OVALBUMIN:

A PHYSICO-CHEMICAL STUDY

by

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To the author's knowledge this thesis contains no material previously submitted for a degree either by himself or by any other person, except when due reference is made in the text.

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SUMMARY

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"Physico-Chemical Studies on Ovalbumin: I. Electrophoretic Fractionation and Characterization by Diffusion" by J.M. Creeth, L.W. Nichol and D.J. Winzor [J. Phys. Chem., 62, 1546 (1958)].

CHAPTER I

INTRODUCTION

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1. General Introduction

The study of the chemistry of proteins has been very greatly aided by the discovery of methods for isolating a given protein from the array of materials with which it is normally extracted from some biological source.[1] With the significant advances in this field there arose a further problem, which is still receiving much attention, namely, the question of protein purity. At first it was believed that crystallisability was a satisfactory criterion, the existence of a protein in crystalline form being regarded as proof of homogeneity. The physical biochemists, by applying the more searching criteria of physico-chemical measurements to these crystalline proteins however, were able to detect heterogeneity in nearly all systems studied: techniques of particular importance included electrophoresis, sedimentation and solubility measurements. By these methods crystallisability was quickly eliminated as a criterion for purity.[2] The next development in the study of protein heterogeneity was the discovery that the satisfaction of one criterion was insufficient proof:[3] a protein which appears homogeneous by one method may be proved to be heterogeneous by another — indeed, a substance may be shown to be both homogeneous and heterogeneous using the same method simply by variation of the conditions used, e.g., alteration of the pH of the solution under investigation.

Unfortunately for the experimentalist, complete homogeneity of a protein is a property which cannot be demonstrated directly,[4]

whereas deviations may be. It is therefore necessary to define a set of criteria as a standard for homogeneity of protein samples. Apart from one or two cases, where only one method of analysis was used, a protein system has yet to be proven pure,[5] even using this operational definition of homogeneity. Thus, while great advances have been made in the study of protein homogeneity, the methods available for such study are continually being improved, and a great deal of present day research is concerned with the quest for, firstly, a set of criteria which will unequivocally prove a protein's homogeneity, and secondly, a protein which will satisfy such requirements.

2. Outline of the Field of Study

Electrophoresis of ovalbumin has revealed the presence of at least three components in samples of the protein which have been repeatedly crystallised.[6-9] These three components, designated A_1, A_2 and A_3 in order of descending negative mobility, appear to be present in all samples of ovalbumin to the same extent, the apparent proportions, as judged from analysis of the electrophoresis patterns, being 80%, 15% and 5% respectively. Of the physicochemical methods available for studying macromolecular systems, only electrophoresis has so far detected this heterogeneity: sedimentation studies,[10,11] for example, have failed to reveal any deviation from a homogeneous system.

In a most elegant study combining enzymatic dephosphorylation experiments with electrophoretic mobility measurements, Perlmann[12,13] has detected a difference in the phosphate content of the three components. After treatment of crystalline ovalbumin with the

dephosphorylating enzyme prostate phosphatase, electrophoresis reveals the complete absence of the A_1 component and a corresponding increase in the amount of protein with the mobility of A_2. By use of another dephosphorylating enzyme, intestinal phosphatase, Perlmann has also been able to convert the A_2 into a protein with the mobility of A_3. Phosphorus analyses, done in conjunction with these experiments, indicate that A_1, A_2 and A_3 contain two, one and zero phosphate groups respectively. Thus she concludes that while minor chemical differences cannot be excluded, it would appear that with the exception of phosphate content these molecules may be identical. If this were the case, then the individual sedimentation coefficients would be indistinguishably close and the mixture should sediment as one species, in agreement with observation.[10,11] This argument should also apply to the diffusion of the ovalbumin mixture.

Optical interference methods of particular value for studying diffusion have been developed by Coulson, Cox, Ogston and Philpot[14] in England, and Kegeles and Gosting[15] and Longsworth[16] in U.S.A.; as a result of the application of these methods a much greater precision in the determination of diffusion coefficients has been attained and thus a more critical test of the identity of diffusion coefficients now appears possible. The procedures developed for the determination of heterogeneity from diffusion measurements on mixed solute systems[17-19] have been shown to be valid for mixtures of simple substances such as sucrose and urea, and have been applied to a few protein systems.[17,19,20] A study of the diffusion behaviour of crystalline ovalbumin and also the

leading electrophoretic component, A_1, is discussed in this work. The author believes that of all the protein systems ever tested for heterogeneity, the A_1 studied in this work may well be among the purest. During the course of the discussion of these diffusion measurements it will become apparent that the interpretation of such results is complicated by two factors:

(i) the possibility of change in the configuration of the protein molecule during diffusion;

(ii) the interaction of flows between the protein and the constituent salts of the buffer solution.

Indeed, before full interpretation of diffusion results obtained with proteins can be realised, both of these effects should be investigated. In the author's opinion, the logical approach is to study the former postulate first.

One of the greatest problems associated with physical measurements on protein systems is the tendency for many proteins to denature, i.e., to change their physical structure (and hence solution properties) but not their chemical composition. This tendency for denaturation may often be correlated with the existence of reactive polar groups of the constituent amino acids of the protein. Of these, the sulphydryl groups of the cysteine residues are the most reactive: two such groups will tend to react together to form a disulphide bridge, either intramolecularly or intermolecularly. Other polar groups may also react, the above example serving only as an illustration of the type of structural rearrangement that may occur. The ease with which proteins

denature varies greatly, and in this respect, ovalbumin is one of the most unstable.[21] With such a labile protein as ovalbumin, there is always the possibility that the material under investigation at the conclusion of a physical measurement differs from that at the commencement of the experiment. This possibility applies particularly to the case of diffusion, where a single experiment extends over several days.

A substance more suitable for physico-chemical measurements may well be obtained if these reactive groups are modified by reaction with some reagent prior to the actual physical measurement. Of course, the method of modification should be such that the modified material resembles as closely as possible the native protein. Since, as has already been mentioned, the sulphydryl group is the most reactive, attention has been focussed mainly on this group. The modification of this group has been the subject of a large number of investigations, the results of which have been summarised in several reviews.[22-24] Of the methods available for modification, the one chosen for this study is the oxidation of the sulphydryl groups with iodine, since this reagent is one of the few reported to react specifically and stoichiometrically with all of the sulphydryl groups of ovalbumin in the native state.[25] The reaction involves the oxidation of sulphydryl to disulphide according to the following equation:

$$2RSH + I_2 \longrightarrow RSSR + 2H^+ + 2I^-.$$

There are five cysteine residues per ovalbumin molecule[26] and, indeed, five atoms of iodine per molecule are involved in the

reaction,[27] this being the number required for five sulphydryl groups to be oxidised according to the above equation. However, because the sedimentation rates of the native and modified materials are essentially identical,[28] Anson concludes that the reaction is entirely intramolecular, since the formation of intermolecular disulphide bridges would lead to the formation of a species with a higher sedimentation rate.

Because the oxidation requires pairs of sulphydryl groups and an odd number appear to exist in the native protein molecule, then for the above reaction to be the sole reaction occurring, at least one intermolecular disulphide bridge must be formed. In view of this anomaly, a comparative physico-chemical study of the native ovalbumin and the iodine-modified material has been undertaken in an endeavour to ascertain the exact nature of the modification.

It was felt moreover, that a comparative study of this kind would provide results likely to be of some significance in the wider field of protein structure; much attention is at present being directed to the "helix-coil" transition (i.e., the change from a peptide chain supposedly conforming in part to the Pauling and Corey "α-helix"[29] to the random coil), and in the further development and critical testing of the theory, the availability of modified products of well defined properties will facilitate progress.

As previously mentioned, the early part of this study is concerned primarily with the diffusion of ovalbumin using the Rayleigh optical system. Accordingly, Chapter II contains a summary

of the theoretical aspects of diffusion measurement by this method; the results of the application of this theory to ovalbumin and the leading electrophoretic component, A_1, are then discussed in Chapter III.

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CHAPTER II

SOME THEORETICAL ASPECTS OF FREE DIFFUSION

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1. General

In studies of biological systems, diffusion is important from two points of view; firstly, knowledge of the laws governing diffusion is basic to an understanding of transport through the walls of living cells and within the cell itself, and secondly, quantitative measurements of diffusion contribute greatly to the knowledge of the molecular weight and characteristic properties of proteins and other molecules of biological interest.[1] It is with the quantitative measurement of diffusion that this study is concerned. The fundamental quantity obtained by such measurement is the diffusion coefficient, D, originally defined by Fick.[2] For diffusion at constant temperature and pressure of two-component systems showing no volume change on mixing, the diffusion coefficient is defined by the relation

$$J_1 = -D\left(\frac{\partial C_1}{\partial x}\right)_t \qquad (1)$$

if only one dimensional transport is considered. In this relation J_1 is the flow of solute and $\left(\frac{\partial C_1}{\partial x}\right)_t$ the concentration gradient at time t. Thus the diffusion coefficient is numerically equal to the flow of solute relative to the cell caused by unit concentration gradient. This D is related to the molar frictional coefficient, f, according to the following equation, which may be deduced by consideration of thermodynamics of irreversible processes.[3]

$$D = \frac{RT}{f}\left(1 + C\frac{d \ln y}{dC}\right) \qquad (2)$$

where y is the activity coefficient of the solute. (Other descriptions of diffusion, leading to alternative but equivalent

expressions, have been given by Ogston.[4] Since the second term in the bracket of equation (2) is zero at zero concentration, extrapolation of the diffusion coefficient to zero protein concentration gives a value, D_o, which is inversely proportional to the frictional coefficient, the exact relation being

$$D_o = \frac{RT}{f}. \qquad (3)$$

Furthermore, since it is most probable that the frictional coefficient encountered in diffusion measurements is identical with that in sedimentation velocity experiments, the molecular weight of the material can be found by combining sedimentation and diffusion data in Svedberg's equation.[5] For a two-component system, it may be shown that

$$s = \frac{M}{f}(1 - \bar{v}\rho) \qquad (4)$$

where M is the molecular weight, $\bar{v}$ the partial specific volume and ρ the density of the solution. Combination of equations (2) and (4) then gives a generalised form of Svedberg's equation, viz.,

$$M = \frac{sRT}{D(1 - \bar{v}\rho)}\left(1 + C\frac{d \ln y}{dC}\right). \qquad (5)$$

Once again the term in brackets becomes equal to unity at zero protein concentration, so that equation (5) reduces to the usual form of the Svedberg equation, viz.,

$$M = \frac{RTs_o}{D_o(1 - \bar{v}\rho)} \qquad (6)$$

where the subscript zero denotes zero concentration.

Thus it is quite obvious that the accuracy of molecular

weights (and frictional coefficients) determined by these procedures is dependent not only on the precision of the measurement of the diffusion and sedimentation coefficients (and $\bar{v}$), but also on the accuracy of the extrapolation of these quantities to zero protein concentration. Also the above equations refer strictly to two-component systems only, and will thus be valid only so far as the system protein-(buffer salts + water) approximates to such a system. From the formal expressions for three-component systems,[6,7] it is to be expected that equation (6) will be adequate so long as the protein is electrically neutral, of fairly high molecular weight (> 10,000), and that the concentration of buffer salts is not much greater than 0.1 molar.

The development of interferometric optical systems for measuring the refractive index distribution, or refractive index gradient distribution, in a cell has been an important advance, as these optical systems have provided the means for a tenfold increase in the accuracy of diffusion coefficients.[8-18] In addition, because of the high accuracy of interferometric measurements, it is possible to analyse the form of the distribution of refractive index (or gradient) to obtain data on the purity of the protein preparation.[12,15,17-19]

The two interferometric optical systems most commonly in use are the Gouy and Rayleigh optical systems, the former giving the refractive index gradient distribution, the latter the refractive index distribution. The theories for the determination of diffusion coefficients of two- and three-component systems,

and also analysis procedures for detecting heterogeneity under certain conditions, have been developed and tested for both systems.[12,15,17,18] Although the Rayleigh method is apparently slightly less accurate than the Gouy method,[1] it is possible from one experiment, to determine the diffusion coefficient at the mean concentration of the experiment and also the concentration dependence of the diffusion coefficient.[17,18] Theoretically, therefore, D_o can be obtained from the results of one diffusion experiment: this is a decided advantage for two reasons -- (i) a saving of protein and (ii) a saving of time. This latter is most important because of the length of time required to perform a diffusion experiment (approximately eight days, four of which are required for the observation of the diffusion process).

2. Determination of Differential Diffusion Coefficients in Two-Component Systems

Although the diffusion of a protein is performed in a buffered solution, and the system therefore approximates to a three-component system, the theory for two-component systems will be given first, as the extension of the theory to three-component systems then becomes more readily understood. As previously mentioned the theory has been developed for both the Gouy[8-10,20,21] and Rayleigh[13,14,16,22] optical systems, but here discussion will be limited to Creeth's interpretation of interferograms obtained from the latter system.[16]

In the development of such a theory, the logical approach is to obtain an expression for the diffusion coefficient in the case

of an ideal diffusion system and then modify the expression for any deviations from ideality.

(a) Ideal Systems

An ideal system is defined as one in which both the diffusion coefficient and the refraction increment are independent of concentration. In diffusion experiments, an initially sharp boundary is formed between two concentrations of solute, C_1 and C_2, the lower being the more concentrated to preserve gravitational stability. This is shown in Fig. II-1(a). With such a system, where concentration is measured as a function of height in the cell, the relevant form of Fick's Law is given by the relation

$$g(C) \equiv \frac{2(C - \bar{C})}{\Delta C} = \frac{2}{\sqrt{\pi}} \int_0^{x/2\sqrt{Dt}} e^{-\beta^2} \, d\beta \qquad (7)$$

where C is the concentration at the point distance x from the initially sharp boundary, $\bar{C}$ is the mean concentration of the experiment $(\frac{C_1 + C_2}{2})$, and ΔC is the difference in concentration between the two solutions, t the time and D the diffusion coefficient corresponding to the mean concentration. The right hand side of equation (7) is frequently denoted by the term H(z), where $z = x/2\sqrt{Dt}$ and H is the probability integral.

The Rayleigh optical system yields a record of refractive index (in terms of interference fringes) as a function of cell displacement (Figs. II-1(b) and (c)). Then for a particular fringe, j, in a system of J fringes, a "reduced fringe number" function, f(j), is defined by the equation

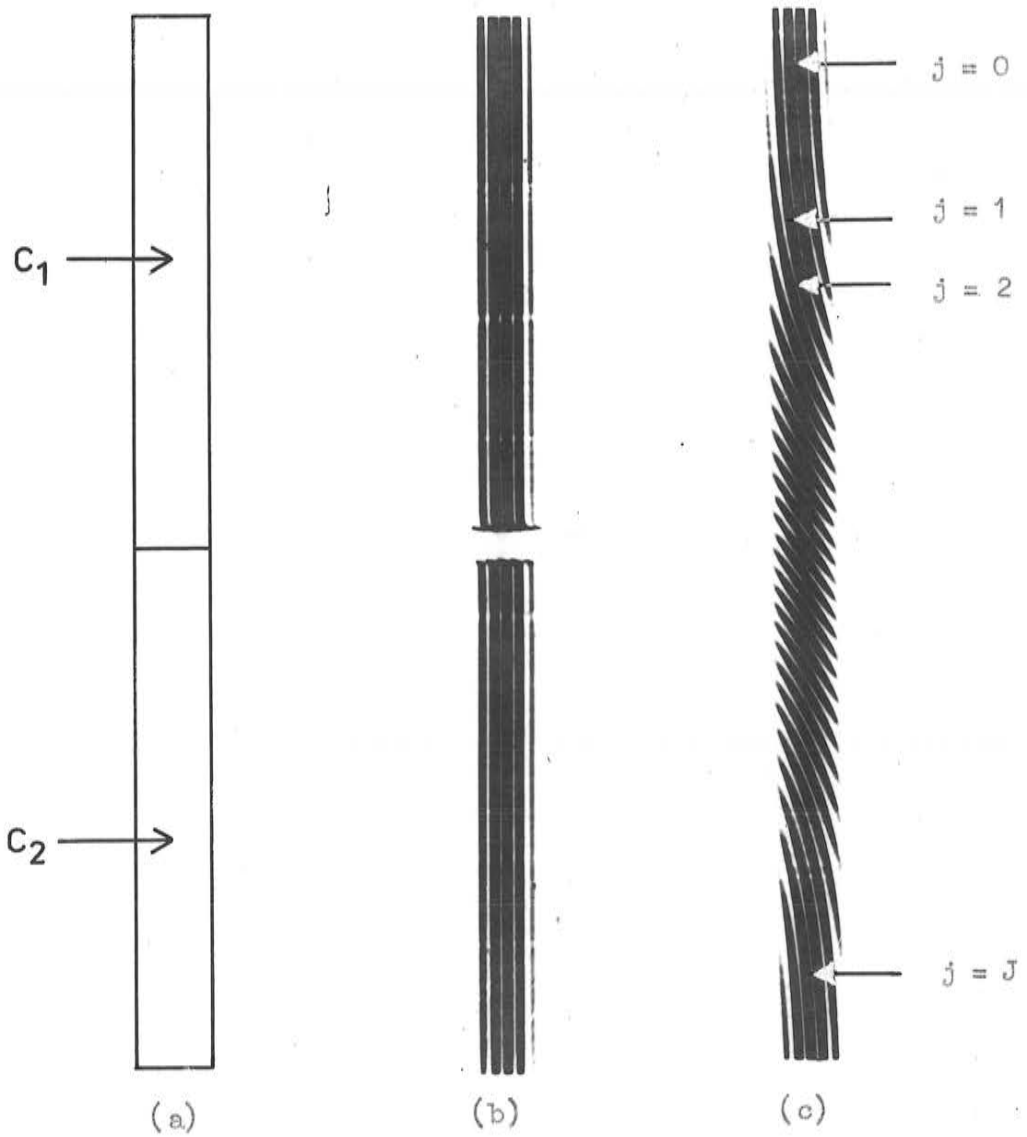

Fig. II-1. (a) Diagrammatic representation of a boundary at the commencement of diffusion.
(b) A Rayleigh interferogram of a boundary at the commencement of diffusion.
(c) A Rayleigh interferogram of a diffusing boundary: the method of fringe numbering is also illustrated.

$$\frac{2j - J}{J} \equiv f(j) \qquad (8)$$

Since by definition of an ideal system the refractive index is proportional to concentration, the function $f(j)$ must equal the function $g(C)$ of equation (7) and accordingly $f(j) = H(z)$. Thus for an ideal system knowledge of j and J enables calculation of the quantity $H(z)$, the corresponding value of z being obtained from tables of probability functions. Combination of this value of z with the experimental quantities x and t then provides a method for the determination of D.

(b) Concentration-dependent Systems

The use of $f(j)$ to obtain values of z is clearly inapplicable if the refractive index (n) is not proportional to concentration, or if D varies with concentration, and the question is therefore raised as to how a significant quantity (such as the differential diffusion coefficient) may be evaluated from the experimental record. This question has been studied,[16] and an answer obtained: the results which are relevant to this investigation will now be summarised.

The theory is derived for the case where the concentration dependence of D and n may be represented by equations (9) and (10):

$$D = D_{\bar{C}} (1 + k_1(C - \bar{C}) + k_2(C - \bar{C})^2 + \ldots) \qquad (9)$$

where D is the diffusion coefficient at concentration C, $D_{\bar{C}}$ that at $\bar{C}$, and k_1 and k_2 are coefficients which can be determined experimentally;

$$n = n_{\bar{C}} + R_{\bar{C}}(C - \bar{C})(1 + a_1(C - \bar{C}) + a_2(C - \bar{C})^2 + \ldots) \qquad (10)$$

where $R_{\bar{C}} = \left(\frac{dn}{dC}\right)_{C=\bar{C}}$, $a_1 = \frac{1}{2!R_{\bar{C}}}\left(\frac{d^2n}{dC^2}\right)_{C=\bar{C}}$ and

$$a_2 = \frac{1}{3!R_{\bar{C}}}\left(\frac{d^3n}{dC^3}\right)_{C=\bar{C}}.$$

Fujita and Gosting[23] have derived expressions relating the reduced concentrations at any point to the parameters k, a, etc., Creeth[16] having made the necessary modifications to these expressions to allow for the requirements of the Rayleigh optical system. The result of the combined treatment may be conveniently expressed by the equation:

$$z = z^* + \frac{\Delta C}{2}[a_1U(z^*) - k_1R(z^*)] + \left(\frac{\Delta C}{2}\right)^2\left[\frac{a_1^2}{2}W(z^*) - a_1k_1V(z^*)\right.$$

$$\left. + a_2U(z^*)H(z^*) + \frac{k_1}{2}S(z^*) - k_2T(z^*)\right] + \ldots \qquad (11)$$

where the symbol z now has the definition

$$z = x/2\sqrt{D_{\bar{C}}t} \qquad (12)$$

and z^* is the argument of the error function defined by

$$f(j) = H(z^*). \qquad (13)$$

The functions $R(z^*)$, $S(z^*)$, $T(z^*)$, $U(z^*)$, $V(z^*)$ and $W(z^*)$, which are themselves functions of the error integral and its derivatives, are defined and, in the case of $U(z^*)$ and $R(z^*)$, tabulated.[16] The most important feature of these two functions is their symmetrical nature about $z = 0$, i.e., $U(z^*) = U(-z^*)$. Therefore first order concentration dependence can be eliminated by comparing fringe displacements

symmetrically about $f(j) = 0$, i.e., the $J/2$ position. Because second order effects are negligible when either $(\Delta C/2)$ or the constants a or k are small, this procedure will give Δz values identical with the corresponding Δz^* values; thus the differential diffusion coefficient $D_{\bar{C}}$ can be calculated directly using equations (12) and (13).

(c) Determination of the Concentration-dependence of D

The concentration-dependence of the diffusion coefficient (or skewness of the boundary) may be obtained from one experiment providing a_1 is negligible and the concentration increment fairly small. Application of equation (11) to two fringes j_1 and j_2 gives the result

$$(z_2 - z_1) - (z_2^* - z_1^*) = -k_1(\Delta C/2)[R(z_2^*) - R(z_1^*)] + \ldots \qquad (14)$$

Thus the required data are (i) knowledge of the fringe number j and the total number of fringes J, (ii) the concentration increment ΔC, (iii) the function $R(z)$ and (iv) the experimentally observed reduced fringe separation Δz, which may be calculated from the known time and $D_{\bar{C}}$.

The procedure adopted does not, however, involve use of t and $D_{\bar{C}}$: use is made of the quantity $\overline{Y}_t$, defined by the relation

$$\overline{Y}_t = 2M\sqrt{D_{\bar{C}}t} = \frac{1}{n}\sum_1^n \left(\frac{X_{J-j} - X_j}{z^*_{J-j} - z^*_j}\right) = \frac{1}{n}\sum_1^n Y_t \qquad (15)$$

where M is the camera-to-cell magnification and X_{J-j} and X_j the comparator readings for the respective fringes. Then if X_1 and X_2 are the comparator readings for fringes j_1 and j_2,

$$\left(\frac{X_2 - X_1}{\overline{Y}_t}\right) - (z_2^* - z_1^*) = -k_1(\Delta C/2)[R(z_2^*) - R(z_1^*)] + \ldots \qquad (16)$$

$$= -k_1(\Delta C/2).\,\Delta R(z^*).$$

Thus a plot of $(\Delta z - \Delta z^*)$ versus $\Delta R(z^*)$, commonly known as a skewness plot, is linear providing the coefficients a_1, k_2, etc. are not significant. Deviation from linearity of this plot indicates that allowances for these terms have to be made.

3. Analysis of Three-Component Systems

Theories for the analysis of three-component systems have been devised for both Gouy[12,15] and Rayleigh[17] interferograms. Although Ogston's method[12] is not restricted to systems containing a small amount of impurity, less use is made of the available information. Consequently preference has been given to the other two analysis procedures, whereby the diffusion coefficient of the major solute component, diffusing in a medium containing a small amount of a second solute, can be calculated providing certain assumptions, to be discussed later, are valid. The theories, which have been checked by application to known mixtures of simple solutes,[12,15,18] have been applied in a few cases to protein systems,[12,15,18,19,24-28] all of which have been shown to contain a small amount of impurity. Since proteins are diffused in a buffered medium, it is necessary to consider the buffer salts as constituents of the solvent, rather than as additional solutes; analysis of the protein system into two protein components of differing diffusion coefficients may then be made.

As before, the discussion of three-component systems which now follows has been specialised to the case of Rayleigh optics;[17] because the results of this analysis have been extensively applied in this work, fuller detail is given.

(a) Assumptions

In order to analyse a mixed solute system for heterogeneity it is necessary to make the following assumptions:

(i) Independence of diffusion of each solute, i.e., the flow of each solute is determined solely by its own concentration gradient.

(ii) Linear dependence of the diffusion coefficient, D_i, of each solute with the concentration, C_i, of that solute, the expression being analogous with that for two-component systems:

$$D_i(C_i) = D_i(\overline{C}_i)\,[1 + k_i(C_i - \overline{C}_i)]$$

where $D_{i(C_i)}$ is the diffusion coefficient of species i at the particular concentration C_i.

(iii) Either independence or linear dependence of the refraction increment of each solute upon concentration of that component as in the case of two-component systems. The refraction increment must also be unaffected by the presence of other solutes. The refractive index, n, of a solution is then given by the following relation:

$$n = n_{(\overline{C}_1,\ldots\overline{C}_q)} + \sum_{i=1}^{q} R_i(C_i - \overline{C}_i)[1 + a_i(C_i - \overline{C}_i)]$$

where $n_{(\overline{C}_1,\ldots\overline{C}_q)}$ is the refractive index of a solution in which all the solute species have their mean concentrations $\overline{C}_i$. It is to be noted that the summation terms extend over solute components

only, the solvent being excluded. The expressions developed on the basis of these assumptions are applicable to systems of two solutes showing significant, but not extreme, effects of concentration dependence. For simplicity, the expressions will be derived for the case in which each component diffuses ideally, i.e., k_i and a_i are negligible: the final expressions derived from the more general procedure, giving due consideration to the k_i and a_i terms,[17] will only be stated.

(b) Theory

An expression is obtained for the difference between two functions which, in the case of a single solute, are identical. They are:

(i) the quantity $H(z^*)$ as given by equation (13). However, whereas for a single solute,

$$f(j) = H(z) + \ldots$$

z being the experimentally observed reduced displacement, the expression for f(j) in three-component systems is as follows:

$$f(j) = \sum_{i=1}^{q} a_i H(z_i) + \ldots \qquad (17)$$

where $z_i = x/2\sqrt{D_i t}$

and $$a_i = \frac{\Delta n_i}{\sum_{i=1}^{q} \Delta n_i}; \qquad (18)$$

(ii) the quantity $H(z_A)$, where z_A is the experimentally observed reduced displacement for the fringe j defined in terms

of the "height-area" diffusion coefficient average D_A.[29]

$$z_A = x/2\sqrt{D_A t} \tag{19}$$

where D_A is defined by the following equation

$$D_A^{-\frac{1}{2}} = \sum_{i=1}^{q} a_i (D_i^{-\frac{1}{2}}). \tag{20}$$

The difference between the two quantities $H(z^*)$ and $H(z_A)$ is the required quantity, i.e.,

$$\Omega = H(z_A) - f(j). \tag{21}$$

The only two classes of two-solute mixtures for which convenient expressions have been obtained are (i) slightly impure solutes and (ii) solutes of similar diffusion coefficients — in the latter case a may be any value, but D_1/D_2 must be close to unity.

(i) Slightly Impure Solutes

If a_1 and D_1 denote the refractive index proportion and diffusion coefficient of the main solute, and a_2 and D_2 the respective quantities for the impurity, then $a_1 = 1 - a_2$ and $z_2 = z_1\sqrt{r_2}$, where $r_2 = D_1/D_2$.

Substitution of these values in equation (17) gives the following expression:

$$f(j) = H(z^*) = H(z_1) + a_2[H(z_1\sqrt{r_2}) - H(z_1)]. \tag{22}$$

Also, equations (19) and (20) may be combined to obtain an expression for z_A

$$z_A = \frac{x}{2\sqrt{D_1 t}} [1 + a_2(\sqrt{r_2} - 1)]$$

$$= z_1 + a_2(\sqrt{r_2} - 1)z_1. \qquad (23)$$

An expression for the quantity $H(z_A)$ may now be obtained from equation (23) by a Taylor's series expansion

$$H(z_A) = H(z_1) + a_2(\sqrt{r_2} - 1)z_1 H'(z_1) + \frac{a_2^2(\sqrt{r_2} - 1)^2 z_1^2}{2!} H''(z_1)$$

$$+ \frac{a_2^3(\sqrt{r_2} - 1)^3 z_1^3}{3!} H'''(z_1) + \cdots$$

$$= H(z_1) + a_2(\sqrt{r_2} - 1)z_1 H'(z_1) - a_2^2(\sqrt{r_2} - 1)^2 z_1^3 H'(z_1)$$

$$+ \frac{a_2^3(\sqrt{r_2} - 1)^3}{3} z_1^3(2z_1^2 - 1)H'(z_1) + \cdots \qquad (24)$$

Then

$$H(z_A) - f(j) = \Omega = a_2[(\sqrt{r_2} - 1)z_1 H'(z_1) + H(z_1) - H(z_1\sqrt{r_2})]$$

$$- a_2^2(\sqrt{r_2} - 1)^2 z_1^3 H'(z_1) + \cdots \qquad (25)$$

It is now necessary to express the quantity z_1 in terms of z^*, which can be found experimentally.

Since z_1 appears in equation (25) as terms at least of order a_2, the expansion of z_1 in terms of z^* need only be accurate to order a_2. Thus, from equation (22)

$$z_1 = z^* + \frac{a_2[H(z^*) - H(z^*\sqrt{r_2})]}{H'(z^*)} + \cdots \qquad (26)$$

whence

$$H(z_1) = H(z^*) + a_2[H(z^*) - H(z^*\sqrt{r_2})] + \cdots \tag{27}$$

Also, from equation (26)

$$H'(z_1) = H'(z^*) - 2a_2 z^*[H(z^*) - H(z^*\sqrt{r_2})] + \cdots \tag{28}$$

and

$$H(z_1\sqrt{r_2}) = H(z^*\sqrt{r_2}) + a_2\sqrt{r_2}\,[H(z^*) - H(z^*\sqrt{r_2})]\,\frac{H'(z^*\sqrt{r_2})}{H'(z^*)} + \cdots \tag{29}$$

Substitution of equations (26)-(29) into equation (25) results as follows

$$\begin{aligned}\Omega = {} & a_2[(\sqrt{r_2} - 1)z^*H'(z^*) + H(z^*) - H(z^*\sqrt{r_2})] \\ & + a_2^2\,[H(z^*) - H(z^*\sqrt{r_2})]\left[\sqrt{r_2}\left(1 - \frac{H'(z^*\sqrt{r_2})}{H'(z^*)}\right) - 2(z^*)^2(\sqrt{r_2} - 1)\right] \\ & - (\sqrt{r_2} - 1)^2(z^*)^3H'(z^*) + \cdots \end{aligned} \tag{30}$$

or $$\Omega = a_2 F(z^*\sqrt{r_2}) + a_2^2 G(z^*\sqrt{r_2}). \tag{31}$$

Thus the difference Ω is now expressed in terms of z^*, a_2 and r_2. The complex functions $F(z^*\sqrt{r_2})$ and $G(z^*\sqrt{r_2})$ have been tabulated for values of z^* and r_2.[17]

The values of z_A may be determined by measuring the separation between fringes symmetrically placed about the J/2 position, since such separations, as shown in the section on two-component diffusion, are free from first order concentration-dependence effects and are hence identical with the separations expected on the basis of ideal diffusion. Then

$$z_A = \frac{x_{J-j} - x_j}{4\sqrt{D_A t}} \tag{32}$$

However, if the system exhibits concentration-dependence, the value obtained is only an approximation to that quantity and consequently it is necessary to define a new function Ω_R such that

$$\Omega_R \equiv H(v_j) - f(j) \tag{33}$$

where v_j is the estimate of z_A obtained by the symmetrical procedure; i.e.,

$$v_j = \tfrac{1}{2}[(z_A)_{J-j} - (z_A)_j] = \frac{x_{J-j} - x_j}{4\sqrt{D_A t}}. \tag{34}$$

In the rigorous derivation of Ω_R, when the concentration-dependence of the diffusion coefficients and refraction increments are considered, it has been shown that the values of Ω_R obtained by this symmetrical comparation procedure are in fact free from first order concentration-dependence effects.[17]

(ii) Solutes of Similar Diffusion Coefficients

In cases where the ratio D_1/D_2 is approximately unity, the expressions for $H(z_A)$ and $f(j)$ are obtained in terms of $(\sqrt{r_2} - 1)$ in lieu of a_2, so that the value for Ω may be determined without restriction on the value of a_2. Expansion of the quantities $H(z_A)$ and $f(j)$ in terms of $(\sqrt{r_2} - 1)$ leads to the following expression for Ω :

$$\Omega = (\sqrt{r_2} - 1)^2 a_2(1 - a_2)(z^*)^3 H'(z^*)$$

$$- (\sqrt{r_2} - 1)^3 \left\{ 2a_2^2(1 - a_2) + \frac{2(z^*)^2 - 1}{3}[a_2 - 3a_2^2 + 2a_2^3] \right\}$$

$$(z^*)^3 H'(z^*) + \ldots \tag{35}$$

(c) Deviation Plots

The experimentally obtained values for the difference function Ω_R are plotted against the corresponding $[H(z^*)]^3$ values, the result of which is called a "deviation plot"; Fig. II-2 is typical of such a plot. Ω_R is plotted against the quantity $[H(z^*)]^3$ because the limiting slope as $H(z) \longrightarrow 0$ is then finite. The abscissa position of the maximum of the deviation plot is determined essentially by the ratio D_1/D_2 because if a_2 is sufficiently small, the $a_2^2 G(z^*\sqrt{r_2})$ term in equation (31) may be neglected: i.e., $\Omega_R \sim a_2 F(z^*\sqrt{r_2})$. An inspection of the table of $F(z^*\sqrt{r_2})$ values[17] will thus give an approximate value of r_2; a_2 may then be determined for this value of r_2. A value for D_1, the diffusion coefficient of the main solute may then be obtained by use of equation (20). However, in practice the position of the maximum is not very sensitive to variation in r_2 and hence upper and lower limits for r_2, and the corresponding values of a_2, are usually given (Fig. II-2).

(d) Practical Aspects of the Analysis Procedure

Having discussed the analysis procedure from the theoretical side, the methods used to determine the various experimental quantities mentioned in the theory, will now be indicated.

(i) Determination of D_A.

This quantity may be obtained by a slight modification of the single solute procedure for determining $D_{\overline{C}}$. Creeth and Gosting[17] have shown that, providing z^* is small, the quantity (v_j/z^*) is proportional to $(z^*)^2$ and approaches unity as $(z^*) \longrightarrow 0$. There-

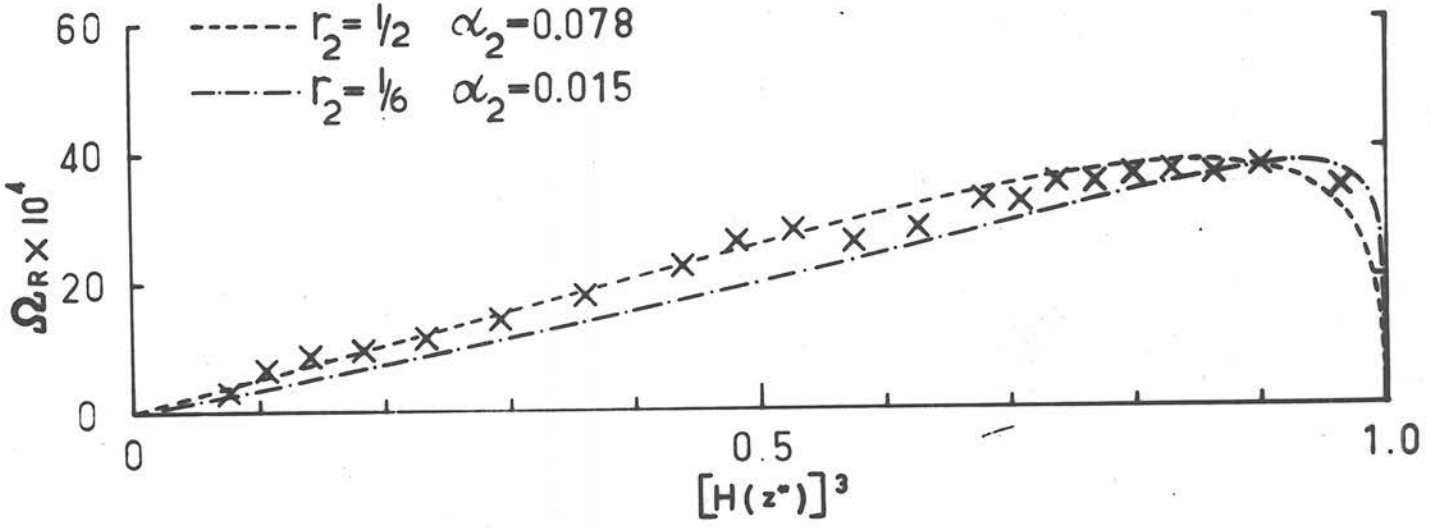

Fig. II-2. A typical "Deviation Plot": the crosses represent the observed deviation, whilst the lines are attempts at curve fitting.

fore, from equation (34) a plot of $(\Delta x/z^*)$ versus $(z^*)^2$ will extrapolate to $4\sqrt{D_A t}$. The actual method used is to plot the quantity $(Y_t/\sqrt{t})$ versus $(z_2^*)^2$, whence D_A is given by equation (36).

$$D_A = \frac{1}{4M^2}\left[(Y_t/\sqrt{t})_{z_2^* \to 0}\right]^2 \qquad (36)$$

where M is the magnification and Y_t is the quantity defined in equation (15).

(ii) Calculation of v_j

Mention should also be made, whilst on the subject of experimental determinations, of the method used for determining v_j. It can be seen from equations (15), (34) and (36) that

$$v_j = \frac{X_{J-j} - X_j}{2(Y_t)_0} \qquad (37)$$

where $(Y_t)_0$ is the extrapolated value of Y_t at $z_2^* = 0$ and time t. However, because $(Y_t/\sqrt{t})_0$ can be obtained with greater accuracy than $(Y_t)_0$, v_j is calculated from equation (38)

$$v_j = \frac{X_{J-j} - X_j}{2\sqrt{t}\,(Y_t/\sqrt{t})_0} \qquad (38)$$

(iii) Determination of Concentration Dependence

As in the case of single solute systems, unsymmetrical pairing of the fringes can be used to give the apparent skewness of the boundary. However, the deviations used in such a plot will include a contribution from heterogeneity, so that the linear relation with $\Delta R(z^*)$ will be lost. The contribution from heterogeneity can be determined and thus the skewness due to the concentration dependence of the diffusion coefficient calculated.

Creeth[18] has shown that

$$\Delta z_A = \Delta z^* + \Delta\left(\frac{\Omega_R}{H'(z^*)}\right) - k_1\left(\frac{\Delta C_1}{2}\right)\Delta R(z^*) + \ldots \qquad (39)$$

where Δz_A is obtained from the pairing of any two fringes, k and l, i.e.,

$$\Delta z_A = (x_k - x_l) / 2\sqrt{D_A t} \qquad (40)$$

and $\Delta\left(\frac{\Omega_R}{H'(z^*)}\right)$ is obtained by pairing the corresponding values of $\Omega_R/H'(z^*)$. Thus the use of D_A is avoided by expressing Δz_A in terms of a difference in comparator readings and the mean quantity $\overline{(Y_t/\sqrt{t})}_0$; equation (41) is used.

$$\frac{\Delta X}{\overline{(Y_t/\sqrt{t})}_0\sqrt{t}} - \Delta z^* - \Delta(\Omega_R/H'(z^*)) = -k_1\left(\frac{\Delta C}{2}\right)\Delta R(z^*). \qquad (41)$$

Thus k_1 may be determined from the slope of a graph of the function on the left of equation (41) versus $\Delta R(z^*)$.

Thus methods have been outlined for the determination of

(a) the "height-area" average diffusion coefficient, D_A;

(b) the deviation of the system from a single solute system in terms of a "deviation plot";

(c) an upper and lower limit for the ratio of the diffusion coefficients and for the amount of impurity;

(d) an upper and lower limit for the diffusion coefficient of the main solute; and

(e) the concentration-dependence of the diffusion coefficient.

However, it must at all times be remembered that such an analysis is possible only if the three assumptions, mentioned at the beginning of this section on three-component systems, are valid. There seems little reason to doubt the second and third assumptions, but the possibility of interaction of flows in diffusing multi-component systems must be borne in mind when using this analysis or its Gouy counterpart.[15]

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CHAPTER III

ELECTROPHORETIC FRACTIONATION AND DIFFUSION OF OVALBUMIN

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1. Introduction

Crystalline ovalbumin has been shown by electrophoresis to contain at least three components,[1-4] designated A_1, A_2 and A_3 in order of descending negative mobility. As mentioned in Chapter I, sedimentation[5-7] and early diffusion[8] studies have failed to reveal this heterogeneity. Perlmann[9,10] has presented evidence which indicates that the only difference between the components may be the possession of two, one and zero phosphate groups by the A_1, A_2 and A_3 molecules respectively. If this were the case, the diffusion and sedimentation coefficients would be essentially identical, and the mixture would diffuse and sediment as one species. Therefore, the crystalline native ovalbumin, henceforth called the ovalbumin complex, and fractionated A_1 should possess identical diffusion coefficients. It was anticipated that a critical test of this conclusion could be made by applying the diffusion theory outlined in the previous chapter to Rayleigh interferograms of the ovalbumin complex and the A_1.

2. Experimental

(1) Preparation of Ovalbumin

It would no doubt be preferable in this kind of investigation to use eggs from one particular hen to ensure that heterogeneity is not being introduced due to genuine minor differences in the molecules produced by different birds (cf. the case of β_1 and β_2 lactoglobulins investigated by Aschaffenburg and Drewry[11]). In an extensive electrophoretic study of egg-white proteins produced by a

number of breeds of fowls, Sibley and Johnsgard[12] have found no significant variation in these proteins from the various breeds. However, they have reported that some protein samples, independent of breed of fowl, showed a distinct tendency of the A_1 peak to separate into two components. The advantages associated with the use of eggs from one hen, however, might well be nullified by the consequent problem of differences in storage time. In this study fresh eggs have been used in preference to eggs from a particular hen.

Crystalline ovalbumin, sample 1, was obtained from fresh hens' eggs by conventional salt fractionation techniques using ammonium sulphate,[13] the sample being recrystallised twice. Electrophoretic analysis, using a "veronal" buffer, ionic strength 0.05 and pH 8.7_0, gave the result shown in Fig. III-1: the apparent proportions of the components are approximately 83% of A_1 and 17% of A_2 (including presumably the small amount of A_3), a result in quite good agreement with the values reported in the literature[4,10] (approximately 80% A_1, 15% A_2 and 5% A_3). Ovalbumin, samples 2 and 3, were identical with sample 1 except that they were freed from a trace of globulin by preparative electrophoresis; the actual method was identical with that used for the fractionation of A_1, discussed below. The apparent proportions of A_1 and A_2, as judged from electrophoresis patterns, were 82% and 18% respectively, i.e., values essentially identical with those for sample 1, since the separation of the components is not good and the values cannot therefore be accurately determined.

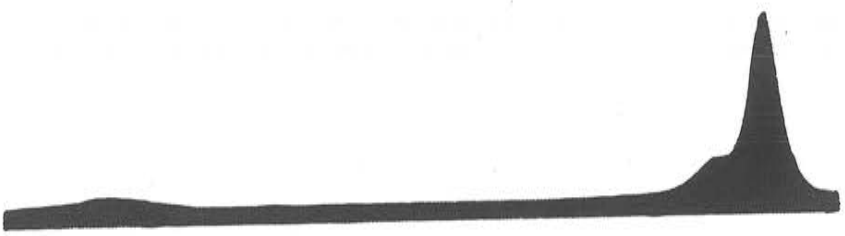

Fig. III-1. Electrophoretic pattern (ascending limb) of the ovalbumin complex at pH 8.7_0 in "Veronal" buffer, ionic strength 0.05, after three hours at a potential gradient of 6.2 volts/cm.

(2) Preparation of Fractionated A_1

The leading electrophoretic component, A_1, was obtained by prolonged electrophoresis of samples of the ovalbumin complex; sample 2 has been used for this fractionation. The preparative electrophoretic technique may be divided into three stages.

(a) Removal of the δ boundary

Since electrophoresis was continued for long times, the boundaries were kept in view by withdrawal of liquid from the region above the descending boundary. As a result of this compensation procedure, the δ boundary would have been pushed into the bottom section and finally into the descending limb, where such a concentration gradient is gravitationally unstable. To avoid the possibility of convective disturbance in the ascending limb, therefore, initial experiments have been performed following the procedure of Charlwood,[14] in which the δ boundary is completely removed by increasing the buffer salt concentration in the supernatant solution. However, it was found that complete removal was unnecessary, a δ boundary of 10% of its original value causing no perceptible convection in the ascending limb during the 24 hour duration of the experiment. This partial removal of the δ boundary required a preliminary electrophoresis experiment on a 2% ovalbumin solution, which had been dialysed against "veronal" buffer, ionic strength 0.05, pH 8.7_0, for three days, using the dialysate as supernatant buffer, a count of the δ boundary fringes and an estimate of the supernatant excess from a previously determined graph. A 2% ovalbumin solution required about a 15% excess concentration for

the supernatant buffer, the actual value not being critical.

(b) Preparative experiment

The bottom section and the descending limb of the centre section of an 11-ml. Tiselius cell were filled with the protein solution which had been dialysed against the 0.05 ionic strength buffer. The remainder of the apparatus was filled with the buffer of the appropriate excess concentration (identical pH). Electrophoresis, using a current of 8mA, was commenced as soon as the boundary was formed at the bottom of the centre section and was continued for 24 hours, with intermittent compensation to keep the ascending boundary in view. Electrophoresis was then stopped and compensation continued until only A_1 remained in the ascending limb. The bottom section was then sectioned off and the protein in the ascending limb removed. The maximum yield for such a preparation was about 16%, no more being obtainable due to the convection in the descending limb caused by the passage through it of the δ and globulin boundaries from the ascending limb. To obtain sufficient protein for a diffusion experiment giving the recommended 100 Rayleigh fringes,[15] four such preparative experiments, each yielding approximately 6.5 ml. were performed consecutively.

(c) Check of purity

The purity of every sample prepared in this way was checked by electrophoresis, the run being performed in an 11-ml. Tiselius cell. The buffer used was again the "veronal" buffer, ionic strength 0.05, pH 8.7_0, the experiment being continued for three hours at a potential gradient of 6.2 volts/cm. In each case the fractionated A_1 was

shown to be free from A_2 within the limits of $\frac{1}{2}$ fringe in 100 fringes (Fig. III-2).

Perlmann[9] has also reported an electrophoretic fractionation to give pure A_1, but no details of method or yield were given. Recently, a method involving the use of a cellulose cation exchanger, carboxymethylcellulose, has been reported for the fractionation of A_1 in high yields.[16] However, in ion exchange and chromatographic procedures, the protein molecule undergoes a large number of transitions between the liquid and solid phases and it remains to be proved whether the forces involved have any marked effect on the protein molecule. Thus the procedure outlined above, although giving a much lower yield, has the advantage that the conditions are much milder and hence less likely to cause any change in the protein molecule.

(3) Diffusion

(a) Standardisation of the apparatus

Diffusion experiments were performed in a commercially available diffusiometer employing Rayleigh optics.[17] It was first necessary to establish both the absolute accuracy of the apparatus and its suitability for heterogeneity estimations. Creeth[18] has suggested the following three criteria for assessing the reliability of a Rayleigh diffusiometer when used with single solutes: (i) constancy of the reduced fringe separations Y_t, as defined by equation II-15, over the whole boundary; (ii) reasonable correlation between the observed and predicted skewness, with a linear relation between the deviations and the appropriate $\Delta R(z^*)$ function; (iii) accuracy

Fig. III-2. Electrophoretic pattern (ascending limb) of the fractionated A_1 at pH 8.7_0 in "Veronal" buffer, ionic strength 0.05, after three hours at a potential gradient of 6.2 volts/cm.

of the differential diffusion coefficient $D_{\overline{C}}$.

The substance used for the standardisation of the apparatus was sucrose, which has been well characterised by diffusion.[19,20] Considerable modifications of the apparatus, which are described in Chapter VIII, were necessary before satisfaction of the above criteria was obtained. At the completion of the alterations, the first of the above criteria was satisfied as was also the third, the discrepancy between the observed value for $D_{\overline{C}}$, and that obtained by Costing and Morris,[19] and also Longsworth,[20] being 0.1%, an allowable margin. Skewness results (calculated from equation II-16), whilst showing considerable scatter, were of the correct sign but of smaller magnitude than predicted. Since this discrepancy has no effect on the determination of differential diffusion coefficients or on the analysis for heterogeneity to be used (as symmetrical fringes are compared in both cases), the apparatus was judged satisfactory; skewness results have therefore been treated qualitatively only.

A final check of the suitability of the analytical procedures, outlined in the previous chapter, to results obtained from this instrument was made by observing the diffusion of a synthetic mixture of sucrose and urea, for which a theoretical result could be calculated (from equation II-31). The agreement between the observed and theoretical results for this experiment, discussed in Chapter VIII, was regarded as satisfactory proof that the instrument could be used for the subsequent diffusion work. However, because of the relatively low camera magnification (~ 1.1),

deviation plots obtained from experiments with $J < 70$ cannot be considered reliable.

(b) Diffusion of protein samples

The diffusion experiments were performed at 0.5 - 1.0°C in accordance with the procedure outlined by Creeth.[15] To minimise complicating factors which arise if the net charge on the protein is finite, diffusion experiments with both the ovalbumin complex and the A_4 were performed at pH 4.59, a value close to the isoelectric point.[2,8,21] For this purpose a potassium acetate-acetic acid potassium chloride buffer, ionic strength 0.16, pH 4.59 was used. Prior to diffusion, all samples were dialysed with constant mild agitation and frequent changes of buffer, for four days at 2 - 3°C; thus with the crystallised ovalbumin complex, diffusion experiments were normally performed within five days of the initial solution of the paste. With electrophoretically prepared materials, approximately ten days elapsed between the time of initial solution of the paste and the commencement of diffusion.

3. Results

The diffusion data obtained on the ovalbumin complex and the fractionated A_4 are summarised in Table III-1. The table is self-explanatory except for the following: (i) the Δt values in column 4 represent the "zero-time" corrections[22] and generally are an indication of the sharpness of the boundary, small Δt values being associated with sharp boundaries. In this particular instrument, which uses a doubled optical path, the values obtained for Δt are some two to five times greater than those reported for

Table III-1

Diffusion Results on the Ovalbumin Complex and Fractionated A_1

1 Expt. No.	2 Material	3 Total no. of fringes = J[a]	4 Δt sec	5 $\mathcal{D}_A$[b] at 1.00°C $cm^2\ sec^{-1} \times 10^7$	6 Ω_R max. $\times 10^4$	7 r_2	8 a_2	9 D_1 $cm^2\ sec^{-1} \times 10^7$
1	AC[d],S1	83.23	60	3.96_0	37	1/2,1/6	.078,.015	3.75,3.86
2	AC,S1[e]	85.64	160	3.84_6	36	1/2	.074	3.69
3	AC,S1	130.97	210	3.92_9	44	1/2,1/6	.093, .018	3.82,3.85
4	AC,S2	34.18	290	3.94_2	-	-	-	-
5[c]	AC,S2	60.15	80	3.92_1	-	-	-	-
6	AC,S3	89.66	0	3.94_2	27	1/2,1/6	.058,.010	3.81,3.89
7	AC,S2	118.05	590	3.92_6	73	1/2,1/6	.146,.029	3.60,3.79
8[c]	A_1	27.05	480	3.95_9	-	-	-	-
9[c]	A_1	47.96	30	3.96_4	-	-	-	-
10	A_1	80.18	530	3.92_5	11	(1/1.5,1.5)	(.059,.055)	3.84,4.02
11	A_1	100.43	250	3.94_4	28	1/1.5,1/6	.155,.011	3.72,3.89

[a] In this apparatus, J = 100 corresponds to a concentration increment of 0.58_2 g./100 ml. for ovalbumin, based on Perlmann and Longsworth's[23] value for the specific refraction increment.

[b] Values corrected for buffer relative viscosity.

[c] Obtained by dilution of a more concentrated sample.

[d] AC stands for albumin complex, S for sample no.

[e] After its initial solution, this material had been stored for three months under saturated ammonium sulphate, and was used without recrystallization.

single path optical systems, in agreement with observations of Longsworth using a similar apparatus.[24] Also, diffusion pictures taken at times such that $\sqrt{Dt} < 0.1$ cm. have proved slightly inconsistent with later exposures. The inability to use these early exposures has resulted in much greater difficulty in defining the zero time accurately, but since t for the experiments is of the order 4×10^5 seconds, even the largest Δt values have negligible effect on the measured height-area average diffusion coefficients. (ii) In column 5, following the nomenclature of Baldwin, Dunlop and Gosting,[25,26] values of $\mathcal{D}_A$ are listed instead of D_A, the reason being that the quantity measured will only be the reduced height-area average diffusion coefficient in the absence of flow interactions and second-order concentration-dependence effects.[18,27] Thus $\mathcal{D}_A$ is the quantity measured by application of equation II-36 to these results. (iii) In column 6 the maximum values of the relative fringe deviation, Ω_R, calculated from equations II-8,33,38 are reported, the actual deviation graphs being shown in Fig. III-3. The crosses represent the mean of four observations. The graphs all show a maximum at the extreme right of the picture, a characteristic of the presence of a fast diffusing impurity. The diagram referring to experiment no. 2, a sample which had been stored in ammonium sulphate suspension after its initial solution, indicates the presence of some slow impurity <u>in addition to</u> the fast, which correlates with the lower value of $\mathcal{D}_A$. On the assumption that the deviations are due solely to heterogeneity, i.e., $\mathcal{D}_A = D_A$, analysis by means of the curve fitting procedure, described in the previous chapter, has been performed, the results of which are given

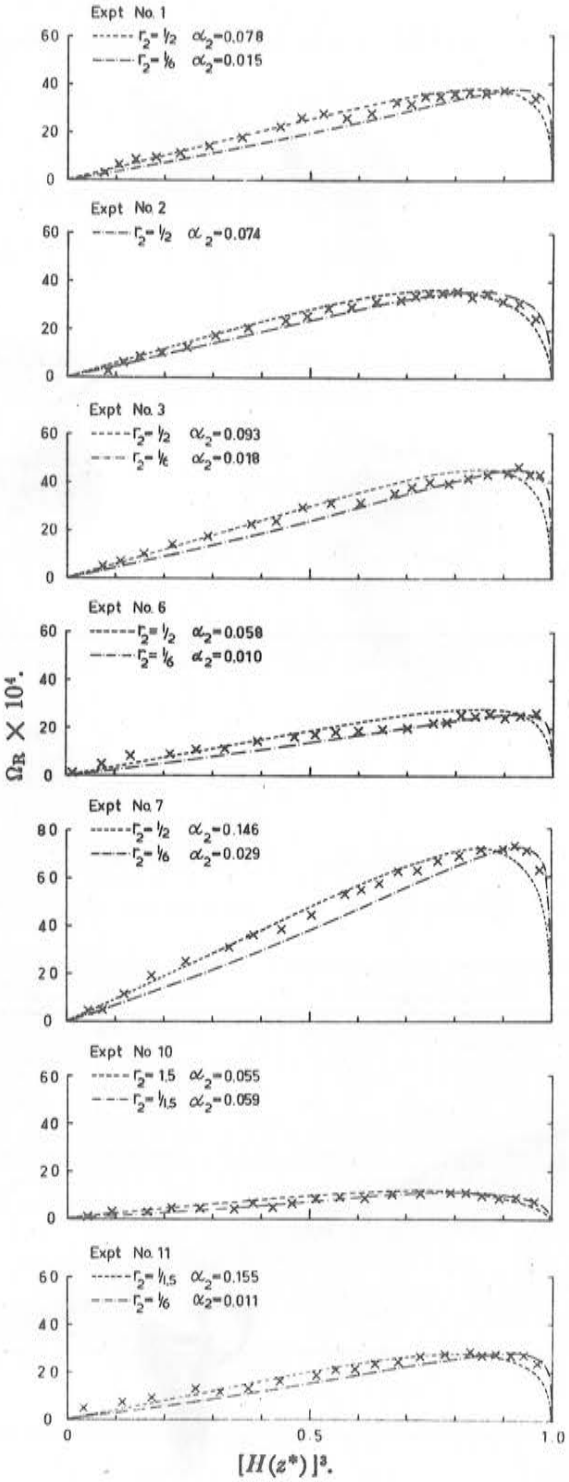

Fig. III-3. "Deviation Plots" of the ovalbumin complex and fractionated A_1, the experiment number referring to Table III-1. The lines are attempts at curve fitting, the results of which are indicated in each case. In expt. no. 2, no unique pair of values α_2 and r_2 can be given for the dashed curve; for $\alpha_2 = 0.5$, r_2 is either 0.83 or 1.17 the corresponding D_1 values being 3.51 and 4.17 respectively, but the material probably contains both fast and slow impurities - see text.

in columns 7-9. On this basis, therefore, the value of D for the main component lies within the limits 3.75 and 3.86 x 10^{-7} cm.^{2} sec.$^{-1}$ for experiment no. 1. It is to be noted that neither the $\mathcal{D}_A$ nor the D_1 values demonstrate any significant concentration-dependence, a result which has been generally confirmed by calculations of the boundary skewness using equations II-39,41: Fig. III-4, an example of the skewness plot, refers to experiment no. 7. It can be seen that the experimental points lie essentially on a line of zero slope, cutting the $[\Delta z_A - \Delta z^* - \Delta(\frac{\Omega_R}{H'(z^*)})]$ axis very close to the origin.

The results obtained with A_1 are shown in the lower part of the table, experiments 8-11, the deviation plots for the more concentrated samples being shown in Fig. III-3. It is immediately obvious that there is no concentration-dependence of $\mathcal{D}_A$ and that there is little difference between the observed values for $\mathcal{D}_A$ of the A_1 and those of the complex. However, the deviation plots are definitely smaller in magnitude, particularly in the third experiment listed, where, moreover, the shape of the deviation plot differs from that of the complex. Whilst the flatter curve is indicative of a greater purity, curve fitting of such a small deviation plot is very difficult because the abscissa corresponding to the Ω_R max is ill-defined. Thus the r_2 values quoted in this case are not so reliable as the others and have been bracketed to indicate this unreliability. While the D_1 values are subject to less uncertainty than the r_2's, values other than the ones quoted are possible if $1 < r_2 < 1.5$ or $1/1.5 < r_2 < 1$. It should be emphasised that an

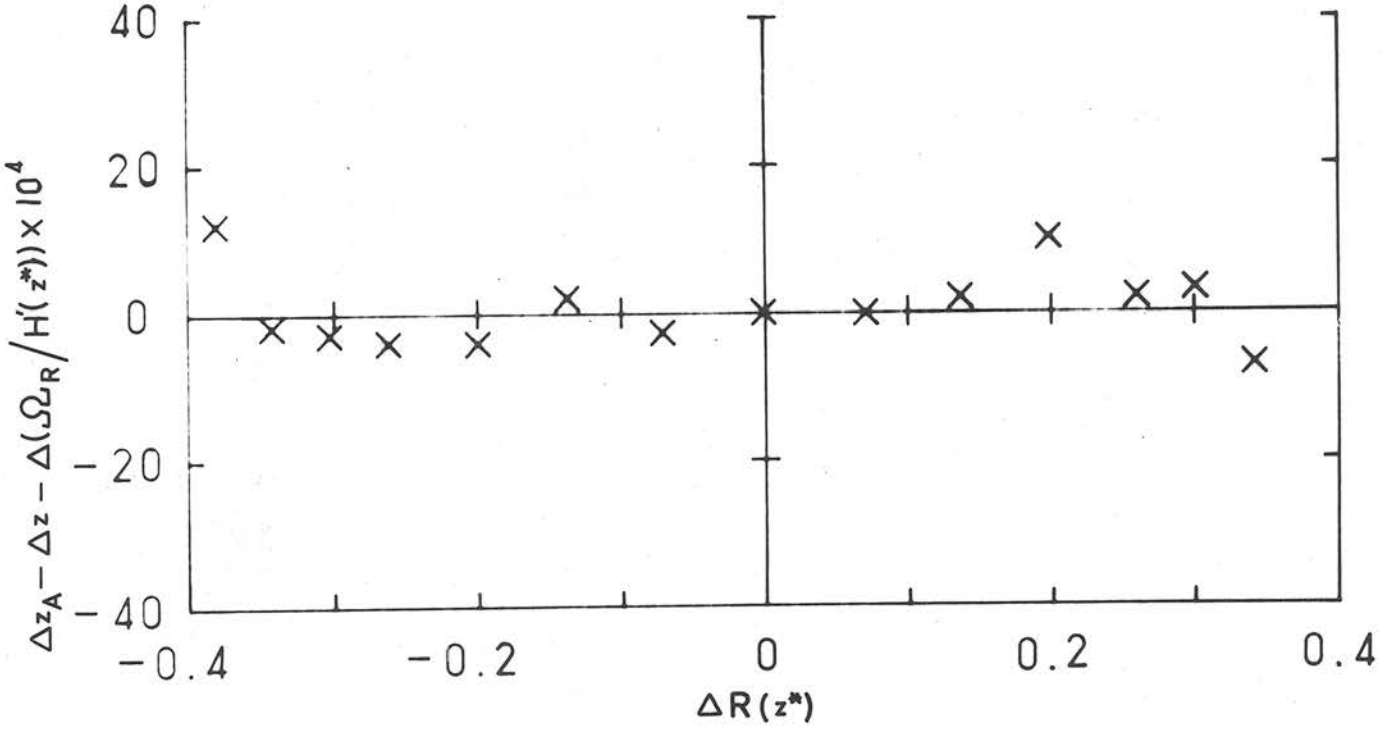

Fig. III-4. Skewness diagram obtained in expt. no. 7. The crosses represent the mean of 4 experimental observations.

error of 3×10^{-4} in the Ω_R values, which is in fact the estimated error in these measurements, for the three points corresponding to the largest $[H(z^o)]^3$ values would result in a curve characteristic of $r_2 = 1/3$, while the D_1 would still be in the range given. The second experiment on A_1, for which analysis is possible, shows the same kind of deviation plot as the ovalbumin complex, the value for D_1 also being essentially identical.

In experiments with substances as labile as ovalbumin, the question of variation of the diffusing protein with time should be considered. Such a change was not reflected in the diffusion results since the later values of (Y_t^2/t) lay on the same line (when plotted versus 1/t) as the early ones and also because the deviation plots showed no dependence on time. However, an electrophoresis experiment performed on one of the A_1 samples recovered from the diffusion cell did show a trace of slower component, which was not present prior to diffusion. This can be seen by comparison of Fig. III-5, which refers to the material after diffusion, with Fig. III-2, the same material before diffusion. As the electrophoresis required dialysis to pH 8.7_0, necessitating further handling of the material and lapse of time, this cannot be taken as unequivocal evidence of any change during the course of the diffusion experiment but is certainly illustrative of the general difficulties encountered in experiments with ovalbumin. Cann and Phelps[28] have recently postulated the occurrence of an isomerisation equilibrium in aqueous ovalbumin solutions, to account for certain features of the electrophoresis behaviour in dilute acetate buffers acid to the isoelectric

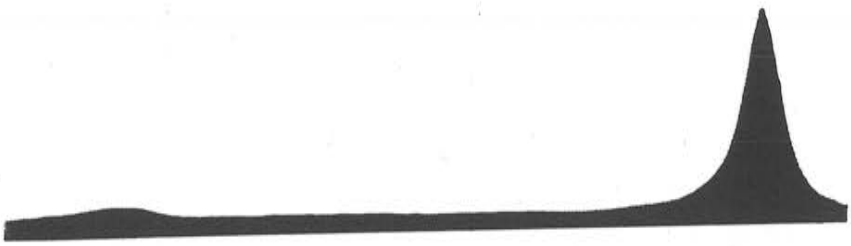

Fig. III-5. Electrophoretic pattern (ascending limb) of fractionated A_1 at pH 8.7_0 in "Veronal" buffer, ionic strength 0.05, after three hours at a potential gradient of 6.2 volts/cm., the material having been recovered from the diffusion cell.

point; there is, however, no evidence to show that such an equilibrium exists under the conditions used in the experiments reported here.

4. Discussion

Within the limitations of the Stokes-Einstein temperature correction[29] the diffusion coefficients reported here agree fairly well with earlier values (corresponding to 1°C): Lamm and Polson[30] found 4.21×10^{-7} cm.^{2} sec.$^{-1}$ (corrected from measurements at 20°) using the scale method, Longsworth[8] found 4.11 to 4.30×10^{-7} (diagonal schlieren) and McKenzie et al.,[31] also using the diagonal schlieren method, obtained the value 4.16×10^{-7} cm.^{2} sec.$^{-1}$ (corrected from measurements at 20°). Champagne,[32] using Scheibling's interferometric technique[33] quotes a range of values in the region of 3.97×10^{-7} cm.^{2} sec.$^{-1}$ (corrected from measurements at 20°) for D_1; she found a slow diffusing impurity ($r_2 \sim 3$) in most samples investigated. The values for the self-diffusion coefficient of ovalbumin found by Wang, Anfinsen and Polestra[34] are at too high a concentration for comparison.

With regard to the similarity of the $\mathcal{D}_A$ values of the ovalbumin complex and the A_1, it should be noted that this may be slightly misleading because $\mathcal{D}_A$ is relatively insensitive to fast impurities — in these experiments the deviation plots are of comparable value to the average diffusion coefficients, and the former show quite a large variation whereas the latter are essentially constant. However, from the general nature of the deviation plots, it can certainly be said that the heterogeneity as indicated by

diffusion for the ovalbumin complex is not in any way related to the heterogeneity detected by electrophoresis. Electrophoresis reveals a 15% impurity, whereas diffusion reveals, for example, 2-4% of an "impurity" with D four times that of the main component. Furthermore, one of the A_1 samples also contained this fast diffusing "impurity". The question is therefore raised as to the significance of the deviation plots, and it is clear that a decision as to their meaning should be obtained before any conclusions can be drawn about the reliability of the D_1 values. There are two possible explanations:

(i) The ovalbumin complex may contain a relatively rapidly diffusing impurity which has the same electrophoretic mobility as A_1; this would result in both the complex and the A_1 containing the impurity. This seems, a priori, most unlikely, and, in addition, the result of experiment 10, showing no such fast impurity, seems to exclude this possibility.

(ii) The only other explanation is that one or more of the basic assumptions of the analysis theory may not be fulfilled. As already stated in the preceding chapter, these assumptions are: (a) the diffusion coefficient of each solute is a linear function (of small magnitude) of its concentration and is unaffected by the presence of other solutes, (b) the refraction increment of each solute is a linear function (of small magnitude) of its concentration and is unaffected by the presence of other solutes and (c) the flow of each solute component is independent.

In this case there is no reason to suspect assumptions (a) and (b) and therefore assumption (c) must be incorrect. The

subject of flow interaction is receiving a great deal of attention at the moment[25,26,35-39] and a recent paper by Dunlop[37] seems pertinent to this case. He found that the diffusion of raffinose in the presence of potassium chloride, there being no gradient of potassium chloride across the boundary, resulted in appreciable deviation from single solute behaviour. Furthermore, the deviation graphs obtained in such systems were identical in form with those indicating heterogeneity; for example, interpretation of the deviation graph of one of his experiments (experiment 2, ref. 37) in terms of an "impurity" would have led to the conclusion that about 0.7% of an "impurity" was present with a diffusion coefficient four times that of raffinose. The fact that the deviation plots in this work have the maximum at the extreme right, indicative of a fast impurity, thus becomes particularly significant: in the light of Dunlop's work it seems probable that the flow of protein has caused a very small flow of buffer salts (essentially KCl). No simple interpretation is possible of the value of the abscissa at which the maximum occurs, as this is determined by a complex function of the diffusion coefficients (equations 9-12, ref. 37; 20-51, ref. 36); however, for this case it is quite certain that the function would be of the same order as $D_{albumin}/D_{KCl}$, or roughly 1/20. Thus the observed deviation plots are qualitatively in accordance with the hypothesis of a flow interaction between protein and buffer. Furthermore, it should be noted that the values of Ω_R are generally smaller in the case of A_1 materials: the diffusion experiments were performed at pH 4.59, the isoelectric point of A_1 in buffers of

ionic strength 0.1.[2,8,10,21] Therefore in the experiments on the complex, some 15-20% of the protein bore a greater positive charge (probably about +1 per molecule). Flow interactions for systems involving ions must generally be expected to occur and would probably be of larger magnitude than those relating to neutral molecules; in this connection, however, it is interesting to note that in a recent study of bovine serum albumin at the isoelectric point and also 1.8 pH units away from it, O'Donnell et al.[40] found essentially identical deviation graphs.

5. Conclusions

The following conclusions may therefore be drawn from this work: (i) the ovalbumin components A_1 and A_2 undoubtedly have very similar diffusion coefficients, in agreement with the suggestion that they differ only in very minor respects; (ii) the curve-fitting procedures adopted to enable D_1 values to be obtained from the observed $\mathscr{D}_A$ values are not justified; however, as the corrections made are small, and in the right direction,[37] it is very likely that the D_1 values quoted are close to the true differential values desired; (iii) in order to prove conclusively that A_1 and A_2 have the same diffusion coefficients, two series of experiments would have to be performed, on both the ovalbumin complex and the A_1 materials. In the first series, $\overline{C}_{protein}$ is varied under constant buffer (b) conditions to enable the limiting value of $\mathscr{D}_A$ as $\overline{C}_{protein} \longrightarrow 0$ to be defined by extrapolation for a particular value of $\overline{C}_b$. This quantity is denoted $(\mathscr{D}_A)_{\overline{C}_p \to 0}$. Repetition of each of these series of experiments varying $\overline{C}_b$ and then

extrapolation of $(\mathcal{D}_A)_{\overline{c}_p \to 0}$ versus $\overline{c}_b$ would then presumably yield the true D for each of the ovalbumin components. Such values could then be used in the Svedberg molecular weight expression. This procedure is in essence that suggested by Dunlop,[37] but would be independent of any pH changes caused by varying buffer composition.

It is quite clear, therefore, that final proof of the identity of diffusion coefficients for the constituents of ovalbumin can only come from the detailed analysis of a large number of experiments. However, prior testing of the procedure suggested in (iii) above with some protein system more stable than ovalbumin would be most desirable.

6. Possible Further Studies

The obvious extension of this work is to attempt the series of experiments outlined in (iii) above, which strictly applies only to the case of a pure protein. At present there does not seem to be a protein system that can withstand all of the physico-chemical tests for purity, chymotrypsinogen being the possible exception.[41] This protein has been reported to be homogeneous by solubility studies,[42] and also chromatographic studies.[43] Electrophoresis at pH 3 of the protein, which has an isoelectric point of 9.1 - 9.5 at ionic strength 0.1,[44,45] shows only one boundary,[42] as does electrophoresis at pH 8.4.[44] In the latter case, however, the boundary is not quite symmetrical, suggesting the presence of a slower moving impurity. Sedimentation studies have revealed the presence of only one component,[46,47] the pattern being quite symmetrical. The concentration-dependence of the sedimentation coefficient and also of the diffusion coefficient, both at pH 4, are such as to suggest

that a slight degree of dimerisation has occurred.[48] In view of this possible dimerisation at pH 4, and the lack of any sedimentation or diffusion data at the isoelectric point, chymotrypsinogen has been by-passed as a possible pure protein for the diffusion studies. It seems therefore that the A_1 component of this study complies with the requirements for a pure protein at least as well, if not better, than any known protein system and therefore appears to be the most suitable substance for the suggested extension of this work. Because large quantities of the protein would be required, the preparative method outlined in this study for obtaining A_1 does not seem a very fruitful source. However, there are two further possible methods for obtaining a pure ovalbumin component.

(a) Use could be made of the dephosphorylating enzymes prostate phosphatase and intestinal phosphatase to convert all of the A_1 and A_2 to A_3,[10] which could then be subjected to the series of experiments.

(b) The recent fractionation of ovalbumin into its three components by use of the cation exchanger, carboxymethylcellulose,[16] means that the series of experiments could be performed with pure A_1 and pure A_2, and thus comparison would not have to be made between a pure protein and a mixture (the complex).

As has already been mentioned, a protein system more stable than ovalbumin is required, and in this respect it is quite possible that a stable derivative could be prepared by modification of the more reactive groups of the ovalbumin molecule. Providing the modification increased the stability sufficiently, diffusion experiments could then be

performed at 25° instead of 1°, with the result that the time required for diffusion would be halved. In view of the large number of experiments needed for the diffusion study, stability at 25° is most desirable.

Therefore, because of the labile nature of the components of ovalbumin, the most logical approach is to modify the ovalbumin molecule in such a manner as to increase the stability, and then use either method (a) or (b) above to obtain a pure, stable protein sample on which the suggested diffusion studies could be performed. Accordingly, the question of modification of proteins is the next problem to be considered.

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CHAPTER IV

CHEMICAL MODIFICATION OF PROTEINS

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1. General

Since the chemical modification of proteins has been the subject of several excellent reviews,[1-3] the following summary contains only those features which, in the author's opinion, need special emphasis and which are relevant to the present study. The requirement of this particular work is a derivative of ovalbumin, which is more stable than the parent material, but which is as little changed in chemical configuration as possible; knowledge of the nature of the reaction causing the modification is also desirable. The topic will therefore be discussed from the following points of view.

(i) the chemical groups most likely to cause the instability of the native protein,

(ii) the reagents available for the chemical modification of such groups,

(iii) the evidence available which might aid the selection of the modification to be finally undertaken.

However, before embarking on this rather specialised outlook, there are some general features of modification which require emphasis.

(a) Definition

The first point to establish is the distinction between the terms "chemical modification of proteins" and "denaturation of proteins". This is perhaps most easily shown if a brief description of the present picture of protein structure is first given.

In the present model of protein structure, there are three fundamental types of structure distinguishable:[4,5]

(i) The primary structure — this is the polypeptide chain

formed by the condensation of the α-amino and α-carboxyl groups of the constituent amino acids.

(ii) The secondary structure — this results from the coiling of the polypeptide chain into a helical form in such a way that each amide group is bonded to the third amide group beyond by hydrogen bonds.[6] This type of coiling allows maximal hydrogen bonding.

(iii) The tertiary structure — these helices are assembled laterally or end-to-end and held together by covalent links such as disulphide bonds, or by hydrogen bonding or some much less permanent interactions between side chains.

In a helical structure, the side chains of the amino acid residues are all located on the outside of the coil and thus are in a favourable position for reacting in a manner similar to that of the parent amino acid. However, the superposition of the tertiary structure, and also the presence of adjacent groups, may affect the reactivity of the side chains of the amino acid residues.

The term "chemical modification" is reserved for non-ionic reactions involving these side chains, without gross alteration of the protein configuration.[1-3] Thus, protein salt derivatives and also materials obtained by hydrolysis of the primary peptide linkages are not included in this category. "Denaturation", on the other hand, is defined as an intramolecular change resulting from the rupture of labile electrostatic linkages;[7] this process is often regarded as the "opening-up" of the molecule by the breaking of the hydrogen bonds responsible for the tertiary structure, and perhaps secondary structure, of the molecule. Denaturation is accompanied

by very marked changes in the physical and chemical properties of the molecule; in fact the process is detected by means of these changes.

The division between the two processes of denaturation and chemical modification is obviously not clearly defined because the reaction of any protein group with a reagent will undoubtedly cause some rearrangement of the atoms in the close vicinity of the reaction site. This rearrangement may well result in the rupture of a few hydrogen bonds without being of sufficient magnitude as to cause a detectable difference in most of the molecule's properties. However, properties which depend critically on the amount of hydrogen bond breaking will most probably undergo considerable change during even a minor modification reaction. Therefore, in order to determine whether a protein has in fact been modified or denatured, it is necessary to discover any changes in the physical and chemical properties that have occurred, and to consider the magnitude of the change in structure necessary to cause such changes. Then, providing such considerations do not indicate any gross changes in configuration, then that protein may be regarded as having been modified.

(b) Group-specific Reagents

In order to know the nature of the modification performed on a protein sample, the use of a modifying reagent specific for one particular reaction on one particular type of group is required. Olcott and Fraenkel-Conrat[1] consider that the ideal reagent should meet the following criteria:

"(i) The amount of reagent introduced or used up should be

measurable.

(ii) The protein group to be reacted should be measurable.

(iii) The amount of reagent introduced or used up should be equivalent to the number of protein groups that have reacted.

(iv) The reaction should proceed under mild conditions, and, preferably be reversible by mild treatment."

A reagent meeting these requirements is said to be specific for the polar group involved, and in the next section methods using group-specific reagents are given preference when the types of modification available for the various polar groups are discussed. For the purpose of this study the need for reversibility of the modification reaction by mild treatment is not essential.

(c) The Groups Available for Modification

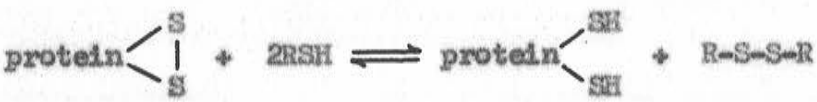
The chemical reactions of a protein are very largely those characteristic of the side chains of the amino acids constituting the protein. As has already been mentioned, these groups are in a favourable position for reaction, but the reactivity is often reduced due to the inaccessibility of the group resulting from the folding or coiling of the protein molecule. The existence of "inaccessible groups" in native proteins has frequently been demonstrated by the detection of the groups in question only after denaturation of the molecule; the first example was Arnold's discovery of sulphydryl groups in egg-white only subsequent to heat coagulation.[8]

Of the polar groups comprising the side chains of the amino acid residues, only the sulphydryl, amino, disulphide and phenolic groups will be discussed. The reason for this choice is

the greater reactivity of the above groups, as is evidenced by the almost exclusive attention accorded them in the search for the active sites of enzymes, hormones, toxins and viruses.[9]

(i) Disulphide Groups

The disulphide linkages, which are attributed to cystine residues, may be reduced to sulphydryl groups under relatively mild conditions, namely 25°C and neutral pH, using as reducing agents any of a variety of thiols, such as cysteine, reduced glutathione and thioglycollic acid.[10-14] The extent and rate of reduction under identical conditions varies for different proteins, and is greatly affected by the degree of denaturation.[12,15] However, since the reaction involved, namely,



is an equilibrium process, the reduced form of the protein exists only in the presence of excess reducing agent. The disulphide bonds of proteins are also attacked by two further "reducing" agents, cyanide[15,16] and sulphite (or bisulphite),[17] with the formation of one sulphydryl group and either a thiocyanate or a thiosulphate group: the process involved is thus not one of reduction, but of addition.

Because the reduction reaction is reversed by the removal of excess reducing agent, the oxidised form of the protein is likely to be more stable than the derivative obtained and hence the possibility of using reduction of the disulphide groups as a method of modification appears most unfruitful in the search for a stable derivative.

(ii) Amino Groups

These groups are usually attributed to the ϵ-amino groups of lysine,[18] although in many cases α-amino groups of other amino acids are free.[19-21] The reagents most commonly used for reaction with amino groups, without attempting differentiation between ϵ- and α-types, are ketene,[22] nitrous acid,[23] phenyl isocyanate,[24,25] formaldehyde[26] and acetic anhydride.[27] However, with the possible exception of acetic anhydride, all of these reagents are known to react with groups other than amino, particularly the sulphydryl and phenolic groups.[3] Acetic anhydride is much more specific in its action and the conditions of reaction are very mild, involving the addition of the reagent to a cold solution of the protein in sodium acetate.[27] The reaction proceeds in accordance with the equation

$$\text{protein} - NH_2 + O\begin{matrix}COCH_3\\COCH_3\end{matrix} \longrightarrow \text{protein} - NHCOCH_3 + CH_3COOH.$$

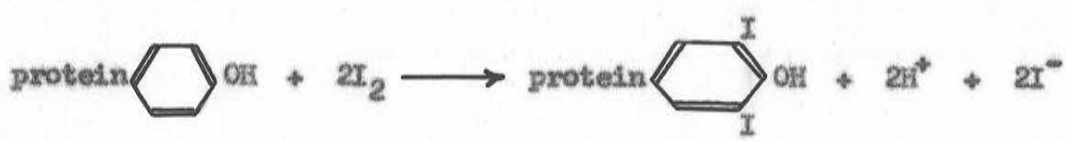
All of the amino groups of ovalbumin and bovine serum albumin appear to react, but reaction does not appear to be complete with all protein systems studied.[1]

The disadvantages of modification of proteins by this method is the doubt that exists about the specificity of the reaction: Olcott and Fraenkel-Conrat[1] present evidence for slight reaction with phenolic groups and also mention the possibility of reaction with readily accessible sulphydryl groups. Acylation of the amino groups has been dismissed as a method of modification suitable for this present study because of the lack of definite proof of the specificity of the reaction involved and also because it is difficult to see any

obvious transformation that the amino groups would tend to undergo in the native protein.

(iii) Phenolic Groups

These groups, which exist in proteins as tyrosine residues, have been studied in great detail because of their apparent essentiality for the activity of many biological systems.[9] An unfortunate aspect of all these studies, however, is the failure to find a reagent specific for the phenolic group. Reagents which react with phenolic groups almost invariably react with sulphydryl groups also: in fact it is only in the absence of the latter that any claims for specificity have been made.[1] Under such circumstances, iodination in neutral or alkaline media yields derivatives in which the tyrosine residues have been iodinated.[3] Di-iodinated and mono-iodinated products can be formed,[28-31] although the existence of the latter was disputed for many years. The overall reaction for the formation of the di-iodinated derivative is written as follows:



Kinetic studies, however, of the reaction with tyrosine suggest that hypoiodous acid may be the primary iodinating agent.[32,33] An objection to the use of iodine, even in the absence of sulphydryl groups, is the possibility of substitution reactions occurring with other amino acid residues: evidence for iodination of imidazole and also indole groups has been obtained.[1]

Therefore, although the phenolic group of proteins is quite

reactive and could quite well be the cause of any instability of the protein molecule, the absence of any reagent capable of modification of this group alone has caused the modification of this group to be by-passed in the search for a stable protein derivative.

(iv) Sulphydryl Groups

More work has been devoted to the chemical reactions of the sulphydryl group, which occurs in the native protein as cysteine residues, than to any other amino acid side chain.[1] The reason for this intense interest in this particular group is three-fold — firstly its great reactivity, secondly its essential presence in many enzyme systems,[9] and thirdly the early discovery of the great differences in its reactivity in native and denatured proteins.[8] Practically all of the commonly used modifying reagents react with the sulphydryl group;[3] several are reported to be specific. These "specific reagents", which all require only mild conditions for reaction to occur, have been divided into three categories:

(i) Mild oxidising agents — these reagents are reported to be specific for the oxidation of the sulphydryl groups to disulphide linkages in accordance with the following equation

$$\text{protein}\begin{matrix}\diagup\text{SH}\\ \diagdown\text{SH}\end{matrix} + \text{oxidant}^{+2} \longrightarrow \text{protein}\begin{matrix}\diagup\text{S}\\ \diagdown\text{S}\end{matrix} + \text{reductant} + 2H^{+}.$$

Reagents for which this specificity of reaction has been claimed include ferricyanide,[34] porphyrindin,[35] iodine,[36,37] iodosobenzoate[38] and cystine.[39]

(ii) Alkylating agents — the two reagents coming under this category, iodoacetamide and iodoacetate,[40,41] react in the following

manner:

$$RSH + ICH_2COO^- \longrightarrow RSCH_2COO^- + HI.$$

(iii) Mercaptide-forming agents — the most frequently used reagent of this class is p-chloromercuribenzoate,[38] the mechanism of action being:

protein — SH + ClHg⟨⬡⟩COOH ⟶ protein — SHg⟨⬡⟩COOH + HCl.

Barron and Singer[42] have criticised the use of the alkylating agents on the grounds that reaction may possibly occur with amino and phenolic groups. In their opinion, the use of oxidising agents is also not desirable because of the need of two sulphydryl groups sufficiently close together for disulphide formation.

The fact that oxidation procedures do appear to give quantitative estimations of the sulphydryl content leads to the conclusion that if the last statement of Barron and Singer's is correct (and it seems logical), the sulphydryl groups must be in positions favourable for disulphide formation. Furthermore, in view of the very mild conditions required, the possibility of spontaneous oxidation seems quite feasible. The cloudiness, general instability and likelihood of some precipitation which is always encountered with ovalbumin solutions could possibly be due to the formation of aggregates (dimers, trimers, etc.) as a result of oxidation of pairs of sulphydryl groups on neighbouring molecules: the great tendency for samples of the simple amino acids cysteine and reduced glutathione to convert to the oxidised forms in air lends support to this postulate of spontaneous sulphydryl-disulphide transformation in the protein molecule. Furthermore, the presence of air may not even be necessary

for the intermolecular disulphide cross-linking, which could result from mercaptan-disulphide exchange: evidence for such cross-linking in denatured ovalbumin solutions has certainly been obtained.[43-45] Also Klotz et al.[45] report that the addition of a disulphide dye to solutions of native ovalbumin results in the slow formation of intermolecular disulphide linkages. Thus it seems quite plausible that dimerisation of the ovalbumin molecule could result from mercaptan-disulphide exchange between a thiol group of one molecule with an intramolecular disulphide bond of another. Therefore, the presence of sulphydryl groups in native ovalbumin has been regarded as the most likely cause of instability in the molecule, and since reagents specific for reaction with this group have been reported, modification using such reagents has been chosen as the method most likely to produce a suitable derivative.

However, it should be emphasised at this stage that, due to the varying degrees of reactivity of the sulphydryl groups in native proteins, not all reagents will react with <u>all</u> of these groups within a molecule. This last statement is probably best illustrated by Hellerman's rather elegant study of the sulphydryl groups of urease:[38] three distinct types are reported:

(i) those which are non-essential for urease activity — all three classes of reagent can be used for their detection;

(ii) a second class which is detectable only by p-chloromercuribenzoate — reaction of these groups causes inactivation, but activity can be restored by reversal of the reaction;

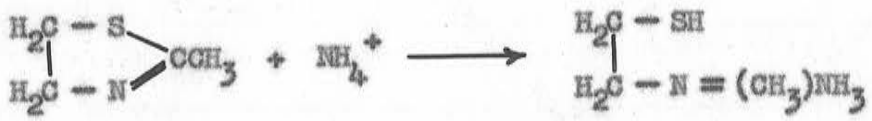
(iii) a third type of sulphydryl group which can only be detected after denaturation of urease.

There are three main theories to account for these differences in reactivity:

(a) The inaccessibility theory — the sulphydryl groups are located in the interior of the native, coiled molecule, and hence are inaccessible to the reagent. Denaturation results in the uncoiling of the molecule and exposure of these "inaccessible" groups.

(b) The bonding theory — this theory attributes the decreased reactivity to the effect of the intra-chain bonding, presumably hydrogen bonds,[46] on the protein groups. Rupture of these bonds on denaturation may well cause a general change in the reactivity of the sulphydryl groups quite apart from any exposure of groups from the interior of the molecule.

(c) The compound theory — this theory assumes that the sulphydryl groups of ovalbumin do not exist as such, but that they are involved in a specific compound, which breaks down on denaturation to release free sulphydryl groups. Linderstrøm-Lang and Jacobsen[47] have suggested that the "inaccessible" groups are actually cyclic structures such as the thiazolidines; such structures are very labile, the ring opening readily in the presence of ammonium salts to give freely reacting sulphydryl groups.



This cyclic structure has received support from Fredericq and Desreux,[48] who have studied physico-chemically the properties of native ovalbumin and the material obtained by modification with chloropicrin. More recently, Smith[49] has proposed that the "sulphydryl" group is

actually present as a thiol ester, i.e., $R-\overset{\overset{O}{\|}}{C}-S-R$. This thiol ester is readily cleaved by mercury or mercurials,[50] reagents which do in fact react with the "sulphydryl" groups of native proteins. He has given preference to this type of structure over the thiazolidine ring structure because the known properties of the latter do not meet the chemical or kinetic requirements for the active site of papain. It is apparent that these covalent bonds between the thiol and carboxyl groups must be maintained by the folding energy of the protein and stabilised by hydrogen bonds, since denaturation apparently breaks these covalent bonds and liberates the groups involved. The fact that the "inaccessible" sulphydryl groups do in fact react with some reagents, for example iodine, p-chloromercuribenzoate and, most important, substrates in the case of enzymes, suggests that these groups are highly reactive rather than "buried". Thus a theory of compound formation seems preferable to theories reliant upon inaccessibility.

The compound theory, in the author's opinion, affords the most logical explanation of the decreased reactivity, so that "unreactive sulphydryl groups" are most probably not sulphydryl groups at all. However, due to the similarity of the reactions of the thiol compound and the free thiol group and also their apparent ease of interconversion, the concept of reactive, slowly reactive and unreactive sulphydryl groups will be retained.

2. Modification of the Sulphydryl Groups of Ovalbumin

In view of the variation in the reactivity of sulphydryl groups within a single urease molecule, it is clearly necessary to establish the number of thiol groups that will react with the various reagents when the particular protein under study is in its native state.

(a) The Availability of the Sulphydryl Groups

The sulphydryl groups of ovalbumin are inaccessible to the majority of the specific reagents, and become available for reaction only subsequent to denaturation. There are a few reagents, however, which react with some, if not all, of the thiol groups: these will now be listed under the same categories as before.

(i) Oxidising Agents

Of these, only iodine is reported to react with the five sulphydryl groups of ovalbumin when the protein is in the native state.[36,37] In fact, no other oxidant mentioned has any reaction with ovalbumin until after denaturation: the value five is then obtained for the number of thiol groups, using any of the oxidising agents. The iodine oxidation of the native material is performed at 0°C in the presence of 1M potassium iodide. The reported specificity of this reagent is rather surprising since iodine is often used for the modification of tyrosine residues. Apparently, reaction of tyrosine is suppressed by high iodide concentrations.[37]

(ii) Alkylating Agents

Iodoacetamide is reported to react with two of the

sulphydryl groups of native ovalbumin at pH 9.[37,51]

(iii) Mercaptide-forming Agents

MacDonnell et al. have prepared a crystalline derivative of ovalbumin by the addition of p-chloromercuribenzoate to a solution of ovalbumin at pH 5.3, crystallisation being promoted by the addition of ammonium sulphate.[52] This derivative has been shown to contain three equivalents of mercury per mole, indicating that only three of the thiol groups have been modified. Boyer,[53] using a spectrophotometric method for determining mercaptide formation, has found that four moles of p-chloromercuribenzoate per mole of ovalbumin react at pH 7. In a study of the uptake by ovalbumin of p-chloromercuribenzoate at a variety of pH values, Cunningham et al.[54] have found that three molecules of reagent react per molecule of protein; this value was obtained using either a spectrophotometric[53] or back titration procedure[52] to determine the extent of reaction. However, in the light of Boyer's results,[53] it does appear that a derivative with four sulphydryl groups modified can be obtained using this reagent.

The question of the sulphydryl content of ovalbumin will be discussed in the next chapter, but it is necessary to mention at this stage that there is evidence, from the determination of the thiol content by a variety of methods on both native and denatured samples, for the existence of only four sulphydryl groups in the ovalbumin molecule,[52-56] so that the latter derivative may well be free of sulphydryl groups. However, the value five for the thiol content has also been obtained in a number of cases,[35,36,38,57-59] and hence the iodine-modified derivative has been prepared in this study in

preference to the p-chloromercuribenzoate derivative. Furthermore, should the existence of only four sulphydryl groups in native ovalbumin be correct, then the reaction of iodine cannot be specific for the oxidation of sulphydryl to disulphide and hence studies on this derivative, with a view to determining the nature of the reaction, could be performed.

(b) The Modification of Ovalbumin with Iodine

With the adoption of this method of modification, the procedures used by Anson will now be discussed in more detail. In his initial experiments on iodine oxidation, Anson performed the reaction in the absence of potassium iodide: the earliest experiments were done at pH 3.2[58] to prevent the iodination of the tyrosine residues, but subsequently[51] it has been found that the reaction can be performed in neutral solution without the oxidation of many of the sulphydryl groups beyond the disulphide stage and without iodination of many tyrosine residues. The actual report of the stoichiometric oxidation of the thiol groups of ovalbumin is only briefly mentioned in a paper concerned with the thiol groups of tobacco mosaic virus:[36] the only requirement definitely stated is the necessity of performing the reaction at 0°C in 1M potassium iodide. From the general context of the paper, it would appear that the reaction was carried out in neutral solution, as the buffer used for the tobacco mosaic virus work was a phosphate buffer pH 7: accordingly, this latter buffer was used in this study.

In all of his work with iodine, Anson has determined the

amount of iodine involved in the reaction titrimetrically, the end-point being detected by the use of starch (or negative nitroprusside test) as indicator. In a study such as this, where the oxidised protein solution is to be used for further physico-chemical measurements, the addition of starch to the solution is clearly impossible: if an indicator is to be used, it would have to be used externally. However, in this work, the iodine uptake has been followed by a potentiometric procedure, thus eliminating the use of an indicator, either internal or external.

(c) Experimental

The procedure adopted for modification was as follows. A solution of 10^{-3}N iodine in 1M potassium iodide was titrated into a solution of ovalbumin which was 1M with respect to potassium iodide and buffered at pH 7 by means of phosphate: the titration was performed in an ice bath to keep the temperature as low as possible. The uptake of iodine was followed potentiometrically using a calomel electrode as reference electrode and a bright platinum electrode, reversible for the reaction $I + e \rightleftharpoons I^-$. The e.m.f. (E) was recorded after each addition of iodine, manual stirring being employed to ensure complete mixing. An approximate end-point of the titration was then determined by plotting a graph of E versus the number of mls. of iodine solution added (V), the end-point being indicated by the point of inflection (point B in Fig. IV-1). A more accurate end-point was then determined by finding the point at which $d^2E/dV^2 = 0$. This was done by obtaining an approximation to the first derivative of the above curve and plotting this quantity

versus V. The first derivative (dE/dV) has been obtained approximately by dividing ΔE, the change in potential between two successive observations, by ΔV, the corresponding difference between the burette readings, and plotting this quantity ($\Delta E/\Delta V$) versus $\overline{V}$, the mean of the two burette readings. The end-point is indicated by the maximum in this curve (point B in Fig. IV-2). Typical titration curves for the titration of ovalbumin with iodine are given in Figs. IV-1 and IV-2.

The protein concentrations were estimated refractometrically by forming a boundary between the ovalbumin solution (to be used), dialysed against water to remove the ammonium sulphate, and the final dialysate; this was performed at 1°C in a 2-ml. Tiselius cell, Rayleigh optics being used to observe the boundary. The number of Rayleigh fringes so obtained was combined with the specific refraction increment for ovalbumin in accordance with the equation

$$C = \frac{J\lambda}{dk}$$

where C = protein concentration in g./100 ml., J = number of fringes, d = thickness of solution (twice width of cell), k = specific refraction increment and λ = wavelength of light (= 5,461Å). The value used for the specific refraction increment requires comment: of the three values recently reported for this quantity, two are in agreement,[60,61] the third[62] (and earliest observation) being 2.3% higher. The results of this latter paper for the variation of the specific refraction increment with temperature and wavelength of light have been applied to the more recent observations of Halwer et

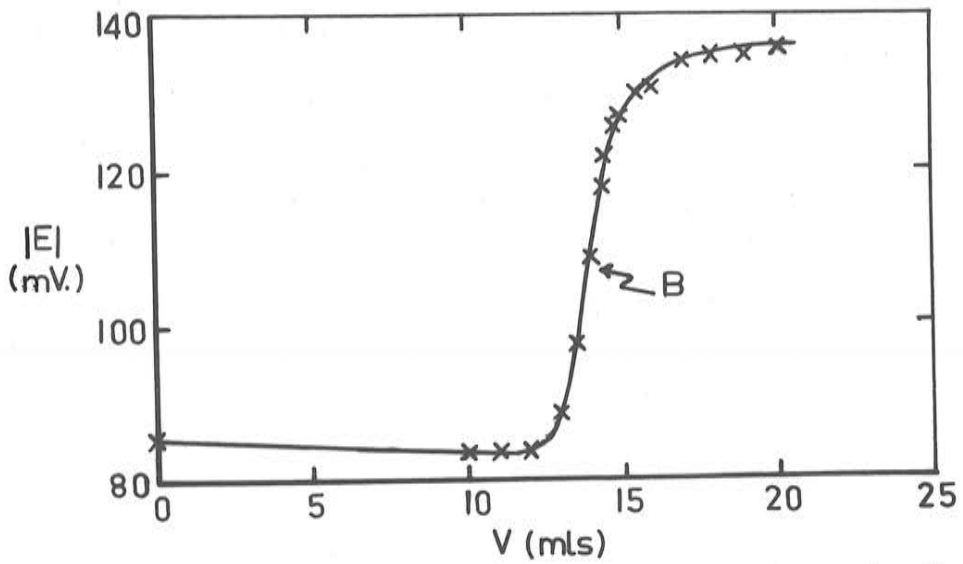


Fig. IV-1. A typical potentiometric titration curve of ovalbumin being titrated with iodine - integral curve.

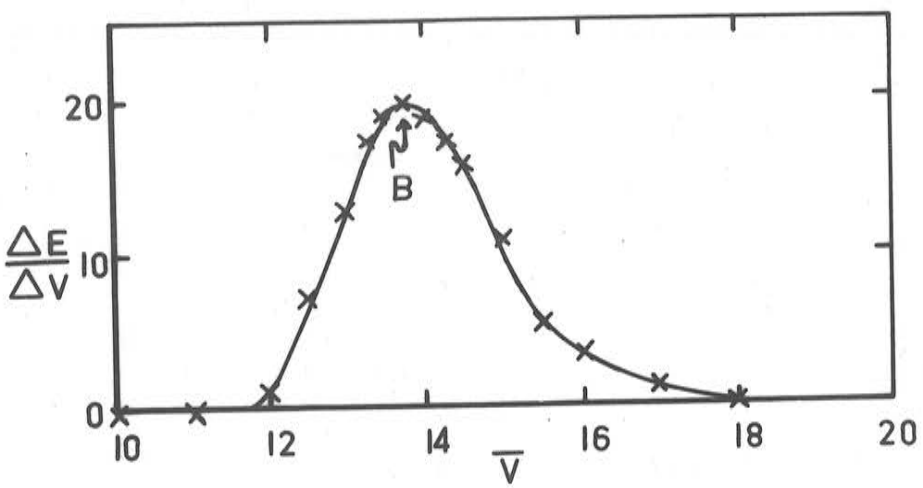


Fig. IV-2. A typical potentiometric titration curve of ovalbumin being titrated with iodine - first derivative of the integral plot.

al.[60] and Charlwood[61] at 25°C. A value 0.1845×10^{-6} was used for the specific refraction increment at 1°C.

The results obtained for the amount of iodine involved in reaction with ovalbumin are listed in Table IV-1.

Table IV-1

Protein preparation[a]	No. of moles protein x 10^6	No. of atoms iodine x 10^6	No. of iodine atoms per molecule[b]
AC,S2	6.53	32.3	4.93
AC,S3	3.76	19.0	5.07
AC,S3	3.76	18.6	4.96
AC,S3	3.76	18.6	4.96
AC,S3	7.51	37.8	5.03
		Mean	4.99 ± 0.06 (S.D.)

[a] The nomenclature used here to designate ovalbumin samples is that used in Chapter III.

[b] These calculations are based on a molecular weight of 45,000 for ovalbumin.

These results using a potentiometric procedure for determining the iodine uptake substantiate Anson's claim[36] that five iodine atoms are involved in the reaction with one molecule of ovalbumin if the reaction is performed at 0°C and in the presence of 1M potassium iodide. However, the value obtained in this study is much more conclusive than Anson's value of 4.4, obtained using either the negative starch test or the negative nitroprusside test subsequent to denaturation as indication of the end-point of the

titration. The similarity between the results reported here and those of other workers using different conditions appears to contradict Anson's observation that low temperature and high iodide concentrations are necessary to prevent oxidation beyond the disulphide stage. Hess and Sullivan,[63] who performed iodine titrations at pH 3.5 and 20°C (no potassium iodide), obtained values in the range 5.1-5.3 for the iodine uptake; this value was obtained with both native and denatured samples. This identity of values in the presence and absence of denaturants is in agreement with Anson's observation that no further iodine uptake occurs after denaturation.[57] MacDonnell et al.,[52] however, present results which indicate that at pH 3.2 (no potassium iodide), four iodine atoms react with the native molecule, a further atom of iodine reacting after denaturation: a value 5.1 was obtained for the number of atoms per molecule reacting with denatured ovalbumin. The essential identity of the results reported in this study and those of these other workers, although using different conditions, is indicative that the same reaction may be occurring.

In order to complete the preparation of the iodine-modified ovalbumin, there remains the problem of the removal of the excess iodine and also the potassium iodide. This was done by dialysis. Exhaustive dialysis against water was not possible because of the limited solubility of the derivative in salt-free solutions. Therefore the titration mixture was dialysed against phosphate buffer pH 7, ionic strength 0.04. The modified protein was kept in solution at all times, the above dialysed solution being stored at approximately

4°C until required for further experimentation. It is of interest to note that this solution could be stored for several months without any obvious signs of denaturation — the solution remained clear throughout the time and electrophoretic patterns showed no variation with time as is evidenced in Fig. IV-3. The drawings were obtained by differentiation of Rayleigh interferograms.

Before commencing a detailed diffusion study such as that outlined at the conclusion of the preceding chapter, it is necessary to obtain information on the following characteristics of the material to be studied:

(i) solubility

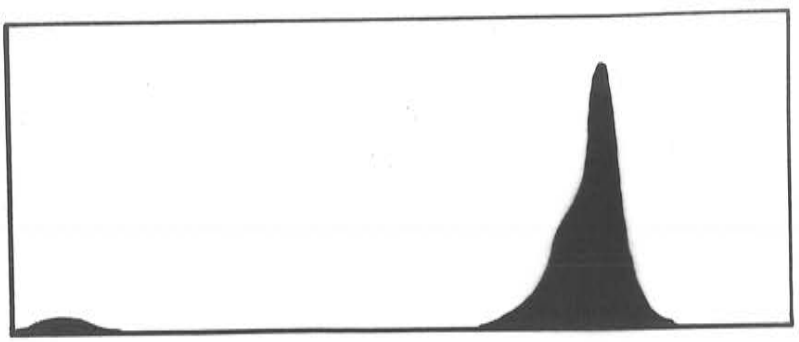
(ii) heterogeneity with respect to charge

(iii) heterogeneity with respect to size and shape

(iv) isoelectric point.

During the course of this characterisation of the iodine-modified ovalbumin, evidence was obtained which indicated that oxidation of sulphydryl groups to disulphide linkages was not the sole reaction occurring. Because of the desirability of knowledge of the nature of the reaction causing modification, emphasis has been placed on this aspect at the expense of the diffusion study. The remainder of this thesis thus comprises essentially a comparative study of the native and iodine-modified ovalbumin in an attempt to determine the nature of the reaction between iodine and ovalbumin.

The first of these studies to determine the nature of the reaction concerns the sulphydryl content of ovalbumin, as five such groups per molecule are necessary for the action of iodine to be



(a)

(b)

Fig. IV-3. Electrophoresis patterns (ascending limbs) of iodine-modified ovalbumin at pH 8.7$_0$ in "Veronal" buffer, ionic strength 0.05, after three hours at a potential gradient of 6.2 volts/cm.: (a) a freshly prepared sample; (b) a sample which had been stored for four months.

specific. However, as has already been indicated, there is evidence for the presence of four and five thiol groups. Consequently, the next chapter contains a survey of previous determinations of ovalbumin's thiol content and also the results of thiol estimations performed by the author.

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The Sulphydryl Content of Ovalbumin

In the preceding chapter on chemical modification of proteins, mention has been made of several reagents reported to react specifically with the sulphydryl groups of proteins: such group-specific reagents should be ideal for the determination of the sulphydryl content of proteins. However, application of a variety of these reagents to the determination of the thiol content of ovalbumin has resulted in an uncertainty as to the exact number of these groups in ovalbumin. The results of many investigations indicate the presence of five such groups, but there is also substantial evidence for only four thiol groups in ovalbumin. Now, whilst differences in the number of sulphydryl groups as determined by the various methods on native samples may be explained on the basis of differing reactivity of sulphydryl groups towards reagents, identical values should be obtained if the protein is first denatured, regardless of the method of determination. Variation in the thiol content of the different ovalbumin samples studied, while providing a possible explanation, seems, a priori, most unlikely in view of the essentially identical properties, such as crystalline form, electrophoretic composition, etc., reported for ovalbumin[1-7] prepared by any one of the three most common methods.[8-10] Therefore, it would appear that any variation in the value obtained for the sulphydryl content should be due to one or more of the following causes:

(i) the lack of a sufficiently sensitive method for determining the extent of reaction,

(ii) non-specificity of the reagent used for the determination,

(iii) incomplete liberation of the "masked" sulphydryl groups by the method of denaturation employed,

(iv) presence of an impurity in the protein sample.

1. Survey of the Literature

A survey of the methods used for the determination of the thiol content of ovalbumin, together with the results obtained by such methods, will now be undertaken to ascertain whether consideration of the above factors can plausibly account for the differences in the reported values. Since ovalbumin is so easily purified by repeated recrystallisation, it has been assumed that the discrepancies are not due to the presence of impurities in the samples studied. The results for the number of sulphydryl groups have been interpreted on the basis that the molecular weight of ovalbumin is 45,000.[11]

(a) Methods Involving Heterogeneous Titrations

The earliest estimations of the sulphydryl content of ovalbumin were performed on heat-coagulated or chloroacetic acid-precipitated samples.[12-14] Determination of the amount of cystine,[12] porphyrindin[13] and 2,6-dichlorophenol-indophenol[14] reduced by such suspensions of proteins revealed the existence of only two sulphydryl groups per molecule. Subsequent investigations by Brand and Kassel,[15] in which porphyrindin was employed as reagent under conditions similar to those used by Kuhn and Desnuelle,[13] yielded the value 5.0 for the thiol content of heat-denatured ovalbumin. However, since these determinations involved titrations of suspensions, the results of such investigations will not be compared with those obtained with samples

of denatured ovalbumin in solution because of the uncertainties inherent in heterogeneous systems.

(b) Methods Involving Titration in Homogeneous Media

The application by Greenstein[16] of the porphyrindin titration to a solution of denatured ovalbumin, instead of a suspension, was an important step in the search for methods for the determination of the thiol content of proteins. Subsequently, many procedures have been devised, the majority of which require the presence of a denaturing agent, capable of denaturing the protein without causing precipitation. The denaturants most commonly used are urea, guanidine hydrochloride or a detergent, e.g., Duponol. All of these methods involve a titrimetric procedure, varying amounts of group-specific reagent being added to a known amount of the protein. One major difference between many of the procedures is the method used to indicate the end-point of the titration, and in this summary division of the methods has been made on this basis.

(i) Methods Using a Qualitative Colour Change as End-point

Use has been made, in some instances, of the colour change associated with the reaction of the reagent with thiol groups. Porphyrindin and iodine both yield colourless compounds on reaction, and hence the end-point of titration has been taken as the point at which the reaction mixture becomes slightly coloured.[16,17] The end-point in iodine titrations has also been determined by the use of starch as indicator: Hellerman et al.,[18] using iodosobenzoate as reagent, have estimated the excess iodosobenzoate by conversion to iodine which is estimated with thiosulphate. The colour resulting from the reaction of nitroprusside with thiol groups has frequently

been used as an indicator, the minimum amount of reagent required to cause the disappearance of the nitroprusside colour being regarded as the end-point.[19] A slight variation of this method is the back-titration procedure adopted by MacDonnell et al.:[20] an excess of the group-specific reagent is added, the amount in excess being determined by a back-titration with standard cysteine hydrochloride solution until the nitroprusside test becomes positive. Mention should also be made of a further method utilising a reaction of the thiol groups, namely the reduction of ferricyanide to ferrocyanide: the latter is detected by the blue colour developed on addition of ferric ions. Thus the amount of group-specific reagent reacting with sulphydryl groups has been determined by titration to the disappearance of the Prussian blue colour.[21] Results obtained by application of these methods to ovalbumin are summarised in Table V-1. From these results, it can be seen that values in the range 3.6 to 5.5 have been observed for the thiol content of ovalbumin by these methods. The two extremely high values, 6.8 and 5.9, obtained using the action of iodine on the native form of ovalbumin, are reported to be in error due to the non-specificity of iodine under the operating conditions.[19,21] The original observations of the sulphydryl content of ovalbumin by Greenstein[16] are such as to indicate that guanidine hydrochloride is a better denaturing agent than urea, since an additional thiol group was detected using the former reagent. However, the subsequent results do not show any obvious dependence on the method of denaturation used.

Table V-1

Reagent	Denaturant	Conditions	Indicator	Thiol Content	Reference
Porphyrindin	urea	pH 7, 20°	self	3.7	Greenstein[16]
Porphyrindin	guan. HCl	pH 7, 20°	self	4.8	Greenstein[16]
Iodine	urea/HCl	pH 3.5, 20°	self	5.1	Hess[17]
Iodine	20% HCl	pH 3.5, 20°	self	3.6	Hess[17]
Iodine	6N H_2SO_4	pH 3.5, 20°	self	5.3	Hess[17]
Iodine	-	pH 3.5, 20°	self	5.2	Hess[17]
Iodine	-	pH 3.2, 37°	-nitropruss	6.8	Anson[21]
Iodine	-	pH 6.8, 0°	"	5.9	Anson[19]
Iodine	-	pH 6.8, 0°, 1M KI	"	4.4	Anson[22]
Iodosobenzoate	guan. HCl	pH 7, 20°	starch	4.8	Hellerman[18]
Ferricyanide	guan. HCl(1)	pH 6.8, 20°	-nitropruss and pruss blue[a]	4.4	Anson[19]
Ferricyanide	urea (2)	"	"	4.4	Anson[19]
Ferricyanide	Duponol (3)	"	"	4.4	Anson[19]
Tetrathionate	(1),(2),(3)	"	"	4.4	Anson[19]
p-CMB	(1),(2),(3)	"	"	4.4	Anson[19]
Iodine	guan. HCl	pH 3.2	+nitropruss	5.1	MacDonnell[20]
Iodine	-	pH 3.2	"	4.2	MacDonnell[20]
p-CMB	guan. HCl	pH 3.2	"	4.1	MacDonnell[20]
p-CMB	guan. HCl	0°C	-nitropruss	3.6	Benesch[23]
p-CMB	urea	pH 7	+nitropruss	5.5	Lontie[24]
p-CMB	heat	pH 5.4, 4.6	"	4.0	Cunningham[25]

[a] End point with prussian blue has also been determined photometrically.

(ii) Methods Involving Photometric Procedures

The estimation of thiol groups by quantitative colourimetric procedures was initiated by Mirsky.[26] He determined the thiol content of proteins by the addition of excess ferricyanide and measurement of the amount of ferrocyanide formed by means of the intensity of the blue colour formed on the addition of ferric sulphate: a similar procedure has been adopted by Bacq and Fischer[27] and Katyal and Gorin.[28] More recently, Horowitz and Klotz[29] have observed the binding of an azomercurial to the thiol groups of ovalbumin by following the removal of the dye (as indicated by changes in optical density) from an immiscible non-aqueous solvent in equilibrium with the protein-containing aqueous phase. The third method in this category involves the observation of the increase in absorbancy in the 250 mμ region accompanying mercaptide formation with p-chloromercuribenzoate;[30] the increase is found to be a linear function of the amount of sulphydryl present. A summary of the results obtained using these procedures, which have the advantage that the progress of the sulphydryl group removal can be followed, appears in Table V-2. From these results, for which the end-point should be more accurate, there is much better agreement between the different methods than is the case in Table V-1, most of the results being consistent with a value 4 for the sulphydryl content. However, the value 4.4, obtained by Bacq and Fischer[27] is indicative of more than four thiol groups per molecule. Furthermore, the evidence of Horowitz and Klotz,[29] whilst proving the existence of at least four thiol groups, does not eliminate the possibility of five sulphydryl groups per molecule.

Table V-2

Reagent	Denaturant	Conditions	Thiol Content	Reference
Ferricyanide	guan. HCl	pH 6.8, 25°	3.5	Mirsky[26]
Ferricyanide	urea	pH 6.8, 25°	3.5	Mirsky[26]
Ferricyanide	Duponol	pH 6.8, 25°	3.6	Mirsky[26]
Ferricyanide	Texapon	pH 6.8	4.4	Bacq[27]
Ferricyanide	detergent	pH 7	4.0[b];4.1[c]	Katyal[28]
Chloropicrin	Texapon	pH 5,7,8	4.4	Bacq[27]
Azomercurial[a]	-	pH 9.1, 25°	4	Horowitz[29]
p-CMB	-	pH 4.6	4.0	Boyer[30]

[a] The reagent used was 4-(p-dimethylaminobenzeneazo)phenylmercuric acetate.

[b] Spectrophotometric determination.

[c] Colorimetric determination.

(iii) Amperometric Procedures

Procedures involving the determination of the end-point of titration amperometrically have been devised for the estimation of the thiol groups of ovalbumin, using as group-specific reagents iodine,[31] silver ions[23] and ferricyanide ions.[28] The reaction of silver ions with sulphydryl groups is as follows:

$$R\text{-}SH + Ag^{+} \longrightarrow R\text{-}SAg + H^{+} \qquad (1)$$

or, since the titration is usually performed in ammoniacal medium

$$RSH + [Ag(NH_3)_2]^{+} \longrightarrow RSAg + NH_4 + NH_3. \qquad (2)$$

Briefly, the principle of the amperometric method is as follows:[32] an electrode assembly is set up in the protein solution such that the titrating reagent is electro-reducible at the cathode. An e.m.f. is then applied such that the reagent being added is the only entity in the titration mixture that will undergo reduction. Thus until an excess of the reagent has been added, the current produced by the system is essentially zero: addition of excess reagent causes an increase in the current. Furthermore, in the presence of a high concentration of a salt that is not reducible at the particular applied e.m.f., e.g., KCl, the current is proportional to the concentration of the reagent in the reaction mixture. (The high salt concentration is not used in the ferricyanide titration.[33]) Thus the procedure adopted is to plot a graph of the current produced versus the amount of reagent added, the titration being continued beyond the end-point. The end-point is then determined by extrapolation to zero reagent concentration in the solution. A summary of the results obtained using amperometric procedures appears in Table V-3.

Table V-3

Reagent	Denaturant	Conditions	Thiol Content	Reference
Iodosobenzoate) Pot. iodide)	-	pH 6.6	4.1	Larson[31]
Iodosobenzoate) Pot. iodide)	heat	pH 6.6	3.9	Larson[34]
Iodine + KI	-	pH 2-3	5.6	Larson[31]
$[Ag(NH_3)_2]^+$	ethanol	pH 9	5.0	Benesch[23]
$[Ag(NH_3)_2]^+$	guan. HCl	pH 9	3.0	Benesch[23]
$[Ag(NH_3)_2]^+$	Duponol	pH 9	3.3	Benesch[23]
$[Ag(NH_3)_2]^+$	-	pH 9.25	5.6	Lontie[24]
$[Ag(NH_3)_2]^+$	urea	pH 9.25	4.1	Lontie[24]
$[Ag(tris)_2]^+$	-	pH 7	5.0	Benesch[35]
$[Ag(tris)_2]^+$	urea	pH 7	4.3	Benesch[35]
Ferricyanide	detergent	pH 7	4.0	Katyal[28]

The data of Larson and Jenness[31,34] require comment — the method involves the addition of excess iodosobenzoate to the protein solution, the excess being determined by titrating the iodine liberated upon addition of acid and iodide. They have found that only 10% of the oxidation is performed by the iodosobenzoate and that the remainder is due to the action of the iodine in acid medium.

Thus, even by this very accurate amperometric method values in the range 3.9 to 5.6 are reported for the sulphydryl content of ovalbumin: the low values observed by Benesch and Benesch[23] have not been included in the range because they were obtained with protein solutions in which precipitation had occurred.

From this summary of the results obtained for the sulphydryl content of ovalbumin it is at once apparent that the method of detection of the end-point of the titration is not the reason for the discrepancies between the reported values. That the possibility of non-specificity, whilst not disproved, does not appear to account for the variation in the results, is shown by consideration of the results obtained with the individual reagents: porphyrindin -values in the range 3.7 - 4.8; iodine, 3.6 - 5.6; iodosobenzoate, 3.9 - 4.8; ferricyanide, 3.5 - 4.4; p-chloromercuribenzoate, 3.6 - 5.5; silver ion, 4.1 - 5.6.

One result, which casts great doubt on the specificity of the action of iodine is the difference observed by Larson and Jenness[31] between the thiol content as determined using iodine alone and that obtained when 10% of the thiol groups are first reacted with iodosobenzoate and then the resultant solution is reacted with iodine.

However, the conditions employed differed from those finally postulated by Anson[22] in that the pH of the solution was 3.5 instead of 7. It is interesting to note, in this respect, that values obtained with iodine are generally higher than those found with other reagents. The statement that the possibility of non-specificity does not obviously account for the discrepancies is unaffected by disregarding the values obtained with iodine.

Consideration of the results from the point of view of incomplete liberation of "masked" sulphydryl groups does not appear to give any obvious reason for the scatter of results either. For example, Greenstein's results[16] indicate that denaturation with guanidine hydrochloride results in greater thiol content detection than is the case with urea. However, subsequent workers[19,26] have shown that the number of sulphydryl groups detected is the same whether urea, guanidine hydrochloride or a detergent is used as denaturant. A survey of the results obtained with the particular denaturing agents reveals the following variation: 3.7 - 5.5 for urea; 3.5 - 4.8 for guanidine hydrochloride; 3.6 - 4.4 for detergent and 4.0 - 5.6 for no denaturant. In fact, perhaps the most interesting facet of these results may be the observation in some instances that the sulphydryl content as determined on the native material is greater than that determined, using the same procedure, on denatured samples. Results which indicate this point are:

(a) The values 5.0 and 4.3 obtained with native and denatured samples respectively, using the $[Ag(tris)_2]^+$ ion as reagent.[35]

(b) The values 5.6 and 4.1 obtained using $[Ag(NH_3)_2]^+$ as

reagent.[24]

The former workers[35] attribute the difference to inaccuracies involved in the titration of native samples, but the latter[24] attribute this to spontaneous oxidation of the thiols to disulphides in the denatured protein — the process of denaturation allows orientation of the thiol groups into positions favourable for spontaneous disulphide formation. They also state that the spontaneous oxidation is strongly dependent on pH, the reaction proceeding much more quickly at pH 9 than at neutral pH, as is evidenced by the lower value, 4.1, obtained with the silver ion titration at pH 9 as compared with the value 5.5 obtained with p-chloromercuribenzoate at pH 7 on denatured ovalbumin samples. However, the finding by Benesch et al.[35] and MacDonnell et al.[20] of a value 4 for the thiol content of denatured ovalbumin using p-chloromercuribenzoate at pH 7 and 3.2 respectively does not support the postulate that spontaneous oxidation at high pH values is responsible for the observed difference in thiol estimations.

One further point in Lontie's and Becker's conclusions[24] requires comment: they consider that the value 5.5 for the sulphydryl content is possibly due to the different thiol content of the components of ovalbumin. Their suggestion is that one component contains five, the other six thiol groups and hence the value 5.5 has been obtained. However such an interpretation would require the existence of two components in equal proportions in crystalline ovalbumin: this is not indicated by electrophoretic evidence, which gives the apparent proportions of A_1 and A_2 as approximately 80% and 20% respectively.[1-7]

Therefore, from this survey of the results of sulphydryl estimations, it can be seen that there is evidence for the existence of either four or five thiol groups per molecule: the author feels that the value 4 is probably correct because, apart from Lontie and Becker's higher value,[24] all of the more recent determinations, using the potentially more accurate procedures of colorimetry, spectrophotometry and amperometric titration, have indicated this value.

2. Experimental

As was indicated at the conclusion of the preceding chapter, the claim that iodine is a specific reagent for the sulphydryl groups of ovalbumin relies on the supposition that there are five such groups in the molecule. Therefore a further study of the question of the sulphydryl content of ovalbumin has been performed because of the lack of unequivocal proof of the existence of either four or five thiol groups per molecule. In addition experiments have been performed on an iodine-modified sample so that whilst the absolute thiol content values may be in error, comparative values should be reliable. An estimate of the disulphide content has also been obtained.

(a) Method of Estimation

The method used has been an amperometric titration procedure using a dropping mercury electrode as indicator electrode and mercuric chloride as reagent. The procedure adopted in this study is similar to that outlined by Stricks et al.:[36] the electrode assembly comprised a dropping mercury electrode as cathode and a saturated calomel electrode as anode (a more detailed description of the apparatus appears in Chapter VIII). The titrations were performed on air-free

solutions in borate buffer pH 9.2, using 0.01 molar mercuric chloride. Titration curves were constructed by plotting the current measured at -0.35 volts versus the volume of mercuric chloride added. However, Stricks et al.'s procedure involved preliminary pepsin or acid hydrolysis of the protein; this step has been replaced by the addition of 8 molar urea to the protein solution being titrated.[37-39] The method outlined enables the determination of sulphydryl content to be performed on the basis of the reaction between thiol groups and mercuric ions, the stoichiometry of which may be represented:[38,40]

$$RS^- + HgCl_2 \longrightarrow RSHgCl + Cl^-. \qquad (3)$$

The reagent is thus monofunctional.

Since, as already indicated in Chapter IV, disulphide groups may be reacted with sulphite to give sulphydryl and thiosulphate groups, the above procedure may also be used for the estimation of such groups; the reaction mechanism is somewhat complicated, however, and leads to different stoichiometric relations. Briefly, the situation is as follows: in the presence of 0.2M sodium sulphite, disulphide linkages are ruptured in accordance with the following equation,[41,42]

$$RSSR' + SO_3^{2-} \longrightarrow RS^- + R'SSO_3^- \qquad (4)$$

only <u>one</u> sulphydryl group being formed from the two S atoms. The RS^- so formed then reacts with mercuric ions to form a mercaptide and hence the above equilibrium reaction proceeds completely to the right. In the process of mercaptide formation, however, the mercuric chloride now acts as a <u>bifunctional</u> reagent, the reaction being[37,39]

$$2RS^- + HgCl_2 \longrightarrow (RS)_2Hg + 2Cl^-. \qquad (5)$$

It therefore follows that in a straightforward estimation

of sulphydryl groups,

1 SH gp $\equiv$ 1 formula weight of $HgCl_2$

while for the additional sulphydryl groups resulting from sulphite scission of disulphide groups,

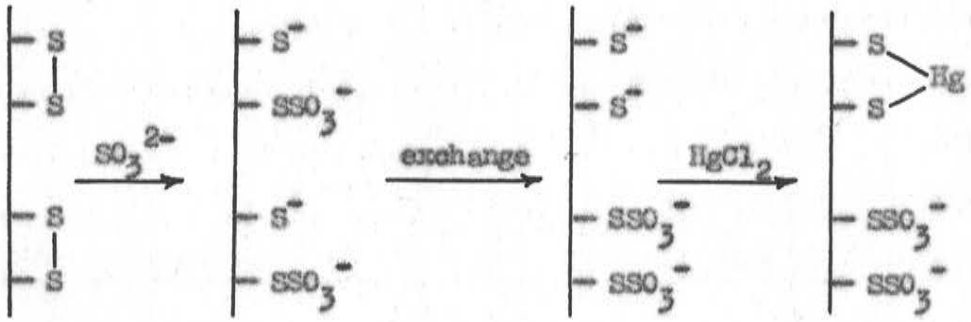
1 SS gp $\longrightarrow$ 1 SH gp $\equiv \frac{1}{2}$ formula weight of $HgCl_2$.

In representing experimental results, therefore, the operational quantity "number of formula weights of $HgCl_2$ reacting per mole of protein" (=Q) will be given, its significance being as follows: the sulphydryl content of the native protein is equal to the value of Q found in the absence of sulphite, whilst the disulphide content is determined from the following relationship.

$$\text{Disulphide Content} = 2\left[Q(SO_3^= \text{ added}) - Q(\text{ no } SO_3^=) \right]$$

To invoke different mechanisms for the reaction of mercuric chloride with naturally occurring sulphydryl groups on the one hand and those arising by sulphite scission of disulphide linkages on the other is at first sight arbitrary and unsatisfactory. However, experimental verification has been obtained with such proteins as bovine serum albumin and insulin for which reliable data on sulphydryl and disulphide contents are available.[36-39,43-45] On purely speculative grounds it seems not at all unlikely that, since naturally occurring sulphydryl groups will be distributed more or less at random, they will react singly with mercuric chloride, which is thus monofunctional in such a case. In order to provide a reason for the formation of a dimercaptide from sulphydryl groups arising by sulphite scission of disulphide groups it seems necessary to postulate an exchange between pairs of groups (SSO_3^-, S^-) either inter- or intramolecularly such

that pairs of sulphydryl groups are in precisely suitable locations for the S — Hg — S linkage. In this respect a similar exchange between sulphydryl and disulphide groups is fairly well established.[42,46] A possible scheme whereby mercuric chloride acts as a bifunctional reagent may be represented diagrammatically as follows:



Such an exchange, while as yet unsupported experimentally, seems at least plausible. However it may be theoretically, the situation seems fairly satisfactory experimentally.

Before embarking on the analyses to be described, experiments were performed on a sample of cystine for which N analysis was available in order to access the accuracy of the procedure. The results of these titrations, which are reported in more detail in Chapter VIII, were such that a purity of 98 ± 2% was indicated, the figure 99.7% being the value calculated on the basis of the N analysis.

(b) Results

The results of the sulphydryl and disulphide contents of both the native and modified ovalbumins are given in Table V-4.

Table V-4

Material	$Q^{a,b}_{(no\ SO_3^=)}$	N[c]	$Q^{a,b}_{(SO_3^=\ added)}$	N[c]	$2[Q_{(SO_3^=)} - Q_{(no\ SO_3^=)}]$
Ovalbumin	3.8 ± 0.1	5	4.9 ± 0.1	5	2.2
Iodine-mod. ovalbumin	1.9 ± 0.1	4	3.0 ± 0.1	4	2.2

[a] The significance of Q is discussed in the text.

[b] Calculated on the basis of 45,000 for the M.Wts. of both materials (see next chapter).

[c] N = number of determinations.

Examination of this table of results reveals the following points:

(i) This determination gives a value for the thiol content of ovalbumin consistent with a value 4.

(ii) Two sulphydryl groups are apparently unreacted after iodine modification.

(iii) The disulphide contents of the native and modified ovalbumins are identical.

(c) Discussion

The major criticism that can be levelled at these amperometric titration results is the use of the bifunctional reagent mercuric chloride, which forms with thiol groups either RSHgCl or $(RS)_2Hg$. However, since this is essentially a comparative study of the native and modified materials, the comparison should be valid even in the event of the absolute values being in error. In this respect it should be noted that the value 4 obtained for the sulphydryl

content of ovalbumin indicates no such error in thiol estimations. Furthermore, the identity of the disulphide contents of the two materials would still be valid should the method of calculation be in error. Repetition of these estimations with a monofunctional reagent, methyl mercury iodide, which is planned as soon as experimental difficulties arising from the very low concentration of reagent employed (5×10^{-4}M) and the consequent increase in the required sensitivity of the polarograph have been overcome, should confirm the conclusions drawn from this study.

Assuming that the correct interpretation of mercaptide formation has been used, the following points require comment:

(i) Firstly, these results indicate that ovalbumin contains four cysteine and two cystine residues per molecule whereas values 5 and 1 respectively are indicated by amino acid analyses.[47] However, since the value 4 has frequently been found for the sulphydryl content of ovalbumin, it is possible that the amino acid analysis is in error. Amino acid analyses methods do suffer from the disadvantage that the protein is first hydrolysed and hence the possibility of a cystine to a cysteine transformation seems quite feasible. This does not provide a complete answer as the system with two cystine and four cysteine residues contains an extra sulphur atom, eight half-cystine residues being required instead of seven, the value most recently found by Akabori et al.[48,49]

(ii) The second point that requires comment is the detection of two residual thiol groups in the iodine-modified ovalbumin: this is rather unexpected as Anson has reported a negative nitroprusside

reaction for urea-denatured samples of the derivative.[19] However, Halwer,[50] in a study of intermolecular mercaptan-disulphide exchange in denatured ovalbumin, has found that intermolecular cross-linking still occurs to a slight extent in denatured samples of iodine-modified ovalbumin. His suggestion that this is due to the incomplete removal by iodine of sulphydryl groups is borne out by the findings of this study. Presumably, the failure of nitroprusside to reveal sulphydryl groups detected by an amperometric titration technique is simply an indication of the relative sensitivities of the two methods for detecting such groups.

(iii) The third and most important point as far as this study is concerned is the fact that, although two sulphydryl groups are involved in the modification process, no additional disulphide linkages have been detected in urea-denatured samples of the protein subsequent to modification. Therefore it must be assumed that oxidation beyond the disulphide stage has occurred, presumably to the sulphenic, sulphinic or sulphonic acid stages.[51,52]

A discussion of possible reaction mechanisms for the iodine modification of ovalbumin will be deferred until after various physico-chemical differences between the native and modified materials have been presented in the next chapter.

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CHAPTER VI

A PHYSICO-CHEMICAL COMPARISON OF NATIVE AND IODINE-MODIFIED OVALBUMIN

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A Physico-chemical Comparison of Native and Iodine-modified Ovalbumin

Physico-chemical studies on both the native and iodine-modified ovalbumins have been performed in an endeavour to elucidate the exact nature of the modification. Whereas the preceding chapter was concerned with changes on a molecular level, the work to be reported here deals with the changes on a macroscopic level, i.e., changes in solubility, size, shape and charge. The techniques used for this study are electrophoresis and sedimentation.

1. Solubility

The solubility characteristics of the modified material in aqueous solution differ quite markedly from those of the parent material, and although no quantitative studies have been made of solubility, the following differences between the native and modified proteins have been observed qualitatively during electrophoretic investigations of the two materials. The parent material, ovalbumin, is soluble over the pH range 4 - 8.5 at least to the extent of 3% w/v at 1°C; the iodine-modified material is also soluble to the extent of at least 3% at pH values 7 and greater. However, at values of pH less than 5.5, a much decreased solubility is observed; in fact, at pH 4.6 the solubility is of the order 0.03% at 1°C. To test whether the decreased solubility in acid solution is due to denaturation of the modified material at these acid pH values, the following series of experiments has been performed: two aliquot samples of iodine-modified ovalbumin (12 mls. of approximately 0.5%) were dialysed against "veronal" buffer pH 8.5, ionic strength 0.10

(sample A) and acetate buffer pH 4.6, ionic strength 0.10 (sample B), respectively, for two days at 4°C, at the end of which time, most of the sample in the acetate buffer had precipitated. After centrifugation at 7,000 r.p.m. of this material, the centrifugate was redialysed against "veronal" buffer pH 8.5, ionic strength 0.10 (sample C); the residue from the centrifugation was dissolved in 12 mls. of the "veronal" buffer and dialysed for a further day (sample D). Electrophoresis of the three samples A, C, and D was then performed at 2°C, approximate values for the electrophoretic mobilities of the three samples being calculated from the rate of movement of the maximum of the schlieren peak. The results of these approximate mobility measurements are listed in Table VI-1.

Table VI-1

Sample	Ascending Mobility x 10^5 cm^2/sec/volt	Descending Mobility x 10^5 cm^2/sec/volt	Average
A	6.6	6.5	6.6
C	6.7	6.5	6.6
D	6.6	6.5	6.6

These mobility measurements are only approximate because no precautions have been taken to eliminate any flow through the cell due to the changing pressure head, and also, since the schlieren peaks are slightly skew (see fig. VI-1), the first moment of the boundary should have been used instead of the schlieren maximum for the mobility determination. On the assumption that a major degree of unfolding of the molecule or other loss of secondary structure

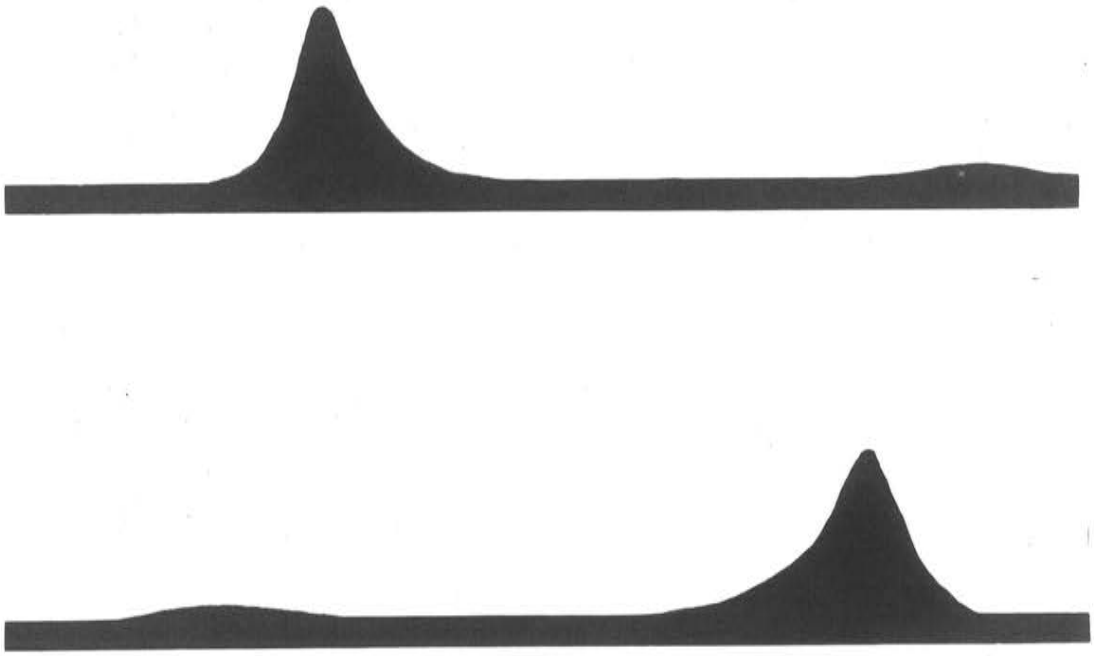


Fig. VI-1. Electrophoretic pattern of iodine-modified ovalbumin at pH 8.5, in "Veronal"-chloride buffer, ionic strength 0.10, after four hours at a potential gradient of 3.4 volts/cm.: upper - ascending limb; lower - descending limb.

would be revealed by a change in mobility, the essential identity of the mobilities of these three samples would seem to indicate either that no denaturation had occurred, or that if denaturation at low pH values had occurred, then the denaturation reaction is reversed by reversion to alkaline pH values. Subsequent sedimentation and electrophoretic mobility studies, to be discussed later, support the postulate that no denaturation occurs at low pH values as long as the temperature is kept low.

The solubility of the modified material at pH 4.6 is greatly enhanced by increasing the temperature to 25°C, an 0.4% solution being obtainable; however, as is the case with the parent material, prolonged exposure of the material to this temperature results in precipitation of the protein, presumably due to denaturation.

2. Electrophoretic Studies

These studies have been of two types; firstly a detailed comparison of the variation of the electrophoretic mobilities of the two materials over the pH range 4 - 8.5 and secondly, a comparison of the apparent net charge on the two proteins at pH 8.5; the reason for this choice of pH for the charge determination will become evident from the results of the mobility determinations. All electrophoresis experiments pertaining to accurate measurement of mobility or charge have been performed at 1.0°C in a Spinco Model H Diffusion-Electrophoresis Apparatus employing Rayleigh optics.

(a) Mobility Measurements

Since the aim of the work is to detect any small differences that may exist between the modified and native ovalbumins, it is

essential that the mobilities be determined with high precision. Consequently the first moment of the boundary has been used to define the rate of migration of the boundary. The use of the first moment for the determination of mobilities is not new, Longsworth,[1,2] for example, having used such a method for obtaining mobilities from schlieren photographs. However, to the author's knowledge, this is the first time that such a method has been applied to mobility determinations from Rayleigh interferograms, and because of the greater accuracy attainable with the latter optical system, it is believed that the method employed here has a greater potential accuracy than any previously used method. Since uncertainties in the definition of the boundary position are one of the sources of error in mobility determinations, the mobility data reported here are probably more accurate than any previously published data. It should be borne in mind, however, that other factors such as the conductivity of the protein solution and the thickness of the cell along the optic axis are also sources of error, and in these respects the measurements reported here may be in error. Since, as already mentioned, the prime interest of this work is the comparison of the two materials, errors in the latter two factors will affect the mobilities of the two materials equally so that the comparison will still be quite accurate even though there may be a consistent error in the absolute mobility values. Consequently, although the absolute values of the mobilities may not be of greater accuracy, the comparison of the two mobilities almost certainly has a greater accuracy than any published data.

(i) Method for Obtaining the First Moment

In the case of the Rayleigh optical system, which gives a

record of the refractive index, n, as a function of cell height, x, the position of the first moment, x_m, is defined by the following equation

$$x_m = \frac{\int_{n_o}^{n^*} x \, dn}{\int_{n_o}^{n^*} dn} \qquad (1)$$

where n_o is the refractive index of the solvent and n^* that of the protein solution, or, expressing n in terms of the number of Rayleigh fringes (J) obtained

$$x_m = \frac{\int_0^J x \, dj}{\int_0^J dj} = \frac{\int_0^J x \, dj}{J} \qquad (2)$$

The problem, therefore, is to find an expression for the area under the curve, $\int_0^J x \, dj$. An approximation to this integral could be made by use of equation (3)

$$\int_0^J x \, dj \sim \sum_{j=0}^{j=J} x\Delta j \qquad (3)$$

However, equation (3) is a reasonable approximation only if x is accurately defined and consequently cannot be used in this case because x tends to infinity at both extremities of the boundary. Accordingly equation (2) is approximated to in the following manner

$$x_m = \left\{ \int_{j=0}^{j=k} x\, dj + \sum_{j=k}^{j=J-k} x\, \Delta j + \int_{j=J-k}^{J} x\, dj \right\} \Big/ J \qquad (4)$$

where k is the value of j at which equation (3) becomes a good approximation to the integral.

For systems of approximately 20 fringes (J), it has been found that for fringes 3 to 17, the integral is represented quite well by $\sum_{3}^{17} x\, \Delta j$ (see Table VI-2). The problem now becomes one of evaluation of the first and last terms in the bracket of equation (4): the method adopted has been as follows:- Experimentally it has been found that for fringes 0 - 3 and (J - 3) to J in a system of approximately 20 fringes, a plot of log j' versus x is linear, j' being counted from the solution side of the boundary in the case of fringes 17 to 20. (Examples of such a plot are shown in Fig. VI-2). Therefore the following expression may be written:

$$\log j' = \log A - \frac{bx}{2.303} \qquad (5)$$

whence $j' = Ae^{-bx}$ and $dj' = -Abe^{-bx}\, dx$.

Therefore $$\int_0^3 x\, dj' = -Ab \int_{\infty}^{x} x\, e^{-bx}\, dx$$

$$= -Ab \left[\frac{e^{-bx}}{b^2} (-bx - 1) \right]_{\infty}^{x}$$

$$= \frac{A}{b} (bx + 1)\, e^{-bx} \qquad (6)$$

That equation (6) is a good approximation to the integrals $\int_0^j x\, dj$

Table VI-2

Expt.	Exposure	$\int_0^3 x\,dj$		$\int_3^{J-3} x\,dj$		$\int_{J-3}^{J} x\,dj$		$\int_0^J x\,dj$	
		Meas.	Calc.	Meas.	Calc.	Meas.	Calc.	Meas.	Calc.
1	1	0.55	0.56	-0.01	-0.01	-0.55	-0.54	0.01	0.01
"	6	1.25	1.26	+0.01	+0.01	-1.11	-1.13	0.15	0.14
"	11	1.47	1.48	+0.03	+0.03	-1.11	-1.13	0.39	0.38
2	1	0.68	0.70	+0.02	-0.01	-0.75	-0.74	-0.05	-0.05
"	6	1.44	1.43	-0.03	-0.02	-1.48	-1.49	-0.07	-0.08
3	1	0.58	0.60	0.00	+0.01	-0.58	-0.60	0.00	0.01
"	6	1.05	1.06	+0.03	+0.03	-1.06	-1.07	0.02	0.02

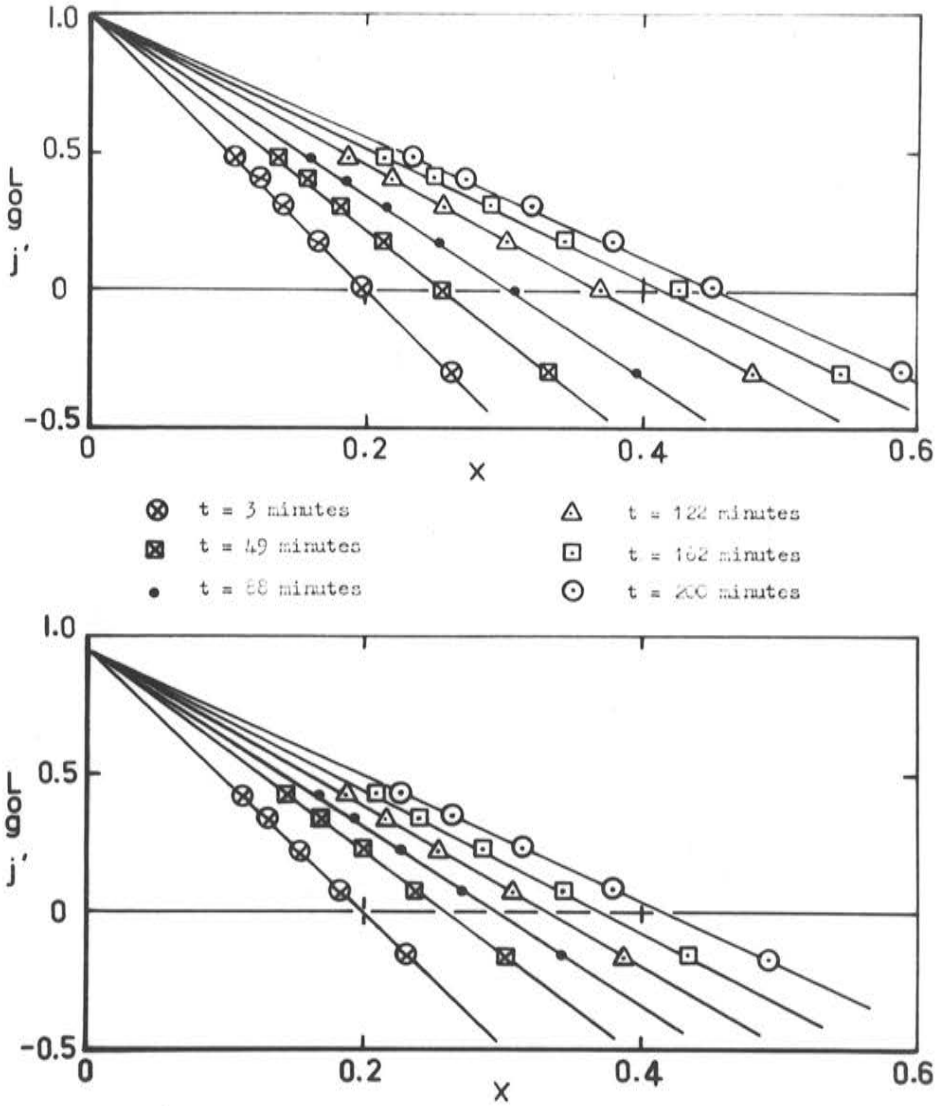


Fig. VI-2. Graphs showing a linear relationship between log j' and x. These graphs have all been obtained from measurements of fringe positions in the ascending boundary during a typical electrophoresis experiment. The upper graph refers to fringes 0-3, whilst the lower graph refers to fringes 13-15.7. in a system where J = 15.7.

and $\int_{J-j}^{J} x\,dj$ can be seen from the close agreement in Table VI-2 between the values of the integrals calculated on this basis and those determined by planimetry. Table VI-2 lists the results of measured and calculated areas for the terms within the brackets of equation (4), both separate and combined. Thus it would appear that equation (4) may be used to obtain the first moment of a boundary.

However, the number of measurements of fringe positions for each exposure is extremely large and therefore a further approximation has been made, namely that the first moment of the boundary is given with sufficient accuracy by equation (7)

$$x_m \sim \sum_{j=k}^{j=J-k} x_j \Delta_j / (J - 2k) \qquad (7)$$

The satisfactory nature of this approximation is evident from a comparison of the values of x_m obtained by use of equation (7) and (4) for experiment 1 (Table VI-3).

From these results it can be seen that the maximum difference between $(x_m)_1$ and $(x_m)_2$ is 0.015 cm., the distance moved during the experiment being about 3 cm., so that the error in the slope of the plot of x_m versus t is less than 0.5%; in fact calculation of the slopes for the above values of $(x_m)_1$ and $(x_m)_2$ reveals a maximum discrepancy of 0.3%.

For the remainder of the experiments the procedure adopted has been to measure the positions of the fringes k to J - k for all exposures and to measure fringe positions in the regions 0 to

Table VI-3

Ascending Boundary		Descending Boundary	
$(x_m)_1$ [a]	$(x_m)_2$	$(x_m)_1$	$(x_m)_2$
5.317	5.323	3.663	3.673
5.916	5.925	3.084	3.098
6.527	6.536	2.471	2.482
7.053	7.061	1.953	1.960
7.650	7.661	1.365	1.369
8.375	8.381	0.651	0.656

[a] $(x_m)_1$ is calculated on basis of equation (4); $(x_m)_2$ on basis of equation (7).

k and J - k to k only for the first and last exposures to assess the inaccuracy involved in using equation (7). The maximum error associated with the determination of the position of the boundary will be quoted with each result.

The actual method by which mobility experiments were performed also requires comment. In accordance with the usual procedure for preventing flow through the 11-ml. Tiselius cell due to the ever changing pressure head, the ascending limb was sealed from the atmosphere. However, such a precaution does not preclude the possibility of flow due to such causes as volume changes within the electrode vessels; in this study the boundaries have been allowed to migrate in one direction for three and a half hours and then the current is reversed for an equal period. Six exposures of each limb were taken at intervals to record the progress of the boundary in each direction. Ideally, the boundary should return to its original

position and thus the calculated mobilities would be identical. In cases where there was a slight variation in the values obtained for the two directions, the average of the two descending mobilities was taken as the mobility of the sample. This method also has the advantage that even very minor cell leaks can be detected by the variation in the relation between the ascending and descending mobilities. Since the mobility is a function of protein concentration to a minor extent, and should, ideally, be extrapolated to zero protein concentration, low concentrations of the native and modified materials (0.03 - 0.10%) have been used in this study; it has then been assumed that the mobilities so obtained are identical with those at zero protein concentration. All experiments have been performed in buffers of ionic strength 0.100 at a temperature of 1.0_0 °C. The conductivity of the protein solutions was also measured at this temperature, so that the results listed in Table VI-4 refer to a temperature of 1°C. In the last column, the accuracy of the boundary location is recorded: the alternate experiments, for which no assessment of the accuracy is given, were calculated in accordance with equation (7) on a preliminary series of experiments, but no attempt was made to determine the x_m position from equation (4). It is most probable that the errors inherent in those results will be of the same order as those determined for the duplicate experiments.

These results are also represented diagrammatically in Fig. VI-3, together with the results of Longsworth[1] and Perlmann[3] for ovalbumin. Their data are not strictly comparable with that

Table VI-4

Buffer[a]	pH	Ovalbumin		Iodine-modified Ovalbumin	
		$\omega \times 10^5$	Error	$\omega \times 10^5$	Error
.04M NaV, .01M HV, .06M NaCl	8.5_4	-5.9_0	0.6%	-6.4_5	0.3%
"	"	-5.9_5[b]		-6.5_7[b]	
.01M NaV, .02M HV, .09M NaCl	7.4_8	-5.8_7	0.6%	-6.4_1	1.2%
"	"	-5.9_5		-6.4_9	
.04M NaCac, .01M HCac, .06M NaCl	6.5_8	-5.2_2	0.3%	-5.8_4[b]	0.4%
"	"	-5.3_0		-5.9_3	
.02M NaCac, .02M HCac, .08M NaCl	6.0_8	-4.8_2	-0.2%	-4.9_5	0.6%
.07M NaAc, .01M HAc, .03M NaCl	5.4_6	-3.5_1	0.4%	-3.5_6	1.3%
"	5.4_1	-3.2_2		-3.2_4	
.07M NaAc, .03M HAc, .03M NaCl	4.9_8	-1.8_5	0.7%	-1.7_8	1.0%
.04M NaAc, .04M HAc, .06M NaCl	4.5_9	-0.4_2	1.0%	-0.4_7	1.5%
"	"	-0.3_6		-0.3_9	
.02M NaAc, .08M HAc, .08M NaCl	3.9_5	$+2.4_2$	1.1%	$+2.4_8$	1.5%
"	"	$+2.5_0$		$+2.4_1$	

[a] V = "Veronal", Cac = cacodylate, Ac = acetate.

[b] The current was not reversed in these experiments.

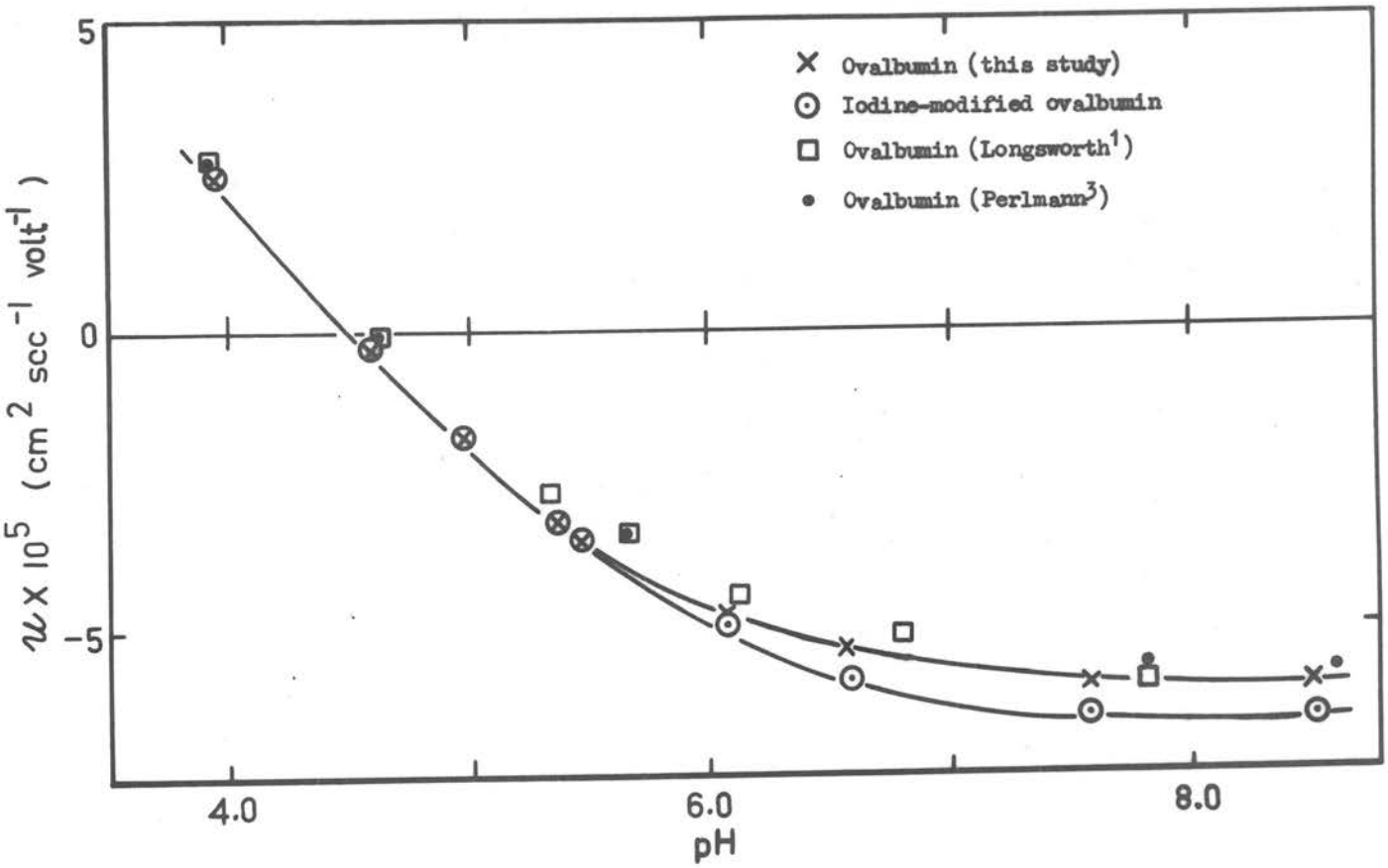


Fig. VI-3. pH-mobility curves of ovalbumin and iodine-modified ovalbumin in buffers of ionic strength 0.10.

reported here, since their conductivity measurements were performed at 0°C, and hence their results are at 0° whereas those reported here are at 1°C. However, for the case of ovalbumin there is a very marked similarity between the mobility - pH plot of this work and their results. It should also be noted that at pH values 5.5 and less, the iodine-modified and native materials have essentially identical mobilities, but that at pH values 6.5 and greater, there is a difference in mobility of 0.6×10^{-5}, the modified material migrating with a slightly greater negative mobility. Such a difference may be attributable to either or both of the following causes: (i) change of charge on the molecule and (ii) change in f, the frictional coefficient. In order to determine which of these effects is responsible for the electrophoretic mobility difference it is necessary to obtain an independent estimate of either the charge or the frictional coefficient of the two materials at some pH value in the range 6.5 to 8.5. Changes in the former have been studied in this work.

(b) Calculation of Charge

Calculations of charge from electrophoretic mobility data may be made by either of two methods:

(i) that due to Abramson et al.,[4,5] wherein use is made of an imperfect microscopic model. They have shown that for a spherical protein molecule the net charge, z, is given by the following approximate relation

$$z = \frac{6\pi\eta r}{f(\kappa r)} \cdot \frac{(1 + \kappa r + \kappa r_i)}{(1 + \kappa r_i)} \cdot \frac{u_p}{1.6 \times 10^{-12}} \qquad (8)$$

where r is the radius of the protein molecule, r_i the average radius

of the buffer ions, η the solvent viscosity, u_p the mobility of the protein in cm.^{2} sec.$^{-1}$ volt^{-1} and κ and $f(\kappa r)$ are the quantities that arise from the Debye-Huckel and Henry treatments of electrolytes.

(ii) The procedure adopted by Charlwood,[6] in which the apparent net charge is calculated from the excess supernatant buffer concentration required to eliminate the δ boundary in electrophoresis. This method, which has the advantage that no postulation of a molecular model is necessary, has therefore also been used in this study even though the Kohlrausch-Charlwood theory, to be discussed later, is known to be imperfect in at least one of its assumptions, with the consequence that results obtained by this method are commonly high by a factor of 1.2 to 2. However, the procedure should be reliable as a means of estimating a maximum charge difference. Furthermore, provided f has not changed, the charge difference must be expected to be a whole number if the measurements are made at pH 8.5, since the mobility difference is constant in the pH range 6.5 to 8.5, indicating complete ionisation of the group or groups responsible for any change in charge.

The Kohlrausch-Charlwood theory will now be presented. The major assumptions of the theory are: (i) the charge operative in membrane equilibria is identical with that on the ion migrating in an electric field and (ii) the mobility of a given ion is independent of its position in the electrophoresis cell. Now whilst the first of these assumptions has received support,[7] Longsworth[8] has shown that the second assumption, which is a requirement of the

Kohlrausch theory for the migration of ions in an electric field,[9] is subject to appreciable error due to pH variation across the boundaries. Since the method involves the elimination of the δ boundary, the theory of the method is probably best explained if the origin of the δ boundary is first indicated.

Kohlrausch,[9] by examination of the differential equations relating to the migration of ions in an electric field, has shown that there is a function B (the Beharrliche Funktion or Regulating Function), defined by the relation

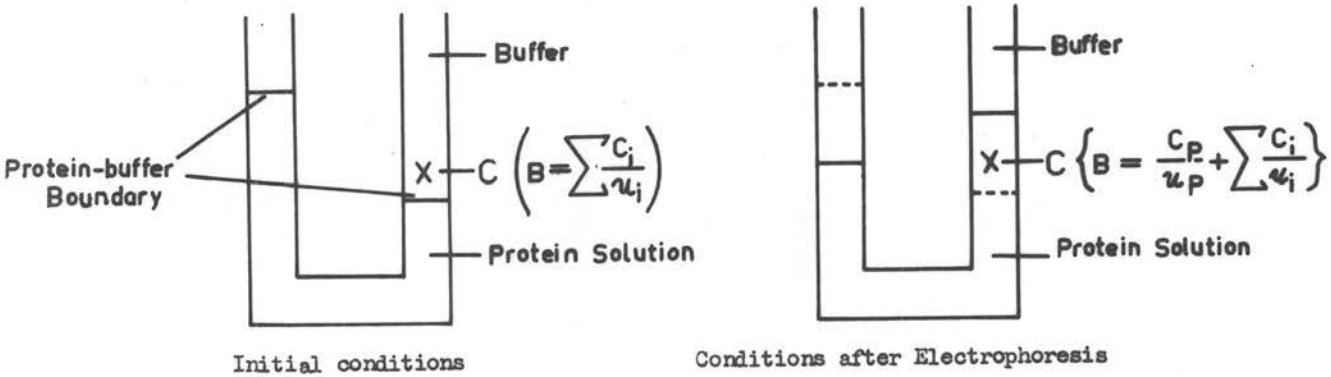
$$B = \sum \frac{C_i}{u_i} \qquad (9)$$

where C_i is the electrochemical concentration and u_i the electrophoretic mobility of species i, such that $(\partial B/\partial t)_x = 0$. Thus, considering an electrophoresis assembly, at a particular point in the cell (C in fig. VI-4), the Regulating Function will not vary. Svensson[7] has shown that the following relationship exists between the regulating functions of the dialysed protein solution (lower) and the solution against which it has been dialysing (dialysate or upper)

$$B_{lower} = B_{upper} + C_P\left[\frac{1}{u_P} - \frac{1}{2I}\sum \frac{z_i C_i}{u_i}\right] \qquad (10)$$

where I is the ionic strength, and C_i and C_P are expressed as e.c.e. per litre. The second term in the bracket is sufficiently small to be neglected, whereupon equation (10) reduces to

$$B_{lower} = B_{upper} + \frac{C_P}{u_P} \qquad (11)$$



Initial conditions

Conditions after Electrophoresis

Fig. VI-4. Diagram illustrating the origin of the δ boundary: in the region into which the boundary migrates, a dilution of the buffer occurs to such an extent that the Regulating Function, B, at any particular point remains unaltered. Thus a concentration gradient results in the region of the initial boundary between protein and buffer.

Since the regulating function does not vary with time, there must be a readjustment of concentrations in the protein solution (the mobility of an ion is almost independent of concentration) as soon as the boundary migrates into the dialysate. Consequently, as soon as the boundary moves into the dialysate there is a dilution of the buffer ions to counteract the C_p/ν_p term in the Regulating Function of the protein solution: this results in a concentration gradient at the point of commencement of electrophoresis, the so-called δ and boundaries.

The method of charge determination used by Charlwood[6] involves firstly an electrophoresis experiment in which the dialysate is used as supernatant buffer. Subsequent experiments are then performed using increasing concentrations of supernatant buffer. A graph is then plotted of δ boundary size versus concentration of buffer: extrapolation of this graph to zero δ boundary yields the concentration of supernatant buffer at which the Regulating Functions of the upper and lower solutions become equal. Thus if it is assumed, first of all, that the concentrations of the various buffer ions are the same in the protein solution as in the dialysate, an approximate value for the concentration of protein in e.c.e. per litre (C_p') can be obtained by equating the two Regulating Functions. If the subscripts 1, 2, 3 are used to denote the concentrations in the protein solution, the dialysate and the supernatant buffer respectively, then the last statement may be represented by the following relation

$$\frac{(c_{Na^+})_2}{\nu_{Na^+}} + \frac{(c_{V^-})_2}{\nu_{V^-}} + \frac{(c_{Cl^-})_2}{\nu_{Cl^-}} \sim \frac{(c_{Na^+})_3}{\nu_{Na^+}} + \frac{(c_{Cl^-})_3}{\nu_{Cl^-}} + \frac{(c_{V^-})_3}{\nu_{V^-}} \qquad (12)$$

in which C_p is the only unknown. Using this approximate value of C_p it is then possible to approximate more accurately the concentrations of the buffer ions within the dialysis bag $(c_i)_1$ by use of the relevant form of the Donnan membrane equilibrium equation. The Donnan equilibrium is most generally expressed in terms of the distribution ratio, λ, for all ions.

$$\lambda = \left[\frac{(c_i)_1}{(c_i)_2}\right]^{1/z_i} \sim 1 - \frac{C_p}{\sum(c_i z_i)_2} \sim 1 - \frac{C_p}{2I} \qquad (13)$$

where I is the ionic strength of the buffer. Thus for a particular ion, i, it can be seen that

$$(c_i)_1 = (c_i)_2 \left(1 - \frac{z_i C_p}{2I}\right) \qquad (14)$$

Substitution of these values of $(c_i)_1$ in place of $(c_i)_2$ in equation (12) then yields the protein concentration, C_p, in e.c.e. per litre. Combination of this value of C_p with the molar concentration, m_p, yields the valence of the protein.

$$z_p = \frac{C_p}{m_p} \qquad (15)$$

Charge Calculations on Ovalbumin and Iodine-modified Ovalbumin

The experimental section of the charge determinations has been performed as follows. Sufficient protein solution for five electrophoresis experiments in an 11-ml. Tiselius cell was dialysed against the veronal-chloride buffer pH 8.5, ionic strength 0.1000

(see Table VI-4) for four days at 4°C, at the end of which time the protein solution was removed from the dialysis bag and stored in five air-tight containers. One of these five samples was then used for an electrophoresis experiment using the dialysate as supernatant buffer: electrophoresis was continued for four hours, a current of 12mA being used. At the end of this time there was sufficient separation between the δ and protein boundaries for the δ boundary size to be determined to within 0.1 of a Rayleigh fringe: the molar protein concentration was also determined refractometrically from this experiment (the molecular weight of both materials was considered to be 45,000). Subsequently, electrophoresis experiments of a similar duration and also employing a current of 12 mA were performed on the remaining samples of protein, the ionic strength of the supernatant buffer being varied in each case to allow the required extrapolation to zero δ boundary to be made. In order to avoid pH changes in the supernatant buffers of differing ionic strength, these buffers were prepared in such a manner that the relative proportions of the three constituents were always identical. The results of such a series of experiments for ovalbumin and iodine-modified ovalbumin are reported in Table VI-5 and are represented graphically in Fig. VI-5.

The extrapolation of the two graphs results in the values 0.1032_0 and 0.1034_5 being obtained for the values of the ionic strength at which the δ boundary is eliminated for ovalbumin and iodine-modified ovalbumin respectively. Substitution of the corresponding values for $(c_1)_3$ in equation (13) and the subsequent

Table VI-5

Ovalbumin ($m_p = 1.38 \times 10^{-4}$)[a]		Modified Ovalbumin ($m_p = 1.47 \times 10^{-4}$)[a]	
Supernatant I	J_δ	Supernatant I	J_δ
0.1000	$10._2$	0.1000	$10._6$
0.1010	$7._2$	0.1020	$4._6$
0.1020	$3._8$	0.1025	$3._0$
0.1025	$2._2$	0.1000	$10._5$[b]
0.1000	$10._2$[b]	0.1010	$7._7$[b]
0.1015	$5._2$[b]	0.1015	$5._8$[b]

[a] Molecular weight of both materials assumed to be 45,000.

[b] These experiments have been performed with a slightly different protein concentration and the values of J_δ have been adjusted to the concentration of the other experiments.

calculations according to equations (14) and (15) lead to the values -14.0 and -15.5 for the apparent net charge on the native and modified materials respectively. (The values of the buffer ion mobilities used for this calculation are those given by Alberty.[10]) The experimental error in each of these determined charges is ± 0.1 and consequently an apparent charge difference of -1.5 ± 0.2 exists between the two forms. This result will be valid even if a change in frictional coefficient has occurred but will be subject to error if the modification process has involved polymerisation, as the value of the molar protein concentration, m_p, used in the charge calculation, has been calculated on the basis of 45,000 for the molecular weight of both materials.

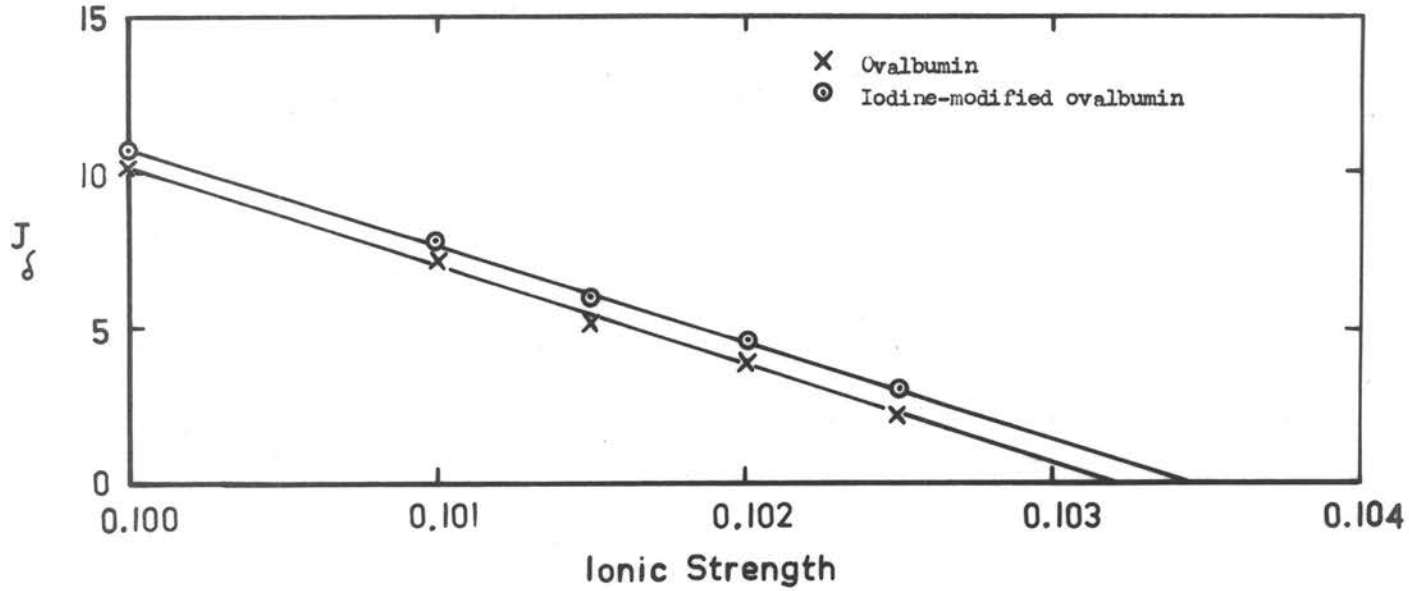


Fig. VI-5. Graphs from which the values of the ionic strengths required to eliminate the δ boundaries in the electrophoresis of native and iodine-modified ovalbumins at pH 8.5, ionic strength 0.100 have been calculated.

If it is assumed that no difference between the frictional coefficients (f) of the two materials exists, then the charge difference may also be calculated by the quantitative interpretation of the mobility difference (equation VI-8). Abramson et al. (p. 153 of ref. 5) have calculated for ovalbumin the relation between the net charge, z, on the protein and the mobility u_p. For a spherical model with sufficient water of hydration to account for its diffusion coefficient, $z = 1.75 \times 10^5 u_p$, where u_p is in cm.^{2} sec.$^{-1}$ volts^{-1}. Insertion of the relevant values of u_p from Table VI-4 in this equation gives a value for the charge difference of -1.1.

Since, as has already been indicated, determination of charge by the Charlwood procedure commonly yields high results, the two values for the charge difference show fairly good agreement. Furthermore consideration of the assumptions made in the two methods of calculation shows that the difference in mobility is probably due solely to a charge difference: calculations of charge by the Charlwood procedure are independent of f but dependent on the molecular weight (M), whereas those based on mobility data alone are dependent on f but not M. On the basis that no significant change in molecular weight or frictional coefficient has occurred (which is substantiated by the sedimentation studies below) a value of -1 for the charge difference, which must then be a whole number, seems indicated by these studies.

3. Sedimentation Studies

In order to substantiate that no change in molecular weight had occurred in the modification process, ultracentrifugal

studies have been performed on both the native and iodine-modified ovalbumins. Consideration of the forces operative in sedimentation velocity experiments reveals that $M \propto f.s$, where s is the sedimentation coefficient. If reference is again made to the assumptions used in the charge determinations, it can be seen that the charge calculated by the Charlwood procedure involved the assumption that f.s remained constant but that f could vary, whereas the quantitative interpretation of mobility data required the assumption that f remain constant but that f.s could vary. The only way that these two sets of criteria can both be obeyed is if f and s remain unaltered. This of course assumes that the partial specific volumes of the two materials are identical, which seems quite a reasonable assumption in view of the similar values for this quantity found for a large number of widely differing protein systems.[11-13] Thus the two proteins should sediment with identical sedimentation coefficients if the postulate of no change in molecular weight and frictional coefficient is correct.

Sedimentation velocity experiments have been performed on both materials at two different pH values: (a) pH 7.5, where both proteins exist as charged species and (b) pH 4.6, where both are essentially uncharged. The latter conditions should be preferred for a study of molecular weight comparison in view of the difficulties of interpreting with any great accuracy the apparent sedimentation coefficients of charged molecules in multicomponent systems. This has not proved the case in this study, however, as the sedimentation behaviour of native ovalbumin has not been reproducible. The

detailed comparison of the sedimentation coefficients of the two materials has, as a consequence, been performed on electrically charged species. Ultracentrifugal studies were performed at 59,780 r.p.m. in a Spinco Model E Ultracentrifuge employing schlieren optics and the sedimentation coefficients have been corrected to water at 20°C.

(a) Sedimentation at pH 7.5, ionic strength 0.10

To test whether a change in molecular weight had occurred in the process of modification, both materials were sedimented at a series of concentrations in the range 0.1 to 1.0% w/v. The schlieren peaks were quite symmetrical for both the native and modified materials (Fig. VI-6) and consequently the rate of movement of the maximum of the peak has been used to determine the sedimentation coefficients. The results of these two series of experiments are summarised in Table VI-6 and are illustrated in Fig. VI-7.

Table VI-6

Ovalbumin		Iodine-modified Ovalbumin	
c	$s_{20,w}$	c	$s_{20,w}$
0.93	3.20	0.89	3.21
0.70	3.23	0.67	3.24
0.49	3.33	0.47	3.31
0.30	3.33	0.30	3.32
0.10	3.38	0.10	3.42

From the graph (Fig. VI-7) it is evident that there is no detectable difference in the sedimentation behaviour of the protein

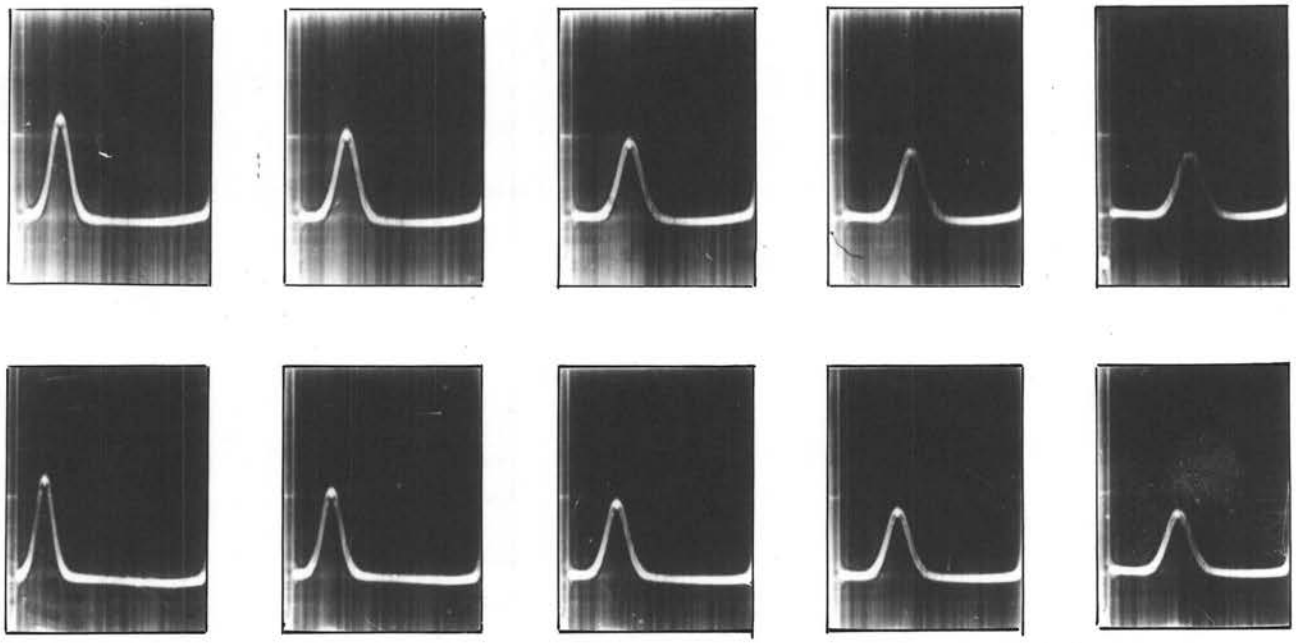


Fig. VI-6. Sedimentation diagrams of ovalbumin (upper) and iodine-modified ovalbumin (lower) in "Veronal"-chloride buffer, ionic strength 0.10, pH 7.5.

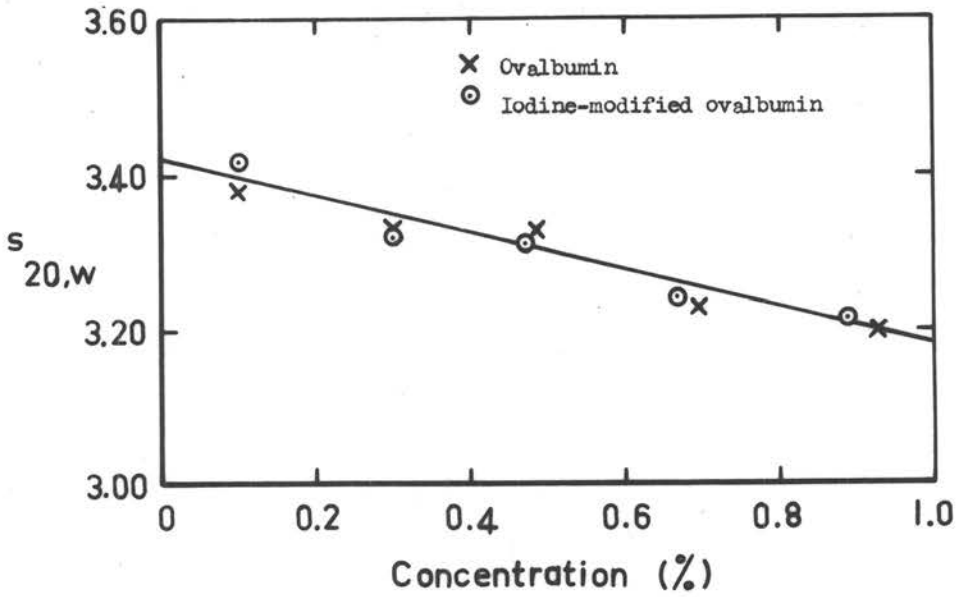


Fig. VI-7. Graph showing the concentration dependence of the sedimentation coefficients of ovalbumin and iodine-modified ovalbumin at pH 7.5, ionic strength 0.10. The line corresponds to the relationship $s_{20,w} = 3.42 - 0.24C$, which is obtained by application of the method of least squares to these results.

before and after modification, indicating that the molecular weight is essentially unchanged. Therefore it would appear that the difference in electrophoretic mobilities at pH values greater than 6 is due to a charge difference alone.

Before passing on to the sedimentation of the two materials at pH 4.6 there are two points which need emphasis:

(i) both the native and modified ovalbumins give essentially symmetrical peaks in the ultracentrifuge, and

(ii) the values of the sedimentation coefficients are in quite good agreement with values reported in alkaline or neutral solution. The values for both the modified and native materials are within 2% of Miller and Golder's values,[14] determined at pH 6-7 in phosphate buffer (their expression for the sedimentation coefficient-concentration relationship becomes, after correction for the adiabatic expansion of the rotor,[15,16] $s = 3.42 - 0.18C$: application of the method of least squares to determine an s - C relationship from the results in Table VI-6 gives $s = 3.42 - 0.24C$, i.e., a slightly greater concentration dependence, which may be a consequence of the lower ionic strength used in this work (0.10 as compared with 0.20).

(b) Sedimentation at pH 4.6, ionic strength 0.16

Whereas essentially symmetrical peaks were observed in sedimentation of both native and modified ovalbumins at pH 7.5, there is a marked difference between the peaks if the sedimentation is performed at pH 4.6. Sedimentation of the modified material again yields a symmetrical peak (see Fig. VI-8a), the values of the sedimentation coefficient agreeing with that calculated on the basis

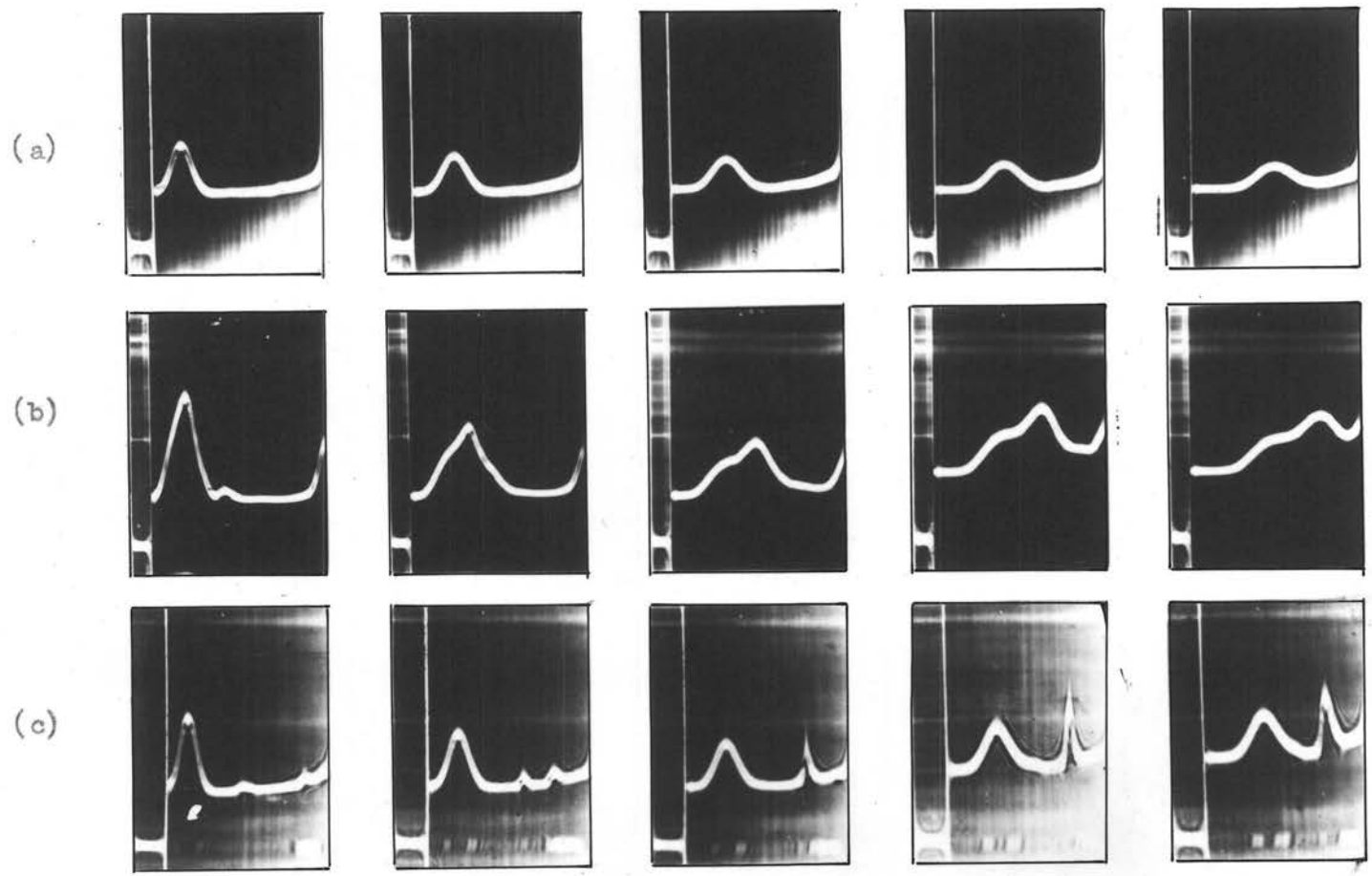


Fig. VI-8. Sedimentation diagrams of ovalbumin and iodine-modified ovalbumin in acetate buffer, ionic strength 0.16, pH 4.59: (a) iodine-modified ovalbumin; (b) native ovalbumin; (c) fractionated A_1.

of the experimentally observed s - C relationship at pH 7.5. However, with native ovalbumin, it was found that the results were not reproducible, either with respect to sedimentation coefficient or appearance of the sedimenting boundary. Thus while some preparations gave nearly symmetrical peaks and s values only slightly greater than those reported by Kegeles and Gutter,[17] in general considerable asymmetry, much higher sedimentation coefficients, and occasionally actual resolution of the peaks were observed. Furthermore, the electrophoretically fractionated A_1 component behaved in a manner entirely analogous to the ovalbumin complex. This behaviour is illustrated in Fig. VI-8: in 8b a quite well defined peak of $s_{20} \sim 6.1$ is present as well as the main peak, the s_{20} of which is approximately 3.6: material of much greater molecular weight has accumulated at the bottom of the cell. It was frequently found that a stationary peak appeared in this region, whose area became greater with time (this is evident in Fig. VI-8c); Kegeles and Gutter[17] reported that many of their preparations gave asymmetric peaks after standing short times at the pH used, which was 4.40, i.e., on the acid side, but quite close to, the isoelectric point 4.59. Also, it should be mentioned that the values for s reported by Kegeles and Gutter are about 9% higher than those at pH 6-7 of Miller and Golder, which is a much greater difference than could be accounted for by experimental error. It appears therefore that high values for s can be expected at values of pH very close to the isoelectric point. At first sight this statement appears to contradict the findings of Charlwood and Ens,[18] who studied the sedimentation coefficient of a 0.35% ovalbumin solution over the pH range 1.9 to 9.7. They have

found s values in the range 1.9 to 3.5 to be constant, but about 3% lower than those in the range 4 - 9.7, where also s was independent of pH. No experiments were reported in the range between pH 4.0 and 5.0, where due to the presence of only a very small net charge, aggregation may be possible, and hence cause the values of s determined at these pH values to be inconsistent with those at other pH values. It may be concluded that aggregation occurs (possibly via a relatively stable unit of only a few molecules) very readily in ovalbumin solutions in the immediate vicinity of the isoelectric point when subjected to high pressures, and accordingly useful results cannot be obtained under these conditions. This aggregation is presumably a pressure effect, since the presence of such aggregates in diffusion would most certainly be detected as a slow diffusing impurity; in direct contrast the diffusion results reported in Chapter III rather indicate the presence of a fast diffusing impurity (if the deviation graphs are considered as estimates of heterogeneity alone).

4. Discussion

From this physico-chemical comparison of native and iodine-modified ovalbumins, the following points require discussion.

(i) The electrophoretic mobility difference of -0.6×10^{-5} cm.^{2}/sec./volt between native and modified materials has been attributed to a charge difference of -1 on the two molecules. In this respect it should be added that Perlmann[19] has interpreted a similar mobility difference as indicative of a difference in charge of -2 in her studies of the differences between the components of

ovalbumin and also the difference between ovalbumin and plakalbumin. Her interpretation is based on Cannan, Kibrick and Palmer's observation[20] that a mobility difference of 0.6×10^{-5} cm.^{2}/sec./volt is obtained when two equivalents of acid are added to a mole of ovalbumin at ionic strength 0.1. However, Abramson et al. (p. 156 of ref. 5) consider that titration curves yield too high a value for the apparent net charge due to the binding to the protein of undissociated acid along with the hydrogen ion: such effects will presumably disappear at zero ionic strength. They have applied a correction to Cannan et al.'s data at ionic strength 0.1 to allow for this binding of acid, with the result that much better agreement is found between values of net charge calculated from titration curves and those from mobility data. This correction is of such a nature that high results would normally be obtained from titration data. Therefore, should Abramson et al.'s interpretation of titration curves be correct, then Perlmann's conclusion that the mobility difference is due to a charge difference of -2 would be in error.

(ii) Whilst on the subject of charge difference it should be mentioned that the appearance of an extra negative charge at pH values greater than 6 subsequent to iodine-modification of ovalbumin is not supported by the results of Fredericq and Desreux.[21] Their investigation has comprised the construction of a pH titration curve for both native and modified materials; the two curves are essentially identical for pH values less than 8.5, then diverge in such a manner that a slightly greater negative charge on the native material is indicated and then become essentially identical at pH values greater than 11.5. However,

a comparison of their results with those of this study should only be made in the pH range of this study (4 - 8.5). Below pH 7.5, Fredericq and Desreux indicate no experimental points for the derivative, presumably because of its low solubility in acidic solutions: their first point is at pH 7.5. Furthermore their next point for the modified material is at pH 9.5, so that a strict comparison of their results with those of this study can only be made at the one pH value, viz., 7.5, where this study indicates a charge difference of -1 and their study indicates no difference. This discrepancy between results may be due to a difference in the materials under investigation: one factor indicative of this possibility is the very limited solubility in salt-free solutions of the derivative used in this study, whereas Fredericq and Desreux's titrations were performed in salt-free solutions in which the final protein concentration was approximately 1%. A difference in materials under investigation does appear quite feasible as Anson[22] considers that oxidation of sulphydryl groups beyond the disulphide stage occurs to a slight extent under conditions similar to those employed by the Belgian workers. (Anson considers that iodine used under the conditions of this study is specific for the oxidation of sulphydryl groups to disulphide linkages.) Anson also states that iodine reacts with the tryptophan residues of ovalbumin if the reaction is performed at acid pH values.[23]

(iii) It would be quite interesting to make a more critical comparison of the shapes of the two molecules. Although the identity of the two frictional coefficients (as judged from this study) indicates relatively little difference, the intrinsic viscosities,

$[\eta]$, of the two substances would give a more sensitive estimate of the differences (the methods employing s and $[\eta]$ have been summarised and compared by Ogston[24]). However, any differences detected by such means will presumably be too small to have a marked effect on any conclusions to be drawn from this work and investigation of this aspect has therefore been deferred. No attempt has been made to combine the data from D, s and d(1/s)/dC measurements in view of Ogston's conclusion that it is not a reliable procedure for determining shape factors.[24]

(iv) One important feature of the sedimentation results at pH 4.6 is the observation that symmetrical peaks are always obtained with the modified material and that the s values are in good agreement with those calculated on the basis of the s - C relationship determined at pH 7.5. Therefore it would appear that the modification process has at least stabilised the ovalbumin against pressure effects. However, the desired stability to heat effects has not been achieved by this modification procedure, as exposure of the modified material at pH 4.6 to a temperature of 20°C causes precipitation after 24 hours: consequently no diffusion experiments have been performed on the modified ovalbumin.

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CHAPTER VII

THE REACTION BETWEEN IODINE AND OVALBUMIN

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CHAPTER VII

The Reaction Between Iodine and Ovalbumin

The two preceding chapters have provided evidence which shows that the reaction between iodine and ovalbumin is not simply an oxidation of the sulphydryl groups to disulphide linkages as has been previously reported.[1] It is the purpose of this chapter to discuss possible reactions which might occur between iodine and ovalbumin in the light of the experimental evidence obtained in this study.

1. Summary of Data Obtained

The following is a summary of the data obtained concerning the reaction between iodine and ovalbumin:

(i) By a potentiometric titration procedure it has been found that the modification process involves 5.0 iodine atoms per molecule of ovalbumin.

(ii) Amperometric titration determinations of the sulphydryl and disulphide content of the native and modified materials have revealed that two sulphydryl groups are lost in the modification but that no additional disulphide linkages are formed.

(iii) Electrophoretic and sedimentation studies have indicated that the iodine-modified ovalbumin possesses an additional negative charge at pH values greater than 6 and also that no significant changes of frictional coefficient and molecular weight have resulted from the modification.

From this data, therefore, the problem is to find a mechanism for the reaction between ovalbumin and iodine wherein two sulphydryl groups react to give some product other than disulphide, the reaction

resulting in a product with almost the same macroscopic features except that it contains an extra ionisable group which dissociates in the region of pH 6: the overall reaction must require 5 iodine atoms per molecule of protein.

2. Implications of the Data

The obvious implication of these results is that oxidation of the sulphydryl groups beyond the disulphide stage has occurred, presumably to the sulphenic, sulphinic or sulphonic acid stages,[2-5] MacLaren, Leach and O'Donnell[5] having expressed the further oxidation of disulphides as follows

$$-S.S- \longrightarrow -SO.S- \longrightarrow -SO_2S- \longrightarrow \ldots 2SO_3H.$$

From the point of view of the number of iodine atoms involved in the modification process, the sulphenic oxidation state would be preferred, two atoms of iodine per sulphydryl group being necessary. A mechanism based on the formation of such compounds could be criticised on the grounds that sulphenic acids are extremely reactive and are usually regarded as unstable intermediates in the further oxidation to the sulphonic acid state. However, evidence suggestive of the existence of the intermediate oxidation states between the disulphide and sulphonic acid stages has been obtained in the case of cysteine oxidation[6-8] and also wool oxidation with hydrogen peroxide,[9] chlorine[10] and peracetic acid.[5,7] Furthermore, in a study of the reaction of tobacco mosaic virus with iodine, Fraenkel-Conrat[11] has isolated a product in which the sulphenic oxidation state exists. A mechanism involving such an oxidation state would therefore presumably be acceptable, especially in view of Hughes and Straessle's

finding that two iodine atoms per sulphydryl group are required for the oxidation of these groups in human serum albumin.[12]

3. The Possibility of Iodination of the Ovalbumin Molecule

So far this discussion has considered only the possibility of oxidation of sulphydryl groups, where all of the iodine involved in the reaction is converted to iodide ions. In view of the similarity between the conditions required for iodination of tyrosine residues[13] and those used for the modification performed in this study, the question of possible iodine incorporation into the molecule has been studied by radiochemical techniques using radioactive samples of iodine (I^{131}).

Briefly, the experimental test of the possibility of iodination of the molecules has been performed as follows (details are given in Chapter VIII): the modification was performed in accordance with the procedure outlined in Chapter IV except that an amount of I^{131}-labelled iodine solution in slight excess of that required on the basis of 5 atoms per molecule of ovalbumin was added, the actual titration not being performed. The resulting solution was then dialysed against phosphate buffer pH 7, ionic strength 0.2 (henceforth called the dialysate[14]) with constant mild agitation and frequent changes of dialysate until no significant amount of radioactivity was detected in the dialysate, the activities of solutions being measured with a scintillation counter.[15] The activity of a sample of the contents of the dialysis bag was then measured and compared with that of a solution of radioactive iodine of known concentration. This solution was prepared at the commencement of

the iodine modification of the protein to avoid any corrections of activities for time effects.

The first measurement of protein radioactivity was taken when the dialysate activity was 200 counts per 100 secs. above the normal background count of 140, the count for the dialysate being used as "background" in the subsequent determinations: at this stage a whole number was not obtained for the iodine content in atoms per molecule of protein (experiments 1 and 2 in Table VII-1). To test whether the fraction of an atom was due to incomplete dialysis a sample was dialysed further against the phosphate buffer until the dialysate and background counts were identical (experiment 3 in Table VII-1). In addition a sample was dialysed further against a veronal-chloride buffer pH 8.5, ionic strength 0.1 to determine whether the activity was due to slow replacement on the protein of closely held iodide ions by the relatively large phosphate ions (experiment 4 in Table VII-1). The results of these experiments are tabulated below (Table VII-1).

Table VII-1

Expt. No.[b]	Standard Solution[a] Sol'n Count[c,d]	Activity/ g.atom[c]	Protein Solution Sol'n Count[c,d]	Activity/ mole[c]	I atoms/ mole
1	24,600	5.17×10^9	16,000	6.44×10^9	1.24
2	24,600	5.17×10^9	16,900	6.79×10^9	1.31
3	17,000	3.58×10^9	3,820	3.83×10^9	1.07
4	17,000	3.58×10^9	3,310	3.33×10^9	0.93

[a] The solution of radioactive iodine of known concentration.
[b] The significance of these expt. numbers is discussed in the paragraph preceding this Table.
[c] Expressed as counts per 100 seconds.
[d] Corrected for background count.

These results indicate the presence of one iodine atom per molecule of ovalbumin and in this respect it should be added that this value is considerably higher than the value 0.25 inferred from Fraenkel-Conrat's results.[11] The methods of modification used in the two studies are essentially identical, the major difference in the subsequent procedures of radioactive iodine removal being that phosphate was used in this study whereas Fraenkel-Conrat used chloride, and in some cases iodide ions, to wash out the radioactive iodide ions. The results of experiments 3 and 4 in the above Table however seem to rule out this difference of technique as a possible reason for the discrepancy between the two results.

It is rather difficult to assess Fraenkel-Conrat's result as no definite information about the actual experiment with ovalbumin is given: the data from which the value 0.25 has been inferred appear in a Table listing the extent of iodine substitution resulting from the iodine modification of a series of proteins.[11] Now although this may not apply to his work with ovalbumin, one point of criticism that can be levelled at Fraenkel-Conrat's radioactivity work in general is the use of inactive iodide in some unspecified cases to remove the radioactive iodine and iodide ions. The calculation of the iodine content of the protein relies upon the fact that the specific activity (activity per gram atom) of iodine in any oxidation state remains constant for the protein sample. Now whilst this condition will most certainly be fulfilled in the case of dialysis against iodide-free solutions, the use of solutions containing inactive iodide ions as dialysate will certainly affect the specific activity of the iodide

ions and could possibly affect the specific activity of the protein. In this respect it should be added that Miller et al.[16] have studied the exchange between covalently bound iodine in di-iodotyrosine and free iodine and also iodide ions: their results show that exchange with iodine certainly occurs, but that exchange with iodide ions is not significant under conditions similar to those employed by Fraenkel-Conrat. However, in view of the known rapid exchange between iodine and iodide, the specific activity of the protein may well be affected during the initial dialysis period when excess iodine is still present in small amounts. The effect would be to lower the specific activity of the protein with the result that a low value for the iodine content would be obtained. This could provide an explanation of the observed discrepancy between Fraenkel-Conrat's results and those of this study, but as already indicated, no definite information about the experiment with ovalbumin was given and it may be that iodide was not used for that particular experiment. Indeed radiochemical techniques may not have been used for the ovalbumin experiment as assays of the modified materials for iodine were also performed in some cases.[11]

4. Further Consideration of Possible Reactions

The first point to establish is the position at which the one iodine atom is attached to the ovalbumin molecule. There appear to be three major possibilities: the iodine could be present as (i) sulphenyl iodide,[11] (ii) mono-iodohistidine[17,18] or (iii) mono-iodo-tyrosine.[12,19-25]

(a) The Possibility of Sulphenyl Iodide Formation

The only protein in which a stable sulphenyl iodide group

has been shown to exist is the iodinated derivative of tobacco mosaic virus:[11] Fraenkel-Conrat has shown that the iodine is liberated upon denaturation of the protein. He also reports that denaturation does not dislodge the small amount of iodine bound in iodine-modified ovalbumin, which is indicative of the iodine being situated on phenol or imidazole rings. Consequently the first of these three possibilities appears to be eliminated.

(b) The Possibility of Mono-iodination of Histidine

Although Roche et al. have detected mono-iodohistidine in iodinated globin,[18] the electrophoretic evidence of this study indicates that such a residue is not formed in the iodine modification of ovalbumin. The pK_a value of the imidazolium group in histidine is quoted as 5.6 - 7.0,[26] this group thus being positively charged in acid solution and the conjugate base bearing no charge in alkaline solution. Therefore any changes resulting from histidine modification would be associated with increase of charge in acid solution, rather than the observed greater negative charge observed with modified ovalbumin in alkaline solution.

(c) The Possibility of Tyrosine Iodination

The iodination of proteins to yield iodotyrosine derivatives is now well established for human serum albumin[12] and thyroglobulin.[22] Furthermore, the electrophoretic mobility and charge data of this study may be explained by postulation of the formation of a mono-iodotyrosine residue in the modification process. The pK_a value of the phenolic hydroxyl group of tyrosine is 10.2,[27] whilst that for di-iodotyrosine is 6.4 - 6.5,[27,28] a value 8.2 being observed for mono-iodotyrosine.[29]

Since the phenolic group bears no net charge on the acid side of the pK_a value, but bears a negative charge in solutions more alkaline, an increase in the dissociation of a phenolic hydroxyl group (indicated by a decrease in pK_a) would result in the appearance of an extra negative charge in the pH range between the new pK_a value and 10.2. If the electrophoretic mobility data of this study can be interpreted on this basis, iodination of a tyrosine has resulted in the depression of the pK_a to approximately 6, a value in much better agreement with that of di-iodo- rather than mono-iodotyrosine. However, the effect of substituents on the pK_a value is very marked, as is evident from the decrease from 8.2 to 6.4 on the incorporation of an additional iodine atom into mono-iodotyrosine, and in this respect the effect of the amino acid residues adjacent to the mono-iodotyrosine may well bring about a similar decrease. Therefore, the electrophoretic mobility data could presumably be explained by postulating the formation of one mono-iodotyrosine residue in the iodine modification of ovalbumin.

Amino acid analyses, however, indicate the presence of nine tyrosine residues per molecule of ovalbumin[30] and the question is therefore raised as to the reason for the preferential iodination of one tyrosine group. Inaccessibility or decreased reactivity of the phenolic groups appears to be the most logical answer, since only a few such groups are detectable in native ovalbumin by titrimetric[31] or spectrophotometric[27] procedures. In this respect it should also be added that whilst the conditions of the iodine modification of ovalbumin are similar to those usually associated with the iodination of proteins, the high concentration of potassium iodide used has been shown to suppress the reaction of tyrosine with iodine[1] and therefore

only a tyrosine residue rather more reactive than free tyrosine due to neighbouring substituents would presumably react under the conditions employed. The postulation of tyrosine iodination might also be criticised on the grounds that the reaction is much slower than sulphydryl oxidation. However, Ramachandran and Sarma[32] have found that the rate of tyrosine iodination is greatly increased if the reaction is performed in phosphate buffer, and thus use of phosphate buffer in this work could be regarded as an explanation of any discrepancy in relative rates of reaction. Finally mention should be made of Charlwood's work on the electrophoresis of a mixture of human serum albumin and iodinated serum albumin in a density gradient,[33] the modified albumin having been iodinated to the extent of one atom per molecule with I^{131} labelled iodine by the method outlined by McFarlane;[34] the iodine is presumably present as iodotyrosine residues.[12] It was found that on electrophoresis of the mixture, the peak of radioactivity did in fact move slightly further than the peak of optical density. Resolution of a faster component was not evident in the schlieren diagrams, but it is not certain that this would have been expected, as no figure was given for the small proportion of iodinated albumin in the mixture. In any case, considerable anomalies must be expected in the apparent proportions of electrophoretic components of closely similar mobility.[35] While no quantitative estimate of the mobility increase for iodinated human serum albumin can thus be obtained from Charlwood's work, it seems quite clear that qualitatively the effect is analogous to the case of iodine-modified ovalbumin.

Of the various possibilities of iodine incorporation into

the ovalbumin molecule, the formation of a mono-iodotyrosine residue has been considered the most likely. Since the formation of such a residue could explain the appearance of the extra negative charge possessed by the modified material, it is necessary to assume that the remainder of the modification has produced no marked effects on the mobility of the molecule in the pH range 4 - 8.5. On the other hand, mono-iodotyrosine has a pK_a value of 8.2[29] and hence it may be that the charge difference is a reflection of the modification to the remainder of the molecule, rather than an increase in the tyrosine hydroxyl dissociation, which may not be apparent until pH values greater than 8.5. Although the lack of any data on the pK_a of sulphenic acids makes any definite conclusions on this point impossible, one factor which is indicative that the charge difference is not due to sulphenic acid dissociation is the value -1 obtained: since two sulphydryl groups have been modified it must be assumed that two sulphenic acid groups would result and hence a charge difference of -2 would be expected, at pH values greater than the pK_a value. This value, although unknown, would be less than 7 and hence the existence of sulphenic acid groups in the modified material seems extremely unlikely. It would therefore seem necessary to postulate that iodine oxidation of the two sulphydryl groups has proceeded beyond the disulphide stage to yield a sulphoxide[5] (R-S(=O)-S-R), which presumably would not ionise. The stoichiometry of the reaction is such that two iodine atoms per sulphydryl group would be required and certainly there is a tendency for sulphoxides to result from the oxidation of sulphydryl groups with peroxides:[36] the mobility data would also be in agreement with such a postulate. It is not possible, however,

to foretell the action of sulphite on such compounds, which, because of their relatively rare occurrence, have not been studied in any great detail.

5. Conclusions

The data reported in this study provide a variety of evdience which indicates that the oxidation of sulphydryl groups to disulphide linkages is not the sole reaction occurring in the iodine modification of ovalbumin. Comparison of the number of iodine atoms used in the reaction with the number of groups modified gives a value 2 for the most likely number of iodine atoms used per thiol group: this would account for four of the five iodine atoms. The finding of one iodine atom in the modified protein molecule does not provide a solution to the problem of the odd number of iodine atoms used in the modification process, as the formation of iodotyrosine will also involve a molecule of iodine, the other iodine atom being liberated as iodide. It would seem therefore that the reaction between iodine and ovalbumin is complicated but that the results reported herein do not provide sufficient evidence for the nature of the reaction to be determined.

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CHAPTER VIII

EXPERIMENTAL - APPARATUS AND METHODS

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Experimental - Apparatus and Methods

1. Preparation of Crystalline Ovalbumin

Crystalline ovalbumin was prepared by the method of La Rosa.[1] The samples were recrystallised twice and stored at -15°C as a paste containing much ammonium sulphate.

2. Diffusion

All diffusion experiments were performed in a Spinco Model H Electrophoresis-Diffusion Apparatus, employing Rayleigh optics alone, and following as closely as possible the general procedures for the conduction of experiments outlined by Creeth.[2,3] The records of the diffusion experiments, which were photographed on "Kodaline C.T.C. Pan" plates,[4] were measured with a two-dimensional comparator[5] accurate to 2×10^{-4} cm., fitted with projection screen. Once again the procedures for the measurement of Rayleigh interferograms suggested by Creeth[2,3] have been followed. The magnification factor, M, for the apparatus has been obtained by placing a horizontally graduated scale in the position equivalent to the centre of the solution (the back cell window in this apparatus which employs a doubled optical path) andphotographing the resultant image. A series of comparator measurements of the scale and corresponding image graduations were then made, M being determined by the regression of image values upon the object values.

(a) Standardisation of the Apparatus

In order to check the performance of the apparatus, a diffusion experiment was performed on sucrose: the Y_t values were certainly constant, but the value calculated for $D_{\overline{C}}$ was 1.2% higher than the

generally accepted value,[6,7] whilst the observed skewness was of the opposite sign to that predicted. In an endeavour to remove these anomalies many modifications to the apparatus were made: these are summarised below.

(i) To minimise vibration the mechanical stirrer mounted on the apparatus was replaced by one attached to a wall, and the mechanically operated camera shutter was suitably modified to allow manual manipulation. The rotating turret holding the cell carriage was locked firmly throughout the experiment.

(ii) To avoid marked disturbance of the boundary the cell carriage was modified so that the centre section remained fixed and the top section could be displaced. Thus the cell centre section could be sealed off during an experiment without itself being moved. Also a pressure head siphon was used for boundary sharpening in preference to the mechanically operated syringe.

(iii) The following alterations were made to the optical system, which is represented diagrammatically in Fig. VIII-1: Wagner and Scheraga's notation[8] for the various optical components of the Spinco Model H apparatus has been used.

(a) A new source slit, 35 microns in width, was constructed from razor blades.

(b) In the Spinco apparatus a doubled optical path is employed whereby the reflected light passes through the cell on its second passage at a different level from that corresponding to its first passage. To minimise this vertical displacement of the light rays, the bath mirror, M2, previously tilted downwards, was adjusted until vertical: this was materially facilitated by replacement of

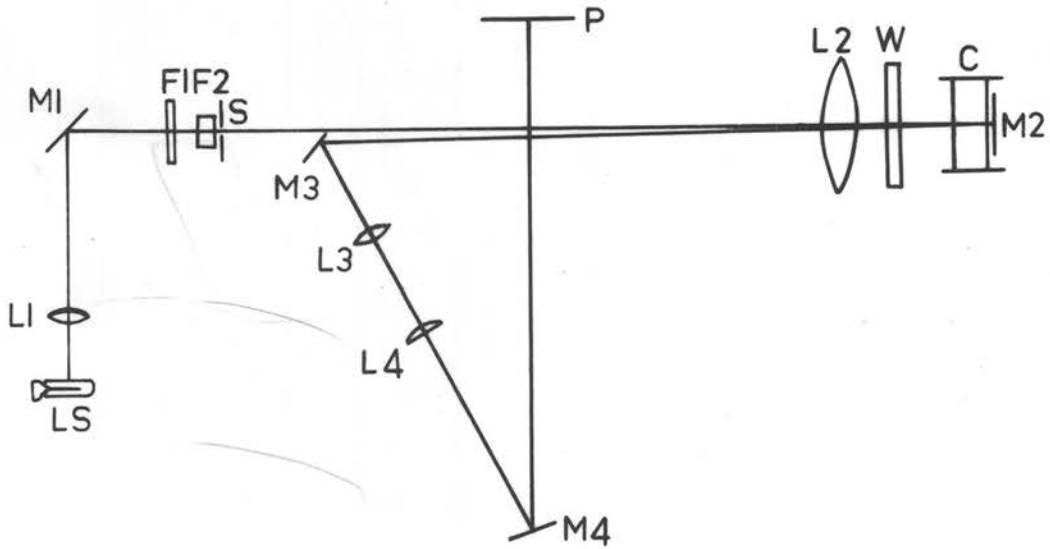

Fig. VIII-1. Sketch of the optical path in the Spinco Model H diffusion apparatus: LS, light source; L1, condensing lens; M1, M2, M3 and M4, first surface mirrors; F1 and F2, light filters; S, slit assembly; L2, schlieren lens; W, bath window; L3, camera lens; L4, cylindrical lens; P, plane of photographic plate (camera). The sketch is not to scale.

the four point suspension device supplied, by a conventional three point dynamic mounting. (The assistance of Dr. J.H. Coates in this respect is gratefully acknowledged.) The source slit was then adjusted vertically until the slit and its reflected image superimposed: a small lateral displacement to the slit then caused it to be slightly off axis sideways but accurately on the optic axis in the vertical plane. This procedure was adopted following the suggestion of Longsworth[9] that the necessary divergence of incident and reflected light is much better accomplished by lateral than vertical displacement. In consequence of the adjustment to mirror M2, the mirror adjacent to the light source, M3, was replaced by the notched mirror (supplied for use with Gouy optics) to enable the light to enter the remainder of the optical system. In order to ensure that light reflected from M3 passed along the principal axes of both the camera and cylindrical lenses, L3 and L4, it was necessary to increase the optical path by displacing M3 by 2.3 cm. The camera, P, was then refocussed on the surface of M2 in accordance again with the procedure adopted by Longsworth.[9]

At the completion of these alterations a further experiment using sucrose (ΔC = 0.7500 g./100 ml., $\overline{C}$ = 0.3750 g./100 ml.) was then performed. Y_t values were again constant and $D_{\overline{C}}$ was 2.411 x 10^{-6} cm.^{2} sec.$^{-1}$ at 1.00°C, compared with Gosting and Morris' value of 2.408 x 10^{-6} cm.^{2} sec.$^{-1}$ obtained with the Gouy method[6] and Longsworth's value of 2.414 x 10^{-6} cm.^{2} sec.$^{-1}$ using the Rayleigh method[7] at this concentration and temperature. Skewness results, whilst showing considerable scatter, were of the correct sign but of smaller magnitude than predicted. It is believed that this discrepancy is due to the

discontinuity in the path of a refracted ray which is a consequence of the use of an external mirror to return the light through the cell: at very long times ($\sqrt{D_A t} \sim 0.7$ cm.) moderately accurate skewness results have been obtained on glycine, which shows much greater concentration-dependence of diffusion coefficient. (The author is indebted to Miss B.E. Peter for conducting the experiment with glycine.)

(b) Test of the Heterogeneity Analysis Procedure

A solution containing both sucrose and urea (0.2250 g. and 7.2040 g. per litre respectively) was allowed to diffuse against pure water: satisfactory agreement was obtained between the predicted and observed values of D_A (D_A calc. = 6.58_3, D_A obs. = $6.59_3 \times 10^{-6}$ cm.^{2} sec.$^{-1}$) and the total number of fringes ($J_{calc.} = 104.82$, $J_{obs.} = 104.83$) and also between the predicted and observed deviation plots, shown in Fig. VIII-2.

(c) Diffusion of Protein Samples

Diffusion experiments on protein samples were performed in an acetate buffer containing 0.01, 0.01 and 0.15 moles per litre of potassium acetate, acetic acid and potassium chloride respectively, the pH of which is 4.59.[10] Prior to diffusion, all protein samples were dialysed with constant mild agitation and frequent changes of buffer, in the cold for four days. The temperatures of the diffusion experiments, all in the range 0.5 - 1.5°C, were periodically recorded using a previously standardised Beckmann thermometer. The relative viscosity of the buffer, used in the correction of all "height-area" average diffusion coefficients to water at 1.00°C, was 1.004.[10]

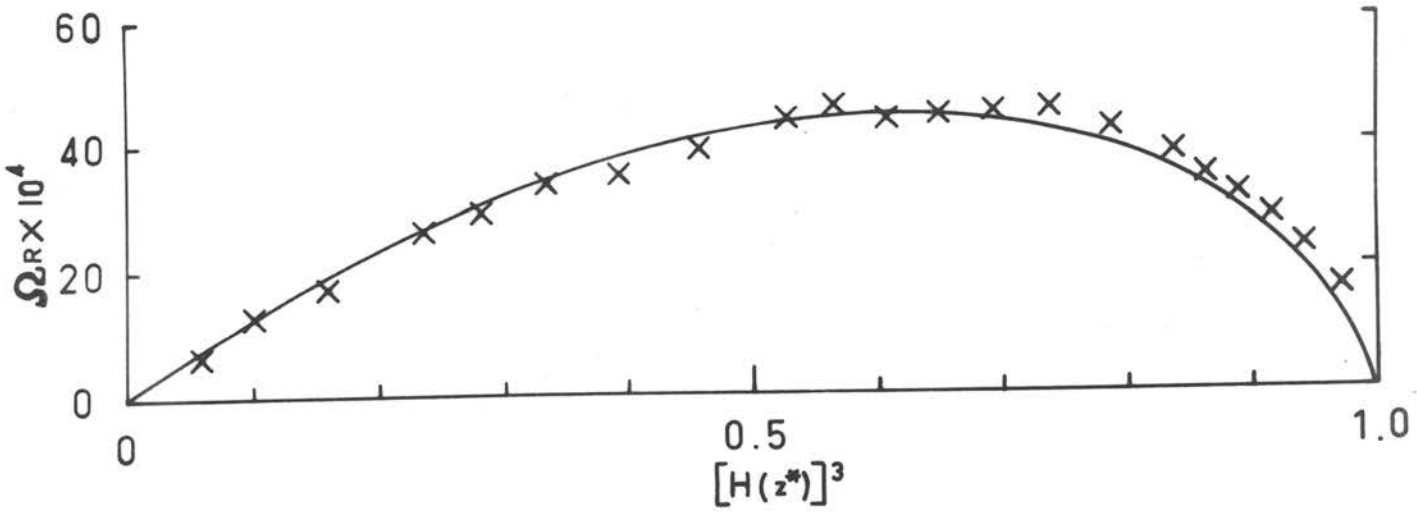

Fig. VIII-2. "Deviation Plot" for a urea-sucrose mixture ($\alpha_{sucrose} = 0.0297$, $D_{urea}/D_{sucrose} = 2.844$). The line represents the predicted deviation, the crosses the mean of 4 experimental observations of the deviation.

3. Modification of Ovalbumin with Iodine

(a) Preparation of Materials

(i) Sufficient crystalline ovalbumin to give a concentration of approximately 3% was dissolved in water and the resultant solution dialysed in the cold for two days against water with constant mild agitation and with frequent changes of dialysate. The concentration of the solution was then determined refractometrically by forming a boundary between the dialysed protein solution and the final dialysate in a 2-ml. Tiselius cell (see Chapter IV).

(ii) The iodine solution to be added to the protein solution was also molar with respect to potassium iodide; the iodine concentration, approximately 10^{-3}N, was determined by titration with a standard sodium thiosulphate solution.

(iii) A phosphate-potassium iodide buffer, pH 7, containing 0.2, 0.2 and 2.0 moles per litre of disodium hydrogen phosphate, sodium dihydrogen phosphate and potassium iodide respectively, was prepared. Equal volumes of this buffer and the protein solution were then mixed just prior to the addition of the iodine solution. Thus the titration mixture was 0.1 molar with respect to both phosphates and 1.0 molar with respect to potassium iodide.

(b) The Potentiometric Titration

The titration assembly comprised a 50-ml. beaker into which dipped a bright platinum electrode and an agar-KCl salt bridge connected to a saturated calomel half-cell. These two electrodes were connected to a Pye potentiometer (portable type), the internal galvanometer being used to determine the potential of the titration

mixture. For the titration, which was performed on 20-ml. samples, the reaction vessel was surrounded with ice to keep the temperature as low as possible. After each addition of iodine solution the e.m.f. was recorded: the titration was continued until the value of the e.m.f. was no longer increased by the addition of further iodine.

(c) Removal of the Excess Iodine

The contents of the reaction vessel were placed in a dialysis bag and dialysed against a phosphate buffer which was 0.01 molar with respect to both phosphates. After dialysis against five portions of 400 mls. of this buffer, the protein solution was stored at 4°C until required.

4. Electrophoresis

All electrophoresis experiments have been performed at 1-2°C in a Spinco Model H Electrophoresis-Diffusion Apparatus: Rayleigh and schlieren optical systems have been employed simultaneously for routine analytical experiments, but for the mobility experiments and the charge determinations the Rayleigh optical system alone was used. Electrophoretic patterns have been recorded on "Process Panchromatic" film.[11]

(a) Analytical Electrophoretic Experiments

Analytical experiments were performed in a "Veronal" buffer, ionic strength 0.05, made by dissolving 10.30 g. of sodium diethylbarbiturate and 1.85 g. diethylbarbituric acid per litre. The pH of this buffer, measured on a Pye Universal pH Meter (direct reading type), was 8.7_0. Electrophoresis was normally continued for a period of three hours at a potential gradient of 6.2 volts per cm. Analyses

of the boundaries have been made by using the schlieren photograph for division of the boundary into components, the relative amounts of the components being calculated from the number of Rayleigh fringes due to each component.

(b) Electrophoretic Mobility Experiments

(i) Buffers - Experiments pertaining to the calculation of electrophoretic mobilities have been performed in a series of buffers, the compositions of which have been given in Table VI-4. The pH of these buffers has been measured on a Doran Universal pH Meter, the electrode assembly of which comprised a glass electrode and a saturated calomel half-cell.

(ii) Protein Solutions - The concentrations of protein solutions used for mobility experiments were in the range 0.05 to 0.15%. A sample of protein was first dialysed against "Veronal" buffer, pH 8.5, for two days at 4°C with constant mild agitation and frequent changes of buffer. Subsequent to the mobility experiment, the protein was salvaged from the electrophoresis cell and redialysed against "Veronal" buffer, pH 7.5 for 15 hours with two changes of buffer and constant mild agitation: this was regarded as sufficient time for equilibrium to be established. Thus the whole mobility curve for each protein was obtained on the one sample of each.

(iii) Conductivities of Solutions - The conductivities of the protein solutions were measured at 1.0$_0$°C using a Philips Conductivity Measuring Bridge PR 9500 and a 2-ml. conductivity cell (Philips PR 9512/01) of cell constant 1.28.

(iv) Electrophoresis Experiments - Electrophoresis experiments

were conducted in an 11-ml. Tiselius cell at 1.0_0°C using a potential gradient of 2.9 - 3.7 volts per cm. for a period of 200 minutes, after which time the current was reversed for an equal interval of time. Six photographs of both the ascending and descending limbs were taken to record the migration of the boundary during each traverse of the cell. Measurement of these records was made on the two-dimensional comparator referred to in section 2 of this chapter.

(c) Charge Determinations

(i) Protein Solutions - Sufficient protein for five electrophoresis experiments in an 11-ml. Tiselius cell at a concentration of approximately 0.6% was dialysed against "Veronal" buffer, pH 8.5, ionic strength 0.1000 for two days, after which time it was stored in five air-tight containers. The five samples were then subjected to electrophoresis using slightly differing supernatant buffer concentrations.

(ii) Buffers - Since the prime aim of these experiments was to determine the ionic strength of supernatant buffer required to eliminate the δ boundary, great care was taken in the preparation of the buffers, all of which have been prepared in the same flask thermostated at 1.0_0°C, the temperature at which the electrophoresis experiments were performed.

(iii) Electrophoresis Experiments - Particular care was taken to ensure that the shapes of the boundaries observed in the electrophoresis experiments were undisturbed by processes other than electrophoretic migration and diffusion. Since the "compensation" process for bringing into view the initial sharp boundary produces a markedly skew integral curve, it was decided to sharpen these boundaries by

the very reliable siphoning procedure used in commencing diffusion experiments. After the initial formation of the two boundaries at the top and bottom of the centre section, liquid was withdrawn from the system until the boundary initially at the top had moved to within approximately two cm. of the bottom of the centre section. Siphoning was then ceased and the bottom section disaligned. A capillary was then inserted to the level of the boundary and intermittent siphoning was commenced to free the region above the boundary of protein. After readjustment of the buffer levels in the two electrode vessels, the bottom section was realigned and the boundary was sharpened from both sides for two or three minutes. The capillary was then withdrawn and the Rayleigh interferogram photographed, after which electrophoresis was commenced in such a direction that the sharpened boundary was the ascending boundary. Electrophoresis was continued for four hours at a potential gradient of 3.4 volts per cm., at which time the interferogram was again photographed. Estimation of the size of the δ boundary to the nearest 0.1 fringes was made using a two-dimensional comparator,[12] reading to 0.01 mm.

5. Sedimentation

Sedimentation velocity experiments were performed in a Spinco Model E Ultracentrifuge at 59,780 r.p.m. and the sedimentation coefficients, calculated in accordance with the general procedure outlined by Charlwood,[13] were corrected to water at 20°C. Photographic records of all sedimentation experiments were taken on "Rapid Process Panchromatic" plates.[14] Initially the temperature was recorded at the start and finish of each experiment, the mean

value, allowing for the adiabatic expansion correction of 0.9°C,[15,16] being taken as the temperature of the experiment. Subsequent to the installation of a Spinco "Rotor Temperature Indicator and Control Unit", the temperatures of the experiments were found by averaging the temperatures at the time of the first and last exposures. The required viscosity and density values for the acetate buffer were those observed by Akeley and Gosting,[10] while the relevant values for the "Veronal" buffer were measured directly using respectively an Ostwald viscometer and a 25-ml. Pyrex pycnometer: the values obtained were 1.022 for the relative viscosity and 1.005 for the relative density, both values referring to 25°C. It was assumed that the value of the partial specific volumes of the native and modified ovalbumins were identical and independent of buffer; Dayhoff, Perlmann and MacInnes's value[17] of 0.7479 was used.

The concentrations of solutions used in the experiments designed for the determination of the concentration dependence of sedimentation coefficients were determined as follows: the concentration of the most concentrated solution in each series was determined refractometrically in the Spinco Model H diffusion cell assembly (see Chapter IV) and the remaining solutions were prepared from this solution, the extent of dilution being determined by weight.

6. Amperometric Titrations

(a) Description of the Apparatus

The electrode assembly for the amperometric titrations comprised a dropping mercury electrode and a saturated calomel half-cell: the cell and electrode assembly, based on that described by

Human and Leach,[18] is represented schematically in Fig. VIII-3. The two electrodes were connected to a manual polarograph,[19] a Scalamp galvanometer[14] being used for the measurement of currents. Oxygen was removed from the solutions with a stream of oxygen-free nitrogen: the atmosphere of nitrogen was maintained over the solution during the titration. A layer of chloroform at the bottom of the cell was used to prevent the mercury metal from reacting with excess mercury in solution.[20] The characteristics of the capillary[20] used were: $m = 2.96$ mg./sec., $t = 3.28$ sec., $m^{2/3}.t^{1/6} = 2.51$ mg.$^{2/3}$ sec.$^{-1/2}$.

(b) Calibration of the Apparatus

A check was made of the voltage calibration of the polarograph by determining the half-wave potentials of solutions of zinc, cadmium and lead ions in 1M potassium chloride: the values obtained, together with the values quoted by Kolthoff and Lingane[21] are given in Table VIII-1.

Table VIII-1

Ion	Half-wave Potential	
	This study	Kolthoff[16]
Zinc	-1.06	-1.02
Cadmium	-0.64	-0.64
Lead	-0.44	-0.44

The voltage calibration was thus regarded as satisfactory.

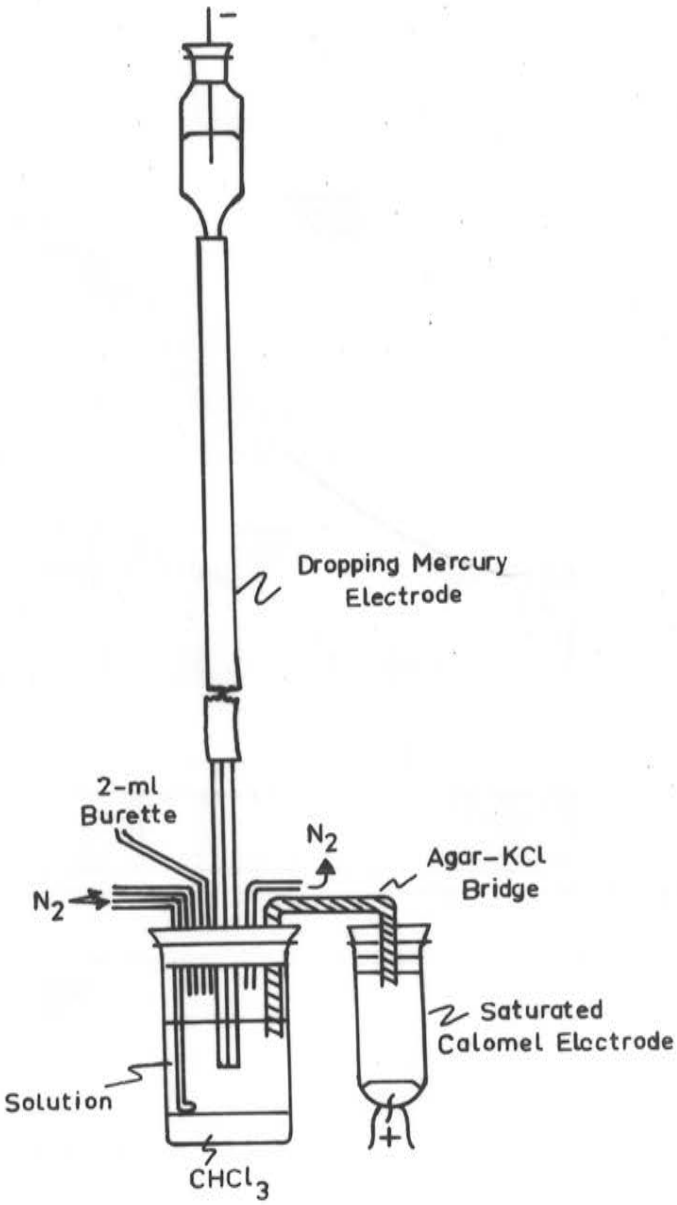

Fig. VIII-3. Diagrammatic representation of the cell and electrode assembly used for amperometric titrations (not to scale).

(c) The Titration Procedure

For all sulphydryl and disulphide estimations a borate buffer of pH 9, which was 0.05, 0.05, 0.5 and 8.0 molar with respect to sodium tetraborate, boric acid, potassium chloride and urea respectively was used: for the latter estimations the solution was also made 0.2 molar with respect to sodium sulphite. The titrations were performed on 25 ml. portions of protein solution containing 2-4 micromoles of -SH, the solutions being titrated with 0.0100M mercuric chloride: the progress of the titrations was followed by observing the current at an applied voltage of -0.35 volts.[20] Fig. VIII-4 shows the current-volume curve of a typical titration, the end point, B, being obtained by extrapolation. Protein concentrations were again determined refractometrically (see Chapter IV).

(d) Standardisation of the Titration Technique

Firstly, in order to show that the extrapolation to zero mercuric ion concentration was valid, a titration was performed in which varying amounts of mercuric chloride were added to the borate buffer and the current at -0.35 volts noted. Fig. VIII-5, which represents graphically the results obtained, shows that such an extrapolation is quite valid for this apparatus.

Titrations were then performed on a sample of L-cystine of known purity (Lot 100101 from the California Foundation for Biochemistry Research). A comparison of the amount of cystine detected by the amperometric titration technique with the amount actually present by weight is given in Table VIII-2.

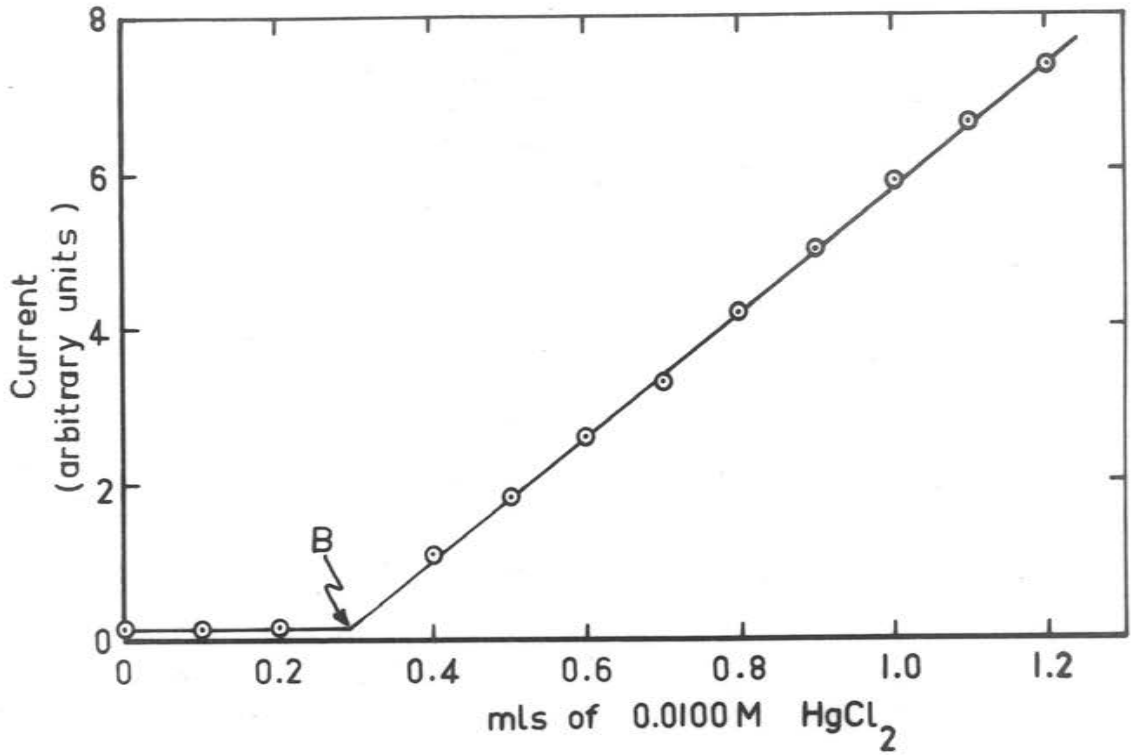

Fig. VIII-4. Current-volume curve obtained in the amperometric titration of ovalbumin with 0.0100M mercuric chloride: the point B is the end point of the titration.

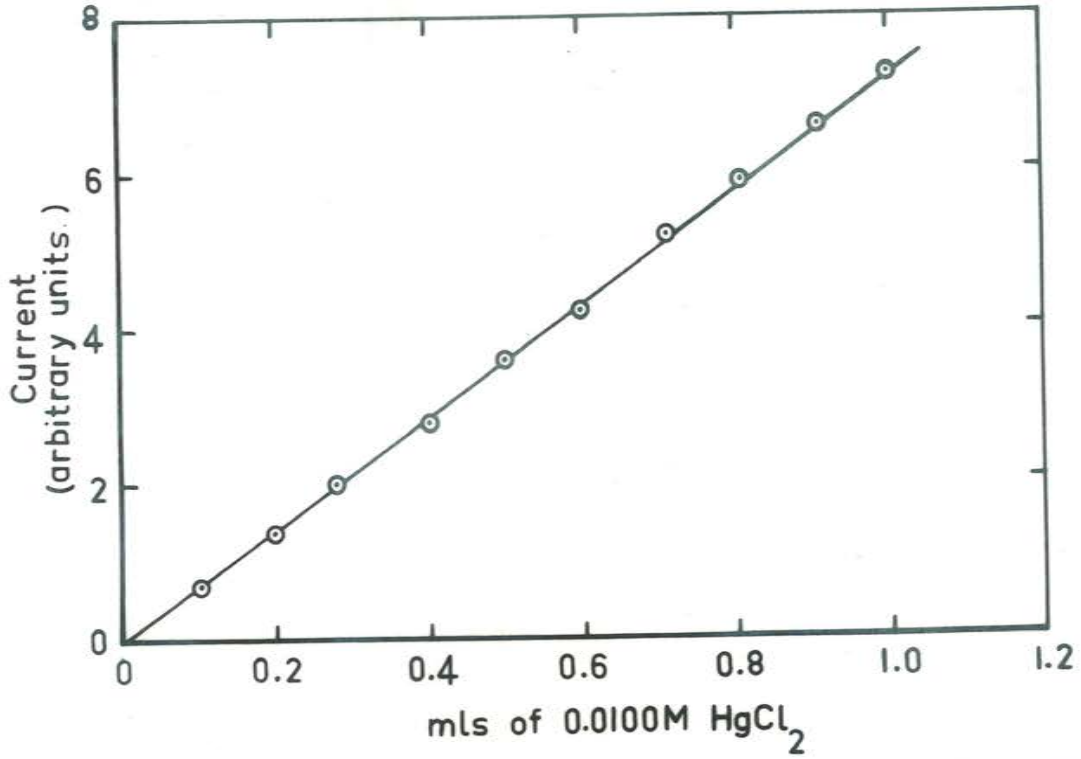

Fig. VIII-5. Current-volume curve obtained for the addition of 0.0100M mercuric chloride to the buffer used in the amperometric titration studies.

Table VIII-2

Cystine by weight mg.	Cystine by Titration mg.	Purity %
1.60	1.59	99
1.60	1.54	98
2.42	2.40	99
2.42	2.31	96

These results estimate the purity of the cystine as 98 ± 2%, whilst on the basis of N analysis the sample is 99.7% pure.

7. Detection of Iodine in the Modified Ovalbumin

The presence of iodine in iodine-modified ovalbumin was detected by using samples of iodine containing the radioactive isotope I^{131}, which was obtained in the form of carrier-free sodium iodide in dilute sodium hydroxide at pH 8-10.[22] The modification was performed under conditions identical with those outlined in section 3 of this chapter, 0.014 g. of iodine dissolved in 4 mls. 1M potassium iodide, labelled to the extent of 0.8 mC. being added to 0.28 g. of protein in 15 mls. of phosphate-potassium iodide buffer, which was 0.1, 0.1 and 1.0 molar with respect to sodium dihydrogen phosphate, disodium hydrogen phosphate and potassium iodide respectively. This solution and also a duplicate solution were then dialysed against phosphate buffer 0.05 molar with respect to both phosphates for 8 days to remove the excess iodine and iodide ions. The protein solutions were then transferred to 25 ml. standard flasks and made up to the mark with the final dialysate. The activities of these solutions

were then determined on 10 ml. samples using an ECKO scintillation counter (Type N550 A),[23] employing crystal source arrangement A, and connected to an ECKO Automatic Scaler (Type N 530D).[23] The activities of the final dialysates were also measured. The standard solution was prepared at the time of commencement of the modification of the protein by diluting 1 ml. of the iodine-potassium iodide solution, which thus contained 0.2 mC. of activity, to 10,000 mls. in two stages.

Subsequently 10 mls. of the above modified solution were redialysed against phosphate for a further three days, whilst another sample, also 10 mls., was dialysed against "Veronal"-chloride buffer containing 0.04, 0.01 and 0.06 moles of sodium diethylbarbiturate, diethylbarbituric acid and sodium chloride respectively, for a similar period. These solutions were then made up to 25.0 mls. as before and their activities, together with the activities of the respective final dialysates, determined.

8. References to Chapter VIII

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Ovalbumin: A Physico-chemical Study

Summary of Thesis presented by D.J. Winzor for the degree of Ph.D. at the University of Adelaide

A diffusion study of crystalline ovalbumin, which contains three electrophoretic components, and also of the component possessing the greatest electrophoretic mobility, A_1, has been performed to determine whether any heterogeneity could be detected in the two systems by a recently developed procedure for analysing Rayleigh interferograms of diffusing boundaries. Even in the case of the pure electrophoretic component a deviation from ideal diffusion behaviour, normally regarded as indicative of an impurity, has been observed. This deviation, however, is believed to be a consequence of an interaction of flows between the protein and the constituent ions of the buffer in which the protein is diffusing. Further studies of the effect of flow interactions on diffusing protein samples have been suggested, but a protein more stable than ovalbumin is required for such studies.

In an attempt to make the ovalbumin molecule more stable, modification of the protein with iodine, claimed to be an oxidation of the sulphydryl groups to disulphide linkages, has been performed to remove the sulphydryl groups, which could be responsible for the protein's instability. In the characterisation of the modified material by electrophoretic, sedimentation, polarographic and radiochemical techniques, data have been obtained which, although apparently insufficient to enable the nature of the reaction to be determined, show that the reaction between iodine and ovalbumin is

APPENDIX

Publication:

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