STUDIES ON PROTEINS BY MEANS OF SALTING-OUT CURVES

II. ESTABLISHMENT OF SALTING-OUT CURVES OF PROTEINS BY SPECTROPHOTOMETRIC MEASUREMENT IN ULTRAVIOLET LIGHT

by

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INTRODUCTION

In the method for establishing salting-out curves of proteins, as described in a previous paper[1], measurements are performed by determining the amount of protein nitrogen remaining in solution by means of micro-Kjeldahl analysis. Although the micro-Kjeldahl method is perfectly suitable for series of analyses it has the drawback of being a lengthy and laborious procedure. Moreover, serious difficulties are encountered in salting out proteins with ammonium sulphate; the removal of the excess ammonium ions complicates and lengthens the procedure, while reducing the experimental accuracy.

We have therefore attempted to replace the chemical method of micro-Kjeldahl determination by a physical method of analysis which would be less time-consuming while not detracting from the precision and dependability offered by the former method.

Preliminary attempts at nephelometric measurement of the increasing turbidity of a series of mixtures with increasing salt concentration, due to the augmentation of the precipitated proteins, did not give satisfactory results. Following H. THEORELL'S suggestion[2], we have therefore tried, with more success, to determine the amounts of protein remaining in solution after removal of the precipitate, by measuring their absorption at a wave-length of 2750 A. This absorption is caused by the aromatic amino acids contained in the proteins, which absorb a broad band of ultraviolet light in this region.

The object of this paper is to give a description of the method employed to establish salting-out curves by means of such spectrophotometric measurements, and to discuss its application to the study of the proteins of blood serum. A preliminary communication on this subject has been presented to the Société de Chimie biologique[3].

EXPERIMENTAL PROCEDURE

The first stages of the establishment of a salting-out curve are performed exactly

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as has been described in a preceding paper[1]. These stages comprise the preparation of the salting-out mixtures, their incubation and their filtration. The clear filtrates are used for the spectrophotometric analysis. The actual measurements have been carried out with a Beckman model DU spectrophotometer at wave-length 2750 A in silica absorption cells of 10 mm depth, against a blank of glass-distilled water.

Before proceeding to measure the absorption by the filtrates, these must first be diluted with glass-distilled water until a suitable concentration has been obtained, because under the conditions prevailing during the establishment of a salting-out curve (see [1]), the protein concentration of the filtrates at the beginning of the curve is much too high to permit measurement. When preparing a salting-out curve of blood serum proteins, for instance, in which the serum has already been diluted ten times, the filtrates must undergo another tenfold dilution before measurement. This dilution must be performed with great care and accuracy: the necessary glass-distilled water is measured slowly from a controlled burette into a perfectly clean test tube, the filtrate is added with a high precision pipette. The contents are mixed with care and the mixture is allowed to stand 1–2 hours before performing the spectrophotometric measurement. The limpidity of the diluted filtrates must be carefully checked, because a slight turbidity will obviously cause erroneous measurements.

The measured optical density of the diluted filtrates must be corrected for the absorption caused by the salt. This is especially important if the salt used is a mixture of primary and secondary potassium phosphate, for these salts absorb considerably at the wave-length employed. Ammonium sulphate hardly absorbs at all under these conditions, so in this case correction is not necessary. To obtain the factor of correction, a small number (8–10) of dilutions of the stock salt solution is prepared, covering the range usually employed for salting-out proteins (*e.g.* from 10 to 90% of the stock salt solution). These mixtures are also diluted ten times and the optical density is measured and plotted against the original salt concentration, before tenfold dilution. From the diagram thus obtained, the correction applicable to any diluted filtrate with a certain salt concentration can readily be deduced.

The corrected optical density of the diluted filtrates (*i.e.* experimentally measured density –density of salt solution) is now plotted against their original salt concentration and the salting-out curve $E = f(C)$ is obtained. Likewise one can construct the derived curve $-\left(\frac{\Delta E}{\Delta C}\right) \cdot C = f'(C)$.

RESULTS

The method of analysis described above has been applied to several blood sera, of human and equine species, and the results obtained have been compared to those found by micro-Kjeldahl analysis.

1. *Horse blood serum*

Fig. 1 shows the salting-out curve $S = f(C)$ of horse blood serum, which has been simultaneously analyzed by spectrophotometric measurement and by micro-Kjeldahl method, according to the routine procedure (salt: 3.5 M $KH_2PO_4 + K_2HPO_4$; pH: 6.5; temp.: 24° C; dilution of serum 1:10).

The results have been expressed in percentages of the initial protein concentration

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(measured as protein N in mg/ml or optical density), after correction for non-protein nitrogen (respectively, residual optical density). It will be observed that, although the breaks are found at the same salt concentrations, indicating that the same groups of protein constituents are revealed, the curve obtained spectrophotometrically declines more rapidly with increasing salt concentration than the curve established by Kjeldahl analysis. In other words, the ratio E/N is not constant, but decreases with increasing salt concentration, as can also be seen in the diagram accompanying the salting-out curve in Fig. 1. This variation of the ratio E/N indicates that the serum proteins have a

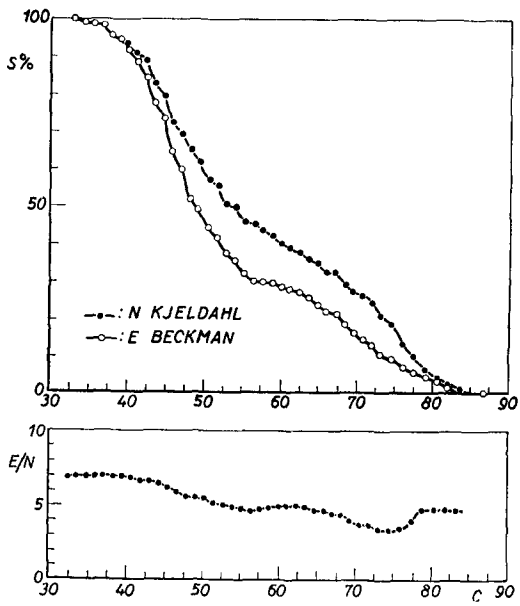

Fig. 1. Above: salting-out curves $S = f(C)$ for horse serum (pH 6.5, temp. 24° C, dilution of serum 1:10). S = amount of protein nitrogen remaining in solution or optical density of solution, expressed in percentage of original protein content. C = salt concentration, expressed in percentage 3.5 M KH_2PO_4 + K_2HPO_4. Below: Variation of ratio E/N with salt concentration.

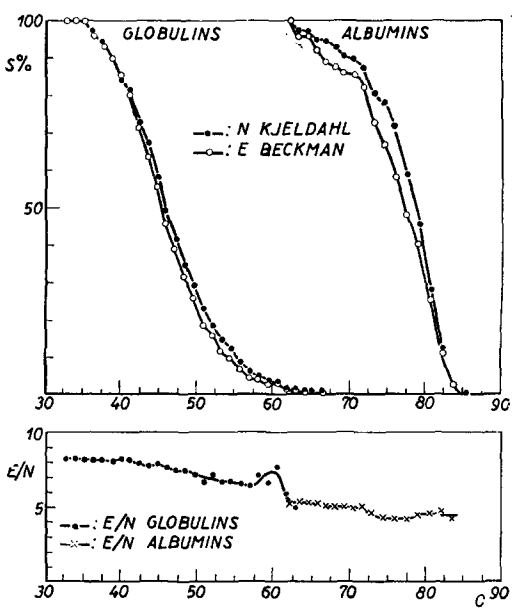

Fig. 2. Above: Salting-out curves for the groups of globulins and albumins separated from the horse serum of Fig. 1. Below: Variation of ratio E/N for both groups with salt concentration. Details as in Fig. 1.

varying content of aromatic amino acids, and notably confirms the well-known fact that the albumins contain less of these acids than the globulins. This has been demonstrated by separating the globulins from the albumins by fractionation of the serum represented in Fig. 1 with half-saturated ammonium sulphate. After dialysis against 0.2 M NaCl to remove the ammonium sulphate, each fraction has again been analyzed by both methods. The results are expressed in Fig. 2. Examination of both sets of curves reveals their similarity; this is especially the case for the globulins. The ratio E/N for the separate groups of globulins and albumins appears to be less variable, and this ratio is distinctly lower for the albumins.

The close resemblance of the results obtained by both methods of analysis can be

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better appreciated by a comparison of the derived curves $-\left(\frac{\Delta N}{\Delta C}\right)\cdot i = f'(C)$ and $-\left(\frac{\Delta E}{\Delta C}\right)\cdot i = f'(C)$, which are given in Fig. 3. It will be seen that, especially in the globulin zone, there is a good agreemant between the curves. All groups of protein constituents brought to light by the Kjeldahl method are also revealed by the spectrophotometric analysis.

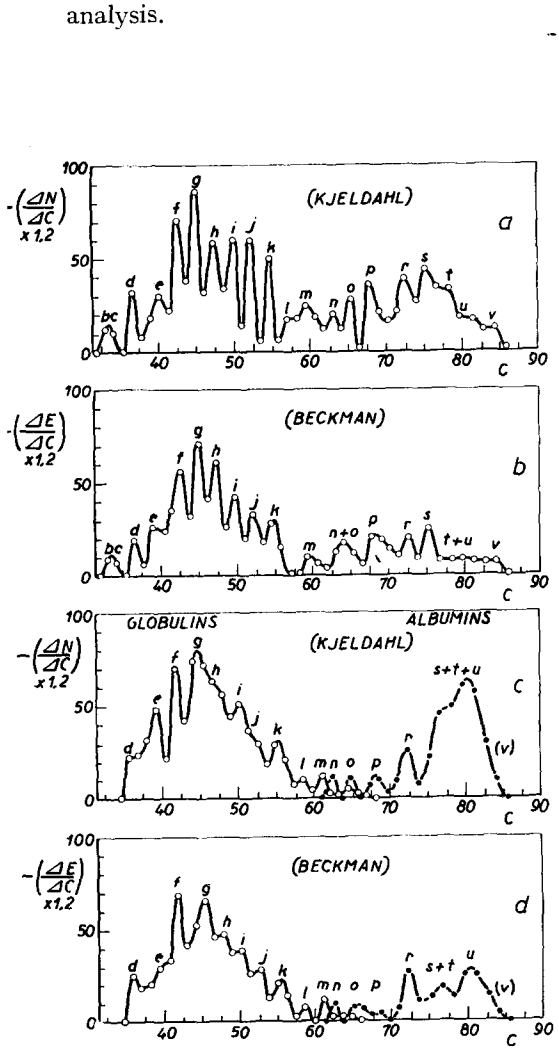

Fig. 3. Derived curves $-\left(\frac{\Delta S}{\Delta C}\right)\cdot i = f'(C)$ for horse serum, calculated from the curves in Figs. 1 and 2. a. curve for total serum established by micro-Kjeldahl analysis; b. same, established spectrophotometrically; c. curves for separated groups of globulins and albumins by micro-Kjeldahl; d. same, spectrophotometrically. Values on ordinate multiplied by 10^3. Abscissa as in Fig. 1.

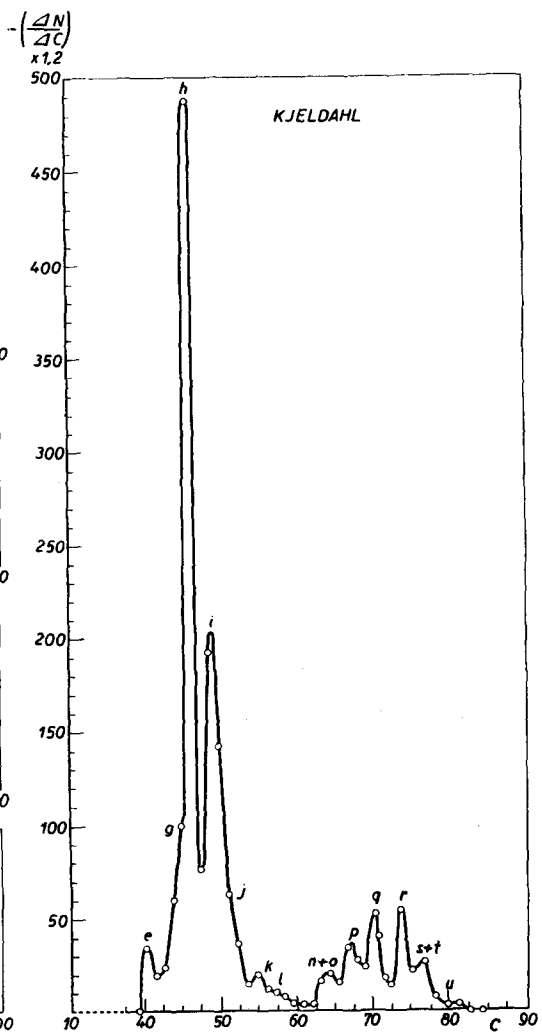

Fig. 4. Derived curve $-\left(\frac{\Delta N}{\Delta C}\right)\cdot i = f'(C)$ for human serum in a case of macro-globulinaemia. Conditions of salting-out as in Fig. 1. Curve established by micro-Kjeldahl analysis. N = mg nitrogen per ml filtrate. Values on ordinate multiplied by 10^3. Abscissa as in previous figures.

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2. Human blood serum

Fig. 4 shows the derived curve $-\left(\frac{\Delta N}{\Delta C}\right) \cdot i = f'(C)$, established by micro-Kjeldahl analysis, of human blood serum of a case of macroglobulinaemia. The analysis has been performed according to the routine method, under the same conditions as described for the horse sera. Before the analysis the serum has been diluted to half its original concentration because of its very high protein content. In the diagram, however, the measured values have been doubled to obtain the standardized graphical representation. The first part of the curve, of interest owing to the excessive proportions of groups of proteins *h* and *i*, has also been analyzed spectrophotometrically. The results can be seen in Fig. 5, which shows that the presence of the extremely high peaks is also clearly revealed by the spectrophotometric method.

The same agreement between both methods of analysis can be observed upon comparing Figs. 6 and 7, which pertain to the serum of another patient, suffering from multiple myeloma.

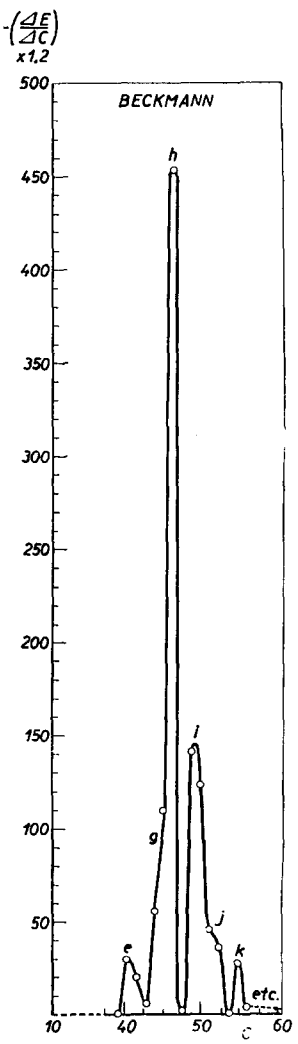

Fig. 5

Fig. 5. Derived curve $-\left(\frac{\Delta E}{\Delta C}\right) \cdot i = f'(C)$ for serum shown in Fig. 4; curve established by spectrophotometric measurements. E = optical density. Further details as in Fig. 4

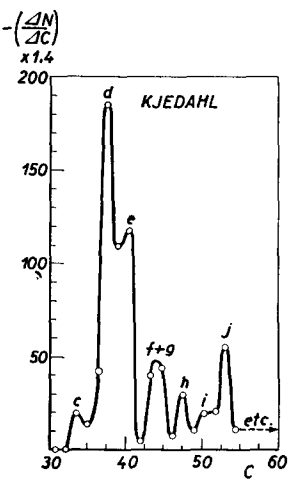

Fig. 6

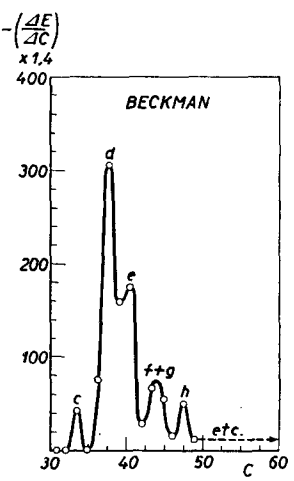

Fig. 7

Fig. 6. Derived curve $-\left(\frac{\Delta N}{\Delta C}\right) \cdot i = f'(C)$ for human serum in a case of multiple myeloma (γ-myeloma). Curve established by micro-Kjeldahl analysis. Further details as in Fig. 4

Fig. 7. Derived curve $-\left(\frac{\Delta E}{\Delta C}\right) \cdot i = f'(C)$ for serum shown in Fig. 6. Curve established by spectrophotometric measurements. Further details as in Fig. 5.

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DISCUSSION

The examples presented above show that in general there is a very satisfactory agreement between the salting-out curves obtained by spectrophotometric measurements and those established by micro-Kjeldahl analysis.

The spectrophotometric method has the advantage of a more rapid execution, while it faithfully reproduces the character of the curve as found by nitrogen determination. Therefore it is also useful as a control for the correctness of the curve established by micro-Kjeldahl analysis. A marked discrepancy between both curves will induce the operator to check the possibility of faulty nitrogen determinations.

Nevertheless, it should be emphasized that the spectrophotometric method also has its limitations. The absorption measured depends upon the composition of the respective proteins, that is to say, upon their content of aromatic amino acids. Indubitably this property is subject to larger variations than their nitrogen content. So, while being satisfactory from a qualitative point of view in revealing the presence of divers groups of protein constituents, it cannot be denied that the quantitative aspect of the salting-out curve as established spectrophotometrically is sometimes seriously distorted. Proteins with a high content of aromatic amino acids will appear in relatively too large amounts, while those possessing but little of these acids will fade into unmerited insignificance. Approximate quantitative determination of the relative amounts of the divers groups of protein constituents, which can be deduced fron the nitrogen curve by calculating the percentage protein nitrogen represented by each segment, cannot be performed with a spectrophotometric curve. However, in sera in which certain protein constituents are pathologically increased, the different aromatic amino acid content of the proteins becomes of minor importance, and such pathological changes can be profitably examined and revealed by spectrophotometric analysis.

This technique also offers a simplified method for comparing salting-out curves of certain proteins, obtained with different salts, including ammonium sulphate. Work on this subject is in progress at present.

The spectrophotometric analysis is of especial importance in the case of ammonium sulphate, the classic salt most widely used for fractionation and purification of proteins. Its application greatly facilitates the establishment of salting-out curves with this salt, so inconvenient if the measurements have to be carried out by micro-Kjeldahl analysis. Salting-out curves with ammonium sulphate, established spectrophotometrically, permit rapid control of the progress of fractionation of proteins with this salt, and of the purity of the final products obtained.

The spectrophotometric technique, which only employs a small amount of filtrate, also has the advantage that less serum is required to establish a salting-out curve, as the volume of the mixtures of serum, water and stock salt solution can be reduced.

ACKNOWLEDGEMENTS

One of us (E.P.S.P.) wishes to thank Professor DERRIEN for his kind hospitality, and the Netherlands Organization for Pure Research Z.W.O. and the French Centre National de la Recherche Scientifique for a grant which enabled participation in this work.

The gift by the Rockefeller Foundation of a Beckman spectrophotometer, used in this research, is gratefully acknowledged.

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SUMMARY

A method is described for establishing salting-out curves of proteins by means of spectrophotometrical measurements of their absorption in ultraviolet light of 2.750 A wave-length. The measurements are performed on the—suitably diluted—solutions of proteins in salt mixtures with increasing salt concentration, after removal of precipitated proteins.

Several samples of horse serum and of pathological human serum have been examined by this method. Comparison of the results with those obtained by micro-Kjeldahl analysis shows a satisfactory agreement between both methods as regards the groups of protein constituents revealed, and also indicates their differences in aromatic amino acid composition.

The advantages and disadvantages of the spectrophotometric method as compared to the Kjeldahl method are discussed. It is more rapid than the latter and useful for qualitative analysis of a protein mixture, but less suitable for quantitative evaluation of the results. It is especially convenient for work with ammonium sulphate.

RÉSUMÉ

Nous décrivons une méthode permettant d'établir les courbes de relargage des protéines par spectrophotométrie en lumière ultraviolette de 2.750 A de longueur d'onde. Les mesures d'absorption sont effectuées sur les solutions — convenablement diluées — de protéines dans des milieux de concentration saline croissante après élimination des protéines précipitées.

Plusieurs échantillons de sérums de cheval et de sérums humains pathologiques ont été étudiés par cette méthode. La comparaison des courbes ainsi obtenues à celles établies par micro-Kjeldahl démontre leur accord très satisfaisant en ce qui concerne l'individualisation des divers groupes de protéines sériques dont elle objective, en outre, la diversité de composition en acides aminés aromatiques.

Les avantages et les inconvénients de la méthode spectrophotométrique comparés à ceux du dosage de l'azote protéique par micro-Kjeldahl sont discutés. La première est la plus rapide. Elle est utilement applicable à l'étude qualitative d'un mélange protéique mais convient moins bien que le second à l'évaluation quantitative des résultats obtenus. Enfin, elle simplifie considérablement l'établissement des courbes de relargage par le sulfate d'ammonium.

ZUSAMMENFASSUNG

Eine Methode zum Zeichnen der Aussalzkurven von Proteinen mit Hilfe spektrophotometrischer Messungen ihrer Absorption in ultraviolettem Licht von 2.750 A Wellenlänge wird beschrieben. Die Messungen werden an den — in geeigneter Weise verdünnten — Lösungen von Proteinen in Salzmischungen mit steigender Salzkonzentration nach Entfernung der gefällten Proteine ausgeführt.

Mehrere Proben von Pferdeserum und von menschlichem pathologischen Serum wurden mit Hilfe dieser Methode geprüft. Ein Vergleich der Resultate mit denen, die mit Hilfe der Mikro-Kjeldahlmethode erhalten wurden, zeigt bezüglich der Zusammensetzung aus verschiedenen Eiweiss-Komponenten für beide Methoden befriedigende Übereinstimmung und weist ausserdem auf den verschiedenen Gehalt an aromatischen Aminosäuren.

Die Vor- und Nachteile der spektrophotometrischen Methode werden im Vergleich zu denjenigen der Kjeldahl-Methode erörtert. Erstere ist rascher und nützlich für die qualitative Analyse von Protein-Mischungen, jedoch weniger geeignet für die quantitative Auswertung der Resultate. Sie ist besonders geeignet für Arbeiten, welche Ammoniumsulfat verwenden.

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Received July 25th, 1951