The physico-chemical differentiation of the constituents of a protein mixture can be achieved by methods which make use of their different constant of sedimentation (SVEDBERG), their different mobility in an electric field (TISELIUS) or their different solubility.

The foundation for the application of the latter property to the study of proteins has been laid by the work of CHICK AND MARTIN, SØRENSEN, COHN *et al.*, and NORTHROP AND KUNITZ. The problem can be approached along different lines, which have been classified by FALCONER AND TAYLOR as: variable solvent solubility test, constant solvent solubility test, and specific property solubility test[1].

The "method of salting-out curves", which is a variable solvent solubility test, has been extensively studied by the present author since 1938. In collaboration with J. ROCHE a number of chromoproteids has been investigated with the aid of this method (hemoglobins of blood and muscle[2,3], hemerythrins[4], hemocyanins[5], cytochromes[6]). It has served as a guide in their purification, has demonstrated their specific solubility, and has shown that their heterogeneity is either due to their composition of a mixture of different pigments (foetal and adult hemoglobin[7]), or to their paucidispersion (hemocyanins[8,9]).

It has made possible the preparation of pure thyroglobulin from thyroid extracts[10], and the study of its chemical and physico-chemical properties[11]. The method has also proved to be of value for the study of the proteins of lens[12], of milk[13] and of blood serum[14,15,16], not only to demonstrate their heterogeneity, but also to control the purification of protein fractions isolated from these media. A first attempt to correlate the protein constituents of blood serum as revealed by salting-out curves with those found by electrophoretic analysis has been published[17].

The purpose of this paper is not to advance new arguments to justify our method of analyzing proteins by means of salting-out curves, but to describe in detail for those interested in this kind of work the procedure of salting-out as it is practised at present by the author and his collaborators. It may also be regarded as an introduction to a series of investigations on the proteins of blood serum which will shortly be published.

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PRINCIPLES OF THE PROCEDURE FOR ESTABLISHING SALTING-OUT CURVES AT A CONSTANT pH, TEMPERATURE AND PROTEIN CONCENTRATION

Without digressing on the historical and theoretical aspects of the salting-out of proteins, which have already been treated at length by other authors[18], the following facts may be briefly mentioned: When a protein mixture is progressively precipitated in a series of media with increasing salt concentration, an equilibrium will be attained between precipitated and dissolved proteins in each medium after a certain time. The amount of protein remaining in solution will depend upon the concentration and the nature of the salt, the concentration and the nature of the proteins, the pH and the temperature of the medium.

So if one proposes to study the variation of the solubility of a protein as a function of the salt concentration only, then protein concentration, pH and temperature must be kept constant. Under these conditions the decrease of solubility S of a pure, monodisperse protein is a logarithmic function of the salt concentration C, as has beenest ablished by E. J. COHN in 1925, who introduced the following expression:

$$\log S = \beta' - k_s' C,$$

in which the salting-out constant k_s' is a characteristic property of the protein in question when employing a certain salt and constant β' registrates the influence of pH and temperature on log S. So the variation of S is a continuous function of C, expressed by an exponential curve (see Fig. 3).

For a mixture of proteins, however, be it natural (*e.g.* blood serum, Figs. 1 and 2) or artificial[19], or for a paucidisperse protein (*e.g.* hemocyanin of Helix pomatia[9]), $S = f(C)$ has the graphical appearance of a discontinuous curve, composed of a number of segments separated by breaks. Each break indicates the appearance of a new solid phase with different solubility.

The principle of the experimental procedure is simple. A number of mixtures is prepared, each with the same pH, temperature and protein concentration, but with increasing salt concentration. After the time required to attain an equilibrium between precipitated and dissolved proteins, the amount of protein remaining in solution is determined. With the aid of these data the curve $S = f(C)$ or the derived curve $-\frac{\Delta S}{\Delta C} \cdot i = f'(C)$ is constructed. These salting-out curves prove to be very sensitive indicators of the heterogeneity of proteins.

However, in order to obtain reproducible curves for a given protein mixture, and to be able to compare the curves of different proteins, the execution requires the highest degree of accuracy, under well-chosen and standardized experimental conditions, as will be described below.

EXPERIMENTAL

1. *Material*

To establish a salting-out curve the following material is required:

a) a number of 50 ml Pyrex glass flasks;
b) a number of Kjeldahl destruction flasks (Pyrex glass);
c) a destillation apparatus according to Parnas and Wagner for micro-Kjeldahl analysis;

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d) 2 burettes, accurately weighed out, content 10 ml, divided into 0.02 ml, length of graduated part: 40–50 cm;
e) a number of highly accurate pipettes of 1, 2, 3, 4, 5 and 10 ml;
f) a water bath that can be regulated to $\pm$ 0.05° C;
g) an apparatus for measuring the hydrogen ion concentration with a glass electrode;
h) reagent tubes and funnels;
i) filter paper disks, diameter 9 cm, analytical grade, that hold back fine precipitates yet do not filter too slowly (a convenient paper is, for instance, Schleicher and Schüll 589[5]);
j) Reagents:
A concentrated stock solution of the chosen salt.
Glass-distilled water.
Concentrated sulfuric acid.
Catalyst for Kjeldahl destruction according to Dumazert and Marcelet[20].
40% sodium hydroxide.
A stock solution of exactly $N/7$ sulphuric acid.
A stock solution of exactly $N/7$ sodium hydroxide.
Before use, the latter two solutions are diluted ten times to give $N/70$ solutions, adding some methyl red as indicator.

2. *General procedure*

Apart from the consideration of the experimental conditions obtaining in each special case (choice of salt, of range of salt concentration, etc.) which will be discussed later, the following steps are always taken when establishing a salting-out curve:

1. The protein concentration of the solution to be analyzed is determined (by micro-Kjeldahl method or with a refractometer) to ascertain the degree of dilution required to obtain, in the experimental mixture of water, salt solution and protein solution, a total protein nitrogen concentration of 0.5–1 mg per ml. For blood serum, which we shall take as example, this usually amounts to a ten-fold dilution. If necessary, the pH of the protein solution is adjusted to the value obtaining in the salt solution.

2. A series of 50 ml flasks is numbered and with the aid of the two 10 ml burettes n ml of concentrated salt solution (n may vary from 0 to 9 ml) and 9-n ml of glass-distilled water are measured with great care into each flask, in such a manner that a series of mixtures of increasing salt concentration is obtained. After mixing the two fluids, exactly 1 ml of protein solution is introduced into each flask. The flasks are stoppered and the contents mixed with care. They are placed in the thermostat for the time required to establish a definite equilibrium between the proteins in solution and those that have been precipitated. To obtain the best results, one operator should not prepare more than 20 to 25 mixtures at a time. Protein solutions with a wide range of precipitation will thus have to be analyzed in more than one experimental series. For control purposes the next series should commence with a repetition of the last four points of the preceding series (see Figs. 1 and 2 in which the first series of experimental data are represented by dots and the second by crosses).

3. After taking the flasks from the thermostat, the contents are immediately filtered, the clear filtrates being collected in a series of reagent tubes. With the aid of the high precision pipettes an aliquot of each filtrate is measured into a Kjeldahl destruction

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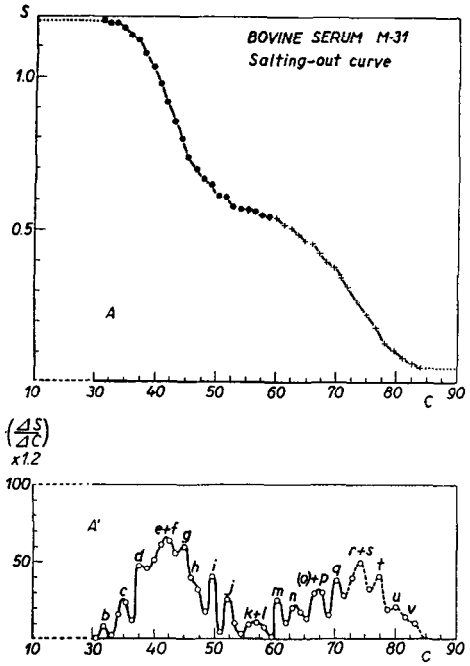

Fig. 1. A: salting out curve S = f(C) of bovine serum proteins. Dilution of serum = 1/10; pH = 6.5, temperature = 24° C.

C = salt concentration, expressed in percent by volume of the stock salt solution (equimolecular mixture of 3.5 *M* mono- and di-potassium phosphate). S = nitrogen in mg per ml filtrate.

A': Derived curve $-\left(\frac{\Delta S}{\Delta C}\right) \cdot 1.2 = f'(C)$; ΔS = protein nitrogen (expressed in γ) precipitated for each increment ΔC of the salt concentration. ΔC = 1.2 from C = 31.2 to C = 72.0 and 1.5 from C = 72.0 to C = 84.0.

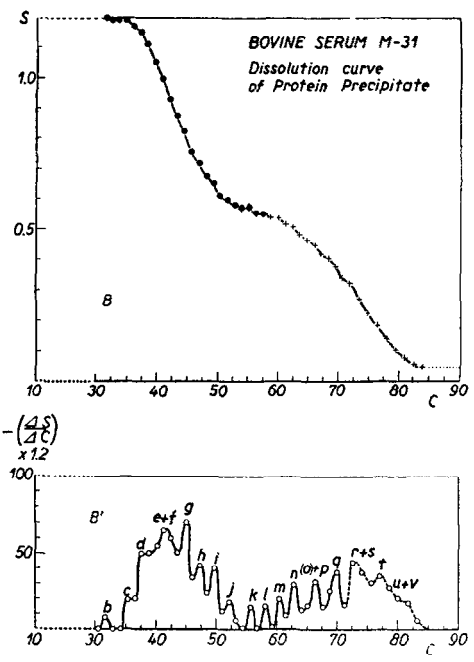

Fig. 2. B: Precipitate dissolution curve S = f (C) of bovine serum proteins. Dilution of serum = 1/10; pH = 6.5, temperature = 24° C.

C = salt concentration expressed in percent by volume of the stock salt solution (equimolecular mixture of 3.5 *M* mono- and di-potassium phosphate). S = nitrogen in mg per ml filtrate.

B': Derived curve $-\left(\frac{\Delta S}{\Delta C}\right) \cdot 1.2 = f'(C)$; ΔS = protein nitrogen (expressed in γ) dissolved for each increment ΔC of the salt concentration. ΔC = 1.2 from C = 31.2 to C = 72.0 and 1.5 from C = 72.0 to C = 84.0.

flask, the volume depending upon the estimated nitrogen content of the filtrate*. About 50 mg of catalyst and 3 ml of concentrated sulphuric acid are added to each flask, and the contents are heated for 1–1½ hours, during which time destruction is usually complete. In each series of Kjeldahl determinations a "blank" is included. For this blank a flask is prepared containing sulphuric acid and catalyst only, and treated in the same manner as the other samples. The stock salt solution is of course tested for ammonia impurities.

4. After quantitatieve transfer of the content of a Kjeldahl flask into the Parmas and Wagner apparatus, 15 ml of 40% sodium hydroxide are added and the ammonia formed is distilled and received into 10 ml of *N*/70 sulphuric acid. The excess acid is titrated with *N*/70 sodium hydroxide, using a 10 ml burette, and observing the precautions indicated by NICLOUX[21], which will be discussed below.

5. The nitrogen content in mg per ml of filtrate is calculated from the amount of *N*/70 sulphuric acid neutralized by the ammonia, taking into account the "blank" value (multiply by 0.2, divide by number of ml of filtrate used for the determination). The

* We presume in this example that the salt used is not an ammonium salt. This case will be discussed later.

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results can be expressed graphically by plotting the nitrogen concentration of each filtrate against its salt concentration, and the salting-out curve is obtained.

3. *Observations regarding the procedure outlined above*

In order to reduce the experimental error as much as possible (down to about $\pm$ 0.5%), which is imperative if dependable and reproducible results are to be obtained, certain precautions must be observed during the operations.

1. All volumes must be measured with great precision. Beforehand the time must be established, required to measure exactly the desired volume from the burette, which means that they must be emptied very slowly, in view of the adhesion of the liquid to the wall. The concentrated salt solution, with its higher viscosity, must run more slowly than the water. In the same manner the time must be determined during which the pipettes should be allowed to drain, in order to obtain the exact volume.

2. The time required to establish an equilibrium between precipitated and dissolved proteins depends to some extent on the salt employed. In most cases at least twelve hours should be allowed to ensure a definite establishment of the equilibrium. In practice the flasks containing the protein and salt mixtures are placed overnight in the thermostat and filtered the next morning.

If, however, the protein precipitate is crystalline, as happens sometimes with hemoglobin, equilibrium is reached much more slowly (several weeks). In that case, a dissolution curve of the crystals should be established instead of a salting-out curve of the protein solution[22].

Incidentally, one is sure that equilibrium between the two phases has been attained when the curve obtained by salting-out a certain protein from its solution is identical with that found by dissolving the amorphous precipitate (see below, p. 638).

3. It is imperative that the protein precipitate be separated completely from the filtrate before analyzing the latter. Therefore the filter paper should be selected with great care. The first drops emerging from the filter are usually opaque, therefore they are allowed to run back into the mixture that is to be filtered. After this mixture has once passed the filter, however, the filtrate should be perfectly clear. It is then collected in a reagent tube. The mixture should not be poured upon the filter more than once before final filtration because subsequent passages cause serious changes in the equilibrium between precipitated and dissolved proteins.

4. The first distillation of series of Kjeldahl analyses always gives a value which is too low, owing to dissolution of ammonia in the vapour that condenses in the Parnas and Wagner apparatus. Once this water of condensation is saturated with ammonia, *i.e.* as long as the apparatus is hot, subsequent distillations give 100% recovery. Therefore a series is started by a "blind" distillation of an amount of an ammonium salt, corresponding to the amounts that are to be distilled later. Also, the distillation apparatus should be kept as compact as possible (avoid large bulbs with safety devices, use condenser with smooth inner wall, etc.).

Titration of the excess acid is performed with precision by following the indications of NICLOUX[21]: sodium hydroxide is added to the acid, after heating the latter to about 90° C, until the first change of colour is observed, the mixture is boiled for a few seconds to eliminate all traces of carbon dioxide, cooled in running tap water (methyl red does not give a clear-cut change of colour when hot) and the titration is terminated in the cold fluid until the methyl red has just turned lemon yellow.

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4. *Choice of the experimental conditions*

Before embarking upon the analysis of a protein mixture by salting out the following points should be considered:

a. *pH*

As the amount of protein precipitated by a certain salt concentration varies with the pH, the latter should have the same value throughout the whole salting-out curve. When a mixture of KH_2PO_4 and K_2HPO_4 is used as salt, with powerful buffering capacity, control is not necessary, but with other salts, *e.g.* ammonium sulphate, the pH of each mixture should be carefully controlled. In general the pH must be chosen within the "zone of stability" of the protein in question, to avoid denaturation of the latter.

b. *Temperature*

The temperature at which precipitation is carried out should be kept constant, as it also influences the degree of precipitation. Usually the temperature of the thermostat in which the mixtures are placed is chosen in the neighbourhood of room temperature, i.e. 22° or 24° C, to avoid a shift of the equilibrium during subsequent filtration. When sodium sulphate is employed, however, these operations must be carried out at a higher temperature (34–37°), owing to its limited solubility at room temperature.

c. *Choice of salt*

The choice of the salt will depend upon the experimental circumstances. In most instances a 3.5 *M* solution of equimolecular amounts of KH_2PO_4 and K_2HPO_4 is a very satisfactory agent owing to its strong buffering capacity (pH 6.5) and considerable salting-out power.* Saturated ammonium sulphate has a higher ionic strength and thus a more extensive salting-out capacity, and is very useful when a curve is to be established as a guide to later preparative work. It has the disadvantage that direct Kjeldahl analysis of the filtrates is impossible. The protein nitrogen must first be freed of ammonium ions according to the method of A. ROCHE AND MARQUET[13], which greatly complicates the procedure and reduces the precision of the analysis. It can only be done for curves composed by a limited number of points. This complication can be avoided by spectrophotometric analysis of the filtrates (absorption in visible light by coloured proteins, *e.g.* hemoglobin[2,3], or in ultra-violet (275 mμ), see[24]). Moreover, the use of ammonium sulphate demands an accurate control of pH.

Sodium sulphate can only be used to salt out the less soluble proteins, *e.g.* globulins, also it has the disadvantage that operations must be carried out at 34–37° instead of room temperature, and that the pH must be controlled. Its advantages are that the protein precipitate formed can easily be separated from the filtrate, being less fine than that formed by potassium phosphate or ammonium sulphate, that it can be added as a solid (for purposes of fractionation), and that a large amount can be easily removed from the filtrate by crystallization at 0° C.

d. *Range of salt concentrations*

The range of salt concentrations employed to precipitate a protein should extend a little beyond the zone of precipitation on each side, in order to be sure that the total

* Preparation of 3.5 *M* solution of equimolecular amounts of KH_2PO_4 and K_2HPO_4: Weigh out 476 g KH_2PO_4 and 609 g K_2HPO_4. Dissolve the KH_2PO_4 in about 1 l of glass-distilled water at 80–90°. Add the solution to the K_2HPO_4, add about 600 ml water, heat until dissolved. Make up the volume to 2 l, taking into account the retraction of volume upon mixing.

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salting-out curve is included in the experimental series. In the absence of data regarding the zone of precipitation, a few preliminary tests must be carried out to determine its limits. Thus, a complete salting-out curve begins (at low salt concentration) and ends (at high salt concentration) with a horizontal part. The ordinate values of these two levels correspond respectively to the total nitrogen and to the non-protein nitrogen content of the experimental media. In order to determine these values, when a stock salt-solution of high ionic strength is used (for instance a 3.5 *M* phosphate mixture), two points containing respectively 10% and 90% of this stock solution are always included in the experimental series. Usually the proteins remain dissolved in the first of these media and are completely salted out in the last.*

From these data the protein nitrogen can be calculated and the experimental results can be expressed in percentage of the total protein nitrogen.

e. *Increment of salt concentration*

As shown in a previous paper[16], the choice of the increment of salt concentration, determining the distance between successive points of the salting-out curve, largely determines the sensitivity of the method. In order to appreciate the complexity of the protein mixture under investigation, the increment should be less than 2% of the concentration of the stock salt solution. For routine investigations, an increment of 1.0 or 1.2% is usually employed, the smaller of the two being chosen for protein mixtures with a more narrow range of precipitation. Even then, only complex constituents are generally revealed, but they offer sufficient information without unduly lengthening the procedure. For more detailed investigations the distance between successive points can be reduced (research technique[16]), but limits are imposed by the experimental error. The influence of the increment of salt concentration on the sensitivity of the method will be discussed more extensively in the last section.

f. *The amount of protein and the concentration of the protein solution*

The amount of protein required depends upon the number of points necessary to establish the salting-out curve. This number in turn is determined by the range of salt concentrations in which the protein precipitates and the degree of sensitivity, *i.e.* the increment of salt concentration. Moreover, for a reasonably accurate determination, the total protein nitrogen content of the experimental media should not be less than 0.4 to 0.6 mg per ml for a protein mixture containing few components, or 0.8 to 1.2 mg per ml for a more complicated mixture. So the amount of protein solution required also depends upon its concentration.

Of blood serum, for instance, the proteins of which precipitate between 30 and 90% 3.5 *M* potassium phosphate, about 50 ml are required to establish a curve with an increment of 1.2%, in tenfold dilution, with 1 ml serum for each point. The total requirement can be reduced to about 35 ml by employing smaller volumes, *e.g.* 5 ml (0.5 ml serum + 4.5 ml dilute salt solution) for the first part of the curve. For the last part of the curve the experimental accuracy would be diminished by reducing the volume, owing to the very low nitrogen content of the filtrates and the small amount of filtrate available for analysis.

In cases of hyperproteinaemia it is desirable to dilute the serum to an approximately

* Some proteins of low molecular weight, such as dog myoglobin[3], remain partly dissolved even in saturated ammonium sulphate solution.

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normal protein concentration before salting out, in order to always conduct the operations under comparable conditions.

If the concentration of the protein solution is below 3–4%, then the curve cannot be established with a tenfold dilution, and consequently more of the protein solution must be used for each point.

If the supply of protein is limited, then obviously the sensitivity must be sacrificed to some extent by increasing the increment of salt concentration. So the choice of the latter will also sometimes depend upon the amount of protein available.

5. *The Order of Addition of the Constituents of the Salting-out Mixture*

Regarding the order in which salt solution, water and protein are added to form the salting-out mixture, two cases must be distinguished.

a. The protein in solution crystallizes with difficulty and only forms amorphous precipitates during the twelve hours required to obtain the salting-out equilibrium;

b. The protein crystallizes easily from its solution or is present in crystalline form.

In the first case it does not matter in which order the ingredients are added to one another. Whether water and salt solution are first mixed to obtain the desired salt concentration, and the protein solution is added last (salting-out curve), or whether salt solution and protein solution are first mixed, precipitating all the protein, and the water is added last (dissolution curve), the result obtained in twelve hours, after equilibrium has been established between precipitated and dissolved proteins, is the same. This is illustrated by Figs. 1 and 2, respectively giving the salting-out curve and the dissolution curve of ox blood serum. It will be seen that the curves are identical within the experimental error.

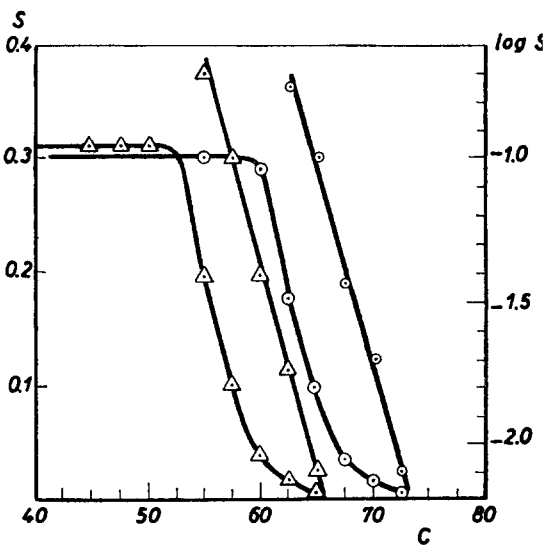

Fig. 3. Variation of the solubility, S (curves) and log S (straight lines), of the same amorphous (circles) or crystalline (triangles) horse serum albumine fraction, at pH = 6.2 and 22° C.
C = percent by volume of saturated ammonium-sulphate solution.
S = protein nitrogen in mg per ml filtrate.

In the second case, the curves obtained by salting out an amorphous protein from its solution or by dissolving its crystals do not coincide at all. This is shown by Fig. 3, giving the salting out and dissolution curves of crystalline serum albumin. The dissolution curve is displaced to the left of the salting-out curve: the cohesive forces in the crystal lattice resist the dissolving forces and render the crystalline protein less soluble.

As has been mentioned previously, it takes several weeks before an equilibrium is attained when a protein precipitates from its solution in crystalline form. Therefore a protein which crystallizes easily cannot be analyzed by salting it out from its solution, because it may partly crystallize during the operations. The best manner to analyze such a protein is by establishing a dissolution curve in the following way:

The protein crystals are suspended in the stock salt solution to a certain concentra-

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tion. The mixtures of water and salt solution are prepared, taking into account the change of salt concentration introduced by adding the protein suspended in the salt solution. After mixing water and salt solution, the desired amount of protein, in even suspension, is added from a straight cylindrical pipette*. The mixtures are further treated as described for the salting out curve.

GRAPHICAL REPRESENTATION

If all precautions discussed above are properly observed, the average experimental error is always less than $\pm$ 0.5%, as has been established by countless experiments performed in duplicate. In such duplicate experiments, the curve $S = f(C)$ of a certain protein mixture, drawn through all experimental points obtained by a routine analysis with an increment of salt concentration of 1.0 or 1.2%, has the same appearance in both cases, and reveals the presence of the same breaks at identical salt concentrations. The reproducibility of such a salting-out curve, obtained in this manner, proves that one is justified in drawing the curve *through* the actual experimental points, and that it would not be correct to construct a graph passing in between these points.

Each break in the salting-out curve $S = f(C)$ indicates that a new protein or group of protein constituents with closely similar solubility approaches its maximum precipitation. As some of these breaks are often not very pronounced, a graphical differentiation is the best way to bring to light the change in slope of the experimental curve[16]. To obtain this graphical representation (in the case of routine experiments only) the amount of protein (as protein nitrogen) precipitated for each salt increment ΔC, *i.e.* the difference ΔS in the amount of dissolved protein nitrogen between two successive salt concentrations, is calculated from the experimental data. The value of the expression: $\frac{\Delta S}{\Delta C} \cdot i$ — in which i is a standard increment of salt concentration (usually 1.2), introduced to obtain comparable standardized curves** — is plotted against the mean value of these two salt concentrations and a flowing line is drawn through the points. In this manner a derived curve: $-\left(\frac{\Delta S}{\Delta C}\right) \cdot i = f'(C)$ is obtained from the curve $S = f(C)$, representing the same experimental data, but more easy to read. Both manners of graphical representation are shown in Figs. 1 and 2.

Each minimum of the derived curve corresponds to the minimal slope preceding a break on the curve $S = f(C)$. The peaks in between the minima represent constituents (or groups of constituents) with maximum of precipitation in the range of salt concentration corresponding to the maximal slope of the segments of the curve $S = f(C)$. The derived curve resembles the representation of data customary in electrophoretic or ultracentrifugal investigations.

That the peaks which appear in a curve $-\left(\frac{\Delta S}{\Delta C}\right) \cdot i = f'(C)$, established with an increment of salt concentration of 1.2%, often do not represent single, pure proteins, but

* Pipettes with a bulb cannot be used as protein particles may be held back in the lower part of the bulb. Burettes are ruled out because sedimentation may occur.

** It is desirable always to mention the value of the increment i in the diagram, to be able to appreciate at a glance the sensitivity employed in the analysis. This value will usually correspond to the increment used to establish either the whole curve or part of it. In the latter case we recommend that the part of the curve determined with a different increment than that mentioned on the ordinate be drawn with a dotted line.

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rather a group of proteins, is revealed by diminishing the salt increment. Under these conditions several new peaks may appear in the range of salt concentrations in which the routine method only indicated the presence of a single maximum[16].

The establishment of a curve with increments of salt concentration smaller than 1.0% is very laborious and requires a large amount of protein. It will therefore only be carried out for research purposes. Results obtained in this manner for blood serum of various species will be presented in another paper. At present, we only wish to remark that in order to preserve the experimental accuracy, the difference in volume between two successive measurements of water or salt solution cannot be reduced at will, but must be maintained at least at 0.05 ml. The desired decrease of increment of salt concentration must therefore be attained by either measuring volumes of a diluted stock salt solution, or by preparing larger volumes of salting-out mixtures, composed of larger volumes of salt solution and water, or by a combination of both resources. In this manner the difference between two successive salt concentrations can be reduced to 0.25% without seriously impairing the accuracy of the measurements.

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SUMMARY

The methods used by the author and his collaborators to establish salting out curves of proteins at constant pH, temperature and protein concentration are described.

RÉSUMÉ

Les méthodes employées par l'auteur et ses collaborateurs pour établir les courbes de relargage de protéines à pH, température et concentration en protéines constants sont décrites.

ZUSAMMENFASSUNG

Die Methoden werden beschrieben, welche der Verfasser und seine Mitarbeiter zur Aufzeichnung der Aussalzungs-Kurven von Proteinen bei konstanten pH, Temperatur und Proteinkonzentration verwendet haben.

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