A PHYSICO-CHEMICAL STUDY OF UREASE

by

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Chapter 1

INTRODUCTION

The complete elucidation of the mechanism of enzyme reactions constitutes a problem of fundamental importance in biochemistry, and which is, moreover, one of considerable complexity. Michaelis and Menten[1] postulated a simple model mechanism in which the enzyme combines with the substrate to form an enzyme-substrate complex, the latter subsequently breaking down to give products and enzyme. This model serves as an excellent basis for the elaboration of more detailed and complex pathways, when these are indicated. Thus, the formulation of this two step reaction permitted a rate expression to be derived, which related the overall rate of the reaction to the initial concentrations of substrate and enzyme.[2] Deviations from this simple behaviour, in many cases, have led to the formulation of modified and frequently complex rate expressions,[3] which are justified in terms of a series of individual steps of the Michaelis-Menten type. A study of the overall kinetics of the reaction, therefore, constitutes a major approach to the general problem of enzyme mechanisms.

However, further consideration of even the simplest set of individual steps reveals that a complete understanding of the mechanism and the related characteristic properties of enzyme reactions, specificity and efficiency,[4] requires a precise knowledge of the type of bonds between enzyme and substrate in the complex, and an explanation of the break-down of the complex in terms of polarization, electron displacement, strain or other distortions[5] of the bonds involved.

By employing a variety of approaches, including kinetic studies with competitive and non-competitive inhibitors and structural determinations on both the enzyme and substrate, it has been shown that the complex forms by the combination of certain chemical groupings on the enzyme, called active sites.[4] Considerable emphasis has been given to the determination of the number and nature of these active sites. This intensive study is both logical and necessary, for without it, the specificity of the enzyme, the bond breakage or rearrangement involved in product formation and the mechanism in terms of individual steps, remain either unexplained or tentative.

The characterization of the enzyme as a protein is also of considerable importance. Thus, even though the active site may constitute only a small part of the total structure, it appears that the tertiary structure[5] of the whole protein is essential for enzymic activity.[6,7] Moreover, in certain cases, a physico-chemical study on the enzyme has shown that a reversible interaction, or aggregation, occurs in solution, resulting in polymeric species of different activities. Clearly, consideration of any such association-dissociation reaction is fundamental in the correct formulation of kinetic results.[8] In a more general sense, estimations of physico-chemical quantities, such as the molecular weight, isoelectric point and the charge on the protein at particular pH values, are invaluable in interpreting results from other approaches. Indeed, while this brief outline of the information required for the complete elucidation of the mechanism of any enzyme reaction indicates that many approaches are necessary, the characterization of the enzyme itself appears to

be of first importance.

This is well illustrated by consideration of the numerous attempts at formulating a mechanism for the enzymic hydrolysis of urea by urease. In contrast with other enzyme systems, the urease action appears to be particularly amenable to study, for the following reasons: (1) methods of estimating the enzymic activity (and hence of performing kinetic experiments) are both rapid and reasonably accurate;[9] (2) the enzyme seems to be absolutely specific for the substrate urea;[10,11] (3) the substrate is readily available and of established structure; (4) there is strong presumptive evidence that the active sites involve sulphydryl groups.[12-14]

Nevertheless, it is clear that the available experimental evidence relating to the mechanism of the hydrolysis, particularly the kinetic results, is frequently contradictory and can be interpreted by several hypothetical sets of individual steps. A brief review of the results exemplifies this and emphasizes the necessity for a physico-chemical study on urease.

Wall and Laidler,[15] by studying the overall rate of the reaction as a function of the substrate concentration in the non-inhibiting buffer tris-hydroxy methyl aminomethane-H_2SO_4, concluded that the simple Michaelis-Menten mechanism was directly applicable, at least at low substrate concentrations (less than 0.3M urea at pH 7). On the basis of this reaction and employing the theory of absolute reaction rates, the entropy of activation was estimated for the bi-molecular reaction between enzyme and substrate. The value obtained indicated a structural change or "opening out" of the enzyme during

complex formation. However, the calculations involved the molar concentration of the initial enzyme solution and, as will be shown in succeeding chapters, the validity of the molecular weight used to obtain this value can be seriously questioned.[16] Therefore, this hypothesis, while perhaps extremely significant, remains unsubstantiated until the molecular weight has been confirmed and the possibility of such a structural change in the protein established.

The results of Kistiakowsky and Rosenberg,[17] which extended to much lower urea concentrations, indicated, on the other hand, that the Michaelis-Menten mechanism was inapplicable at all substrate concentrations. This disparity was not due to inhibition by the supporting electrolyte (as had been the case previously[18,19]), as the ureolytic activity was independent of the sodium maleate concentration, in the range investigated by Kistiakowsky et al.[9] Inhibition by urea was postulated as the cause for the reduction of the rate at relatively high urea concentrations (in this case, greater than 0.17M urea at pH 7). The non-linearity of the rate when plotted against the substrate concentration (at low urea concentrations) was attributed to the existence of either two types of active sites, with different Michaelis-Menten parameters, or pairs of identical sites, with interaction such that the kinetic parameters of a site were altered when the neighbouring site combined with urea. Later results,[20,21] which considered effects due to inhibition by hydrogen ions, urea and thiourea molecules and bisulphite ions,[22] as well as ionic strength effects, favoured the former hypothesis. It was emphasized, however, that the apparent agreement may have been fortuitous, as other

mechanisms would probably have led to rate equations which would equally well fit the data.

To emphasize the tentative nature of this hypothesis further, it should be noted that the derivation relies on the implicit assumption that the specific activity of urease is a linear function of the enzyme concentration: Kistiakowsky and coworkers[17,20] emphatically uphold this dependence, even in view of several reports in opposition.[23-25] This important disparity may be due to experimental conditions, as Kistiakowsky et al. used H_2S as a 'protecting' or 'activating' agent in almost all kinetic experiments.[9,17,21] While anomalous effects have been observed with other such agents in the urease system,[26] no study of their precise effect has been reported, though there is the distinct possibility that the protein itself would be modified.[27] On the other hand, if the specific activity is not a linear function of concentration, the possibility of protein polymerization or aggregation[23,26,28] should be considered.

It is clear that a physico-chemical study on the enzyme, in terms of fundamental properties, is needed before the reliability of the results and hypotheses outlined can be assessed. In particular information on (1) the molecular weight, (2) effects of possible modifying reagents used as 'protecting' agents, (3) possible protein interactions and (4) the structural nature of the active site would be valuable. It is the aim of this thesis to present physico-chemical results on urease, which, it is hoped, will provide a sounder basis on which to interpret data more directly concerned with its action as an enzyme. The major hypothesis to be formulated and vindicated is

that urease chemically interacts in solution to give a series of polymeric species.

The study of interacting protein systems is of great interest at this time, as examination of several protein systems, using the moving boundary methods of sedimentation velocity and electrophoresis, has revealed the existence of various polymeric species in solution.[29] If the interaction of protein molecules to form polymers is reversible the ultracentrifuge and electrophoretic patterns obtained depend largely on the rates of the forward and backward reactions as compared to the rate of separation of the components in the transport experiments. Various possibilities have been discussed qualitatively by Longsworth and MacInnes,[30] in connection with the electrophoresis of interacting systems, and similar considerations are pertinent to the sedimentation of such systems. Two extreme cases are of particular interest.

The case of instantaneous establishment of equilibrium during transport has been treated theoretically by Gilbert.[31] The theory predicts, neglecting diffusion, that more than one moving boundary in the ultracentrifuge would be expected, provided, that if a simple polymerization is involved, polymers higher than the dimer are present. This was in agreement with the sedimentation data found with α-chymotrypsin,[32] where a single peak was evident when monomer and dimer were in equilibrium at high ionic strengths, and two peaks were present when higher polymers (possibly hexamers), were in equilibrium with the monomer at low ionic strengths. Moreover, the theory predicted accurately the observed increase in sedimentation coefficient with protein concentration in dilute solutions.[32,33] A

recent extension of this work by Rao and Kegeles[34] showed that monomers, dimers and trimers could be present as an equilibrium mixture and yet give only one boundary in the ultracentrifuge.

The second case to be considered is that of the slow rate of attainment of equilibrium compared to the rate of ultracentrifugal or electrophoretic separation. It has been pointed out[29] that the pattern obtained in such cases will indicate the presence of each component: the appearance of discrete boundaries in the ultracentrifuge will then be a measure of the equilibrium distribution of the molecular species initially present in the solution. In essence, this case is a trivial example of reversible interaction as, in the extreme, it is indistinguishable from an heterogeneous 'static' system. As in the former instance, there are several proteins, for example l-amino acid oxidaze,[35] ground nut globulins[36] and soy-bean proteins,[37] which exhibit characteristics of this type of interaction. Evidence based largely on chemical modification has established a relation between the discrete boundaries observed in the ultracentrifuge in terms of polymeric species.[37] The failure to resolve the components electrophoretically in some cases is particularly significant and will be discussed later.

An investigation of the existence and nature of an interaction in enzyme systems of either type described is extremely relevant to a study on enzyme mechanisms, because the activity of the polymers could be quite different from that of the monomer. This is certainly the case with trypsin, where the active site is involved directly in the polymerization,[38] and α-chymotrypsin, where the reduced activity

of the dimeric form has a pronounced effect on the rates of the catalyzed reactions.[8] In view of these examples and the difficulty in interpreting and correlating results relating to the action of urease, it appears essential to characterize the exact nature of the chemical interaction of urease in solution. In fact, it is probable that reproducible and meaningful kinetic results will only be obtained by devising a method to prevent or reverse the polymerization, while preserving the activity of the monomer. Certainly, other physico-chemical studies would be greatly facilitated by this. In this respect, the use of chemical modifying reagents has proved invaluable and will be discussed fully. Of the various methods subsequently selected to characterize the species of urease obtained, the 'stabilized monomeric' form, that of diffusion requires special comment at this stage.

The development of interferometric optical systems for measuring the refractive index distribution or refractive index gradient distribution has provided accurate diffusion data,[39] which can be used to obtain a diffusion coefficient. This is related to the frictional coefficient, and also may be employed to obtain the molecular weight of the protein.[40] Moreover the form of the distribution may be analysed to obtain information (subject to certain restrictions) on the heterogeneity of protein samples. Therefore, in relation to a study on urease, where an estimation of the molecular weight, for example, is required in many calculations[13,15,19,22] the diffusion method appears to be potentially valuable. In a general sense, any experimental criterion for assessing protein purity is of considerable interest at this time.[41,42]

The procedures for the determination of heterogeneity from diffusion measurements on mixed solute systems 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.[43-48] Deviations from ideal diffusion behaviour in a manner indicative of heterogeneity have been observed in all protein systems studied, the deviations being represented as a fringe deviation diagram.[44,46] Analysis of the systems into two protein components of differing diffusion coefficients have been made in these cases. (It is implied that the buffer salts, which are constituents of the necessary medium used in protein diffusion experiments, are assumed to be part of the solvent, rather than additional solutes.) While several additional assumptions[45] must also be postulated, it is relevant to point out particularly, that analysis by these procedures assumes independence of flow of each solute component.

The exact nature of the phenomenon of 'interacting flows' in systems containing more than two components is unknown. Thus, although it has been suggested that it results in part from the electrostatic coupling of ion flows[48,49] it has been demonstrated to exist to a measurable degree not only in solutions containing ions, but also in other systems, including those composed entirely of non-electrolytes. If flow interaction effects do exist in protein diffusion experiments, analysis in terms of heterogeneity (or the correction of the experimentally observed diffusion coefficient, referred to as $\mathcal{D}_A$, to a D suitable for molecular weight determinations) could be quite misleading. This is particularly true if the protein must be diffused at a pH different from its isoelectric point due to solubility and stability

limitations, as in the case of urease.[50] In this case, at least the electrostatic coupling of ion flows could be expected to affect the size and shape of the fringe deviation diagram. Consequently, although the application of the diffusion method, in principle, would supply information on the purity, molecular weight and indirectly on the size and shape[51] of the urease molecule, an extensive investigation of the existence of complications, particularly flow interaction effects, must be made before this information may be considered meaningful.

The choice of a suitable protein system for such a study should be guided by the following considerations:

(1) the protein should be readily available as each of the many diffusion experiments required involves at least 25 ml. of an approximately 0.5% protein solution. (This of course varies with the type of optical system used and with the design of the experiment);

(2) ideally, it should be free of any 'impurities', including those due to reversible interaction;

(3) it should be stable <u>at least</u> during the usual two week duration of an experiment. (This includes time for adequate dialysis);

(4) the isoelectric point of the protein should be known and it must be soluble at this pH in the buffer used for the determination. This would enable experiments to be performed where any flow interaction due to electrostatic coupling of ion flows is presumably at a minimum;

(5) some data on the net charge of the protein over a pH range (where the protein must be stable) would be desirable. This would enable buffer gradients resultant on dialysis prior to diffusion, due to Donnan membrane effects, to be allowed for when markedly charged

species are diffused.

While urease, as will be seen, fails to fulfil any of these requirements, crystalline ovalbumin seems in many ways a model system to use. In conformity with expectation, the results to be presented on the diffusion of essentially uncharged species and on charged ovalbumin molecules have proved useful in discussing flow interaction effects in other systems. With this guide, the significance of the diffusion results obtained with urease and of the widely used molecular weight will be discussed.

It can be concluded from this discussion that the overall mechanism of the enzymic hydrolysis of urea by urease would be greatly facilitated by a physico-chemical study on the enzyme as a protein. In Chapter II an investigation of the preparation of urease will be outlined, together with electrophoretic and sedimentation velocity analyses of the samples obtained. The results are used to provide presumptive evidence for the interaction of urease in solution. Chapter III is therefore devoted to direct experimental evidence for the interaction and a discussion of the probable mechanism, in terms of the groups involved. The effect of the polymerization on the enzymic activity is also outlined, with conclusions as to how the system may be stabilized to give an active 'monomeric' form. The sedimentation and electrophoretic properties of the stabilized (or 'modified') material are presented in Chapter IV, and these provide more information on the mechanism of the interaction. It will be shown also that further investigations would be facilitated if reliable diffusion measurements could be made. Chapter V is concerned with a critical appraisal of the diffusion results obtained with urease. This

assessment requires frequent reference to results obtained with ovalbumin, which was employed as a 'model' protein system to test the major assumptions of the diffusion theory. As the ovalbumin material does not contribute directly to the elucidation of the urease system, it has been summarized in Appendix I. Since the diffusion results prove to be somewhat inconclusive, an alternative method of assessing the extent of the heterogeneity of the modified material is presented in Chapter VI. The apparent homogeneity observed in samples, under a specified set of experimental conditions is contrasted with the effect of altering the environmental conditions (with regard to pH and ionic strength). The observations are related to the published kinetic data.

As might be expected from this indirect approach to enzyme action, the results do not permit a formulation of the detailed mechanism of the hydrolysis. Nevertheless, they are illustrative of the general principle that a physico-chemical study on the enzyme is frequently essential before the results from other approaches can be used for this purpose.

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CHAPTER II

THE PREPARATION AND MACROSCOPIC PROPERTIES OF UREASE IN SOLUTION

(1) A review of the physico-chemical properties of urease.

(2) The preparation of urease
   (a) electrophoretic fractionation,
   (b) salt fractionation,
   (c) acetone fractionation.

(3) Electrophoresis of urease solutions.

(4) Sedimentation velocity analyses.

(5) Correlation of electrophoretic and sedimentation velocity data.

(1) A review of the physico-chemical properties of urease.

The preparation of crystalline urease by Sumner[1] represented one of the major advances in enzymology, as it was the first demonstration that an enzyme was not only capable of being studied in a purified form, but also that it was essentially protein in nature.[2] However, the original preparative procedure has subsequently been improved only in one respect (by the modified recrystallization outlined by Dounce[3]), even though the difficulty of obtaining urease is specifically mentioned in several papers.[4-6] An examination of the published data indicates that no more than a 15% yield[4,5,7] can be expected with the Sumner preparation, and as the urease content of the meal (Canavalia ensiformis) rarely exceeds 0.2%[5,8] the difficulty in preparing sufficient enzyme for an extensive physico-chemical study is apparent. This is convincingly illustrated by inspection of the experimental details given by Sumner, Gralen and Eriksson-Quensel[9] in a paper concerned with the establishment of sedimentation and diffusion data. For this reason, little information exists on fundamental properties such as the isoelectric point, the solubility, the stability of resultant solutions and the molecular weight. In addition, the reliability of the available information can often be seriously questioned.

The isoelectric point, for example, was found by a minimal solubility method[10] to be near pH 5.0; but this method, which relied on a qualitative estimation of the "density" of the precipitate formed in various buffers, depended strongly on the absence of other impurities. The workers themselves conceded the possibility of marked error due

to this. Of the other attempts to confirm this value[11,12] only the inhibition technique used by Wills[12] is notable. Wills found that suramin almost completely inhibited urease at pH 5.0 (presumably by a bridging mechanism involving electrostatic linkage between the negatively charged sulphonic acid groups on the suramin molecule with positively charged groups in the vicinity of the active site on the enzyme), while no inhibition occurred at pH 5.3. He concluded that an increase in the net negative charge near the active site, resultant on increasing the pH above 5.0, caused repulsion with the sulphonic acid groups on the suramin and thus prevented inhibition. However the reliability of the method in estimating the isoelectric point of the <u>whole</u> protein molecule is in doubt.

Ideally, a series of electrophoresis experiments designed to enable the construction of a pH-mobility curve would be desirable. The isoelectric point could then be obtained by interpolation. Unfortunately, such electrophoretic data are not available: the only data are those found by Anderson and Alberty,[13] who reported negative values for the mobility of urease at pH 7.4 and 6.4 in 0.01 ionic strength buffers. The system showed reversible spreading in both the ascending and descending limbs, indicative of heterogeneity, but no reliable quantitative measure of this could be made from boundary spreading experiments.

More information is available on the sedimentation velocity characteristics of urease solutions. In all cases reported[9,14,15] urease, prepared by the method of Sumner[1] and recrystallized by the modified procedure of Dounce,[3] has shown at least two peaks in the

ultracentrifuge at pH 7. Thus, Sumner et al.[9] sedimenting several twice recrystallized samples always found a main component of S_{20}[a] 18.6 to 19.9 and a faster sedimenting peak of S_{20} 26-28. A slight decrease in the relative amount of the latter component, achieved by more careful recrystallization, was associated with a small increase in specific activity. This implied that the S_{20} 26-28 material and other components with S_{20} values of 7 and 36, which also occurred in most preparations, were impurities of unknown nature. The enzymic activity was attributed, then, to the material which sedimented with an S_{20} of approximately 19. McLaren et al.[14] also observed two peaks in 0.02M phosphate buffer, using 0.5% protein solutions, with S_{20} values of 18.6 and 28 (after allowing for the adiabatic expansion correction of 0.9°).[16] They also showed that loss in activity induced either by heat denaturation or irradiation by U.V. light was accompanied by a loss of the S_{20} 18.6 material, and thus indirectly supported the hypothesis that this boundary represented the enzymically active urease.

The only attempt to elucidate the nature of the several boundaries usually observed in sedimentation velocity analysis and to confirm the fundamental assumption that the S_{20} 19 component was the enzymically active form was the interesting work of Kuff, Hogeboom and

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[a] The symbols used to denote corrected sedimentation coefficients are as follows:

(1) S_{20}, in buffer at 20°

(2) $S_{20,w}$ corrected to water at 20°

(3) $S^{\circ}_{20,w}$ an extrapolated value at zero protein concentration.

Striebich.[15] A preparative centrifugation in the presence of a sucrose density gradient was employed to separate partially the components, prior to activity measurements performed on samples taken from successive levels in the cell. The fraction of the activity of the sample over the original activity of the undiluted sample was plotted against the average distance from the meniscus and the resultant curve was translated into an increment curve. This indicated the presence of four broad and irregular boundaries without evidence for a plateau region. A critical analysis of the assay results presented reveals that the method was very insensitive, but the result that peaks of S_{20} 19, 28, 36 and 46 were present, all with some enzymic activity, provided the first independent indication that aggregates or polymers of urease coexisted in solution. Other observations, including osmotic pressure determinations[4] and the variation of the ureolytic activity with time,[17,18] temperature[17] and enzyme concentration,[17,19] while suggestive of an associating system, were equally as tentative and unconvincing.

In view of the known ultracentrifugal complexity, the value of the accepted molecular weight,[9] 483,000, requires critical appraisal. It was calculated by inserting a sedimentation coefficient, found at a finite protein concentration and in the absence of sulphite ions, and a diffusion coefficient, found in the presence of sulphite ions, into the Svedberg molecular weight expression. Besides several theoretical objections to this procedure, which will be discussed in later chapters, two fundamental criticisms are apparent immediately. First, as sulphite ions are known to cleave structurally important disulphide linkages in other protein systems,[20] the assumption that

the urease species in sulphite-free media are the same as those present in buffered sulphite solutions was completely unjustified. Secondly, there was no real evidence that the peak with an S_{20} of 18.6 actually represented urease. Certainly, the marked discrepancy between the molecular weight of Sumner et al. and that estimated by Setlow[21] on the basis of deuteron bombardment experiments should have caused reluctance to employ the former value. Nevertheless, it has been used in several calculations, some of which have been used to provide a basis for possible mechanisms.

A discussion of other important properties of urease will be deferred until experimental evidence has been presented on the preparation, electrophoresis and sedimentation velocity behaviour, the solubility and the stability of urease solutions.

(2) The preparation of urease.

While only small quantities of enzyme are needed for kinetic experiments, other physico-chemical measurements would be greatly facilitated if the preparation could be improved to increase the yield. Moreover, in view of the complex nature of the ultracentrifuge patterns obtained with samples prepared by the acetone fractionation method of Sumner,[1] a demonstration that other components with higher or lower sedimentation coefficients than 19 could be eliminated by adopting an entirely different preparative technique would greatly strengthen the hypothesis that these boundaries represent macroscopic impurities.[9] Alternatively, if they persisted, the suggestion that

these other boundaries represented polymers of urease would be strengthened.

The most convenient and rapid method of following the efficiency of any particular fractionation procedure is by the use of assays on the enzymic activity. In order to compare results of different workers standard conditions must be employed[7] and in this respect the Sumner Unit,[22] S.U., is suitable. However, accidental inhibition of urease may lead to misinterpretation of the results,[23] and therefore additional analysis procedures, such as electrophoresis and sedimentation velocity, are invaluable in assessing the relative merits of different preparative procedures.

Examination of the data available[5,22] reveals that the initial extraction in the Sumner procedure, which involves stirring Jack Bean Meal with a 32% acetone water mixture, is extremely inefficient. Indeed, the final recrystallization is achieved by adjusting the pH and adding acetone. An alternative extracting agent, which at least in principle should be more efficient, is a phosphate buffer, as this is employed to measure the maximum activity in the meal. The data in Table (II-1) confirm this and show that the amount extracted is not markedly increased by prolonging the time of extraction. Moreover, the extract obtained is clearly quite stable at room temperatures for several hours. Unfortunately, other materials, some almost certainly protein in nature, are abundant in the buffer extract as is illustrated by the electrophoretic pattern shown in Fig. (II-1). Thus, while initial extraction with a buffer is more efficient in the sense that a smaller loss of enzyme is incurred, the difficulties involved in

Table (II-1)

Extraction of Urease from Jack Bean Meal[a]

Extracting agent	Extract no.	Time of extraction[b] (mins.)	Time after extraction[c] (hr.)	Activity of extract (S.U./ml.)	Maximum % of urease extracted[d]
32% acetone-water mixture (500 ml. used for 100 g. of meal)	1	18		6	20
	2	18		8	30
phosphate buffer	3	18		70	93
0.062M Na_2HPO_4	4	18		65	87
0.015M NaH_2PO_4	5	67		70	93
pH = 7.4	4		48[e]	70	
(200 ml. used for 100 g. of meal)	4		48	65	

[a] The specific activity of the meal used for this comparison was 150 S.U./g.

[b] The time includes the times taken for initial stirring, centrifugation and withdrawal of the supernatant extract.

[c] The time after extraction and before assay was less than 1 hr. when not cited.

[d] The figures cited are the ratios of the total number of S.U. in the initial volume of extracting agent, to the number of S.U. available in 100 g. of meal (15,000 S.U.), expressed as a %. They are therefore independent of the varying volume of extract collected and thus enable a comparison to be made of the relative efficiency of the two extracting agents. However, they represent a theoretical maximum as the meal retains about 40% of the extract.

[e] Extract 4 was left in the diluted state immediately ready for assay. The other assay reported after standing at room temperature for 48 hr. was performed on solutions which were diluted immediately before the assay.

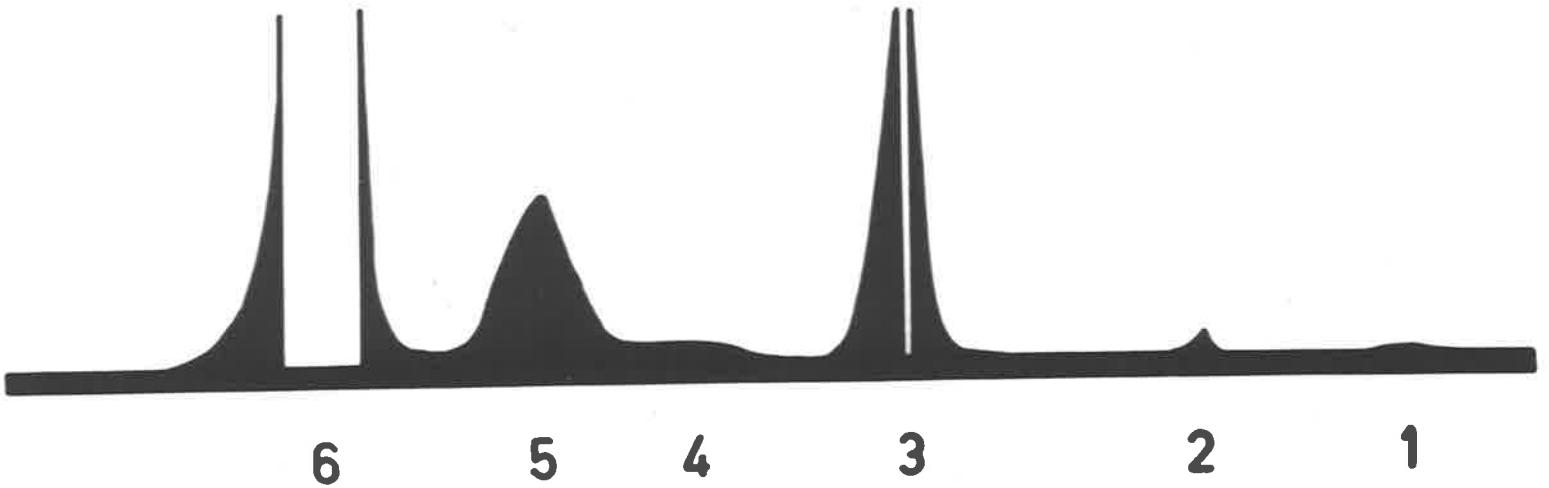


Fig. (II-1). Electrophoretic pattern (ascending limb) of the phosphate buffer extract of Jack Bean Meal. The electrophoresis was performed in phosphate buffer, 0.003 M Na_2HPO_4 and 0.001 M NaH_2PO_4, pH 7.6 and the pattern obtained after 1 hr. at potential gradient of 7.14 volt $cm.^{-1}$

separating the enzyme from the large amount of associated impurity may be greater than in the existing method.

Although an extensive investigation of fractionation procedures was not considered warranted at this stage, a preliminary survey was made of three general methods,[24] salt fractionation, preparative electrophoresis and a modified acetone fractionation. The information obtained from these investigations will now be briefly summarized.

(a) Electrophoretic fractionation

This technique has proved useful in other systems, but is only applicable if the fraction required has either the fastest or slowest electrophoretic mobility. In these cases, conditions are chosen such that the mobility difference between the component desired and that closest to it is as great as possible.[25] Prolonged electrophoresis in an 80 ml. cell of the phosphate buffer extract, previously concentrated by dialysis against gum arabic, permitted a sufficient quantity of the components represented by the leading boundaries 1 and 2 of Fig. (II-1) to be isolated and analysed. As the resulting solution was completely inactive, revealed only one peak of $S_{20} \sim 4$ and contained only species with a net negative charge at pH 4.4, when subjected to electrophoresis, it was concluded that boundaries 1 and 2 did not represent urease. (Mobility results to be presented later in this Chapter indicate that the urease content of the extract contributed, in part, to the unresolved boundary 3 of Fig. (II-1).) It was immediately apparent that unless some prior fractionation was achieved, preparative electrophoresis would be useless.

(b) Salt fractionation

Twice recrystallized ammonium sulphate was used as a precipitating agent and a typical procedure, together with assay data is reported in the schematic flow sheet (II-1). Mention should be made of the step in which the solution was dialysed to pH 5.0, the accepted isoelectric point. This had the effect of precipitating large quantities of material leaving the bulk of urease in solution and, therefore, represents a valuable fractionation step. It also shows that urease is soluble to a limited extent at this pH and in the presence of other impurities. This method gave an active form of urease, the yield being comparable with other preparations. The sedimentation velocity analyses, however, revealed the presence of a large amount of slow sedimenting material, S $\sim$ 3-6, as well as boundaries with a S_{20} of 19, 28 and 36. The recurrence of the faster sedimenting boundaries, while not in any way conclusive evidence, does suggest that they may be closely related to urease.

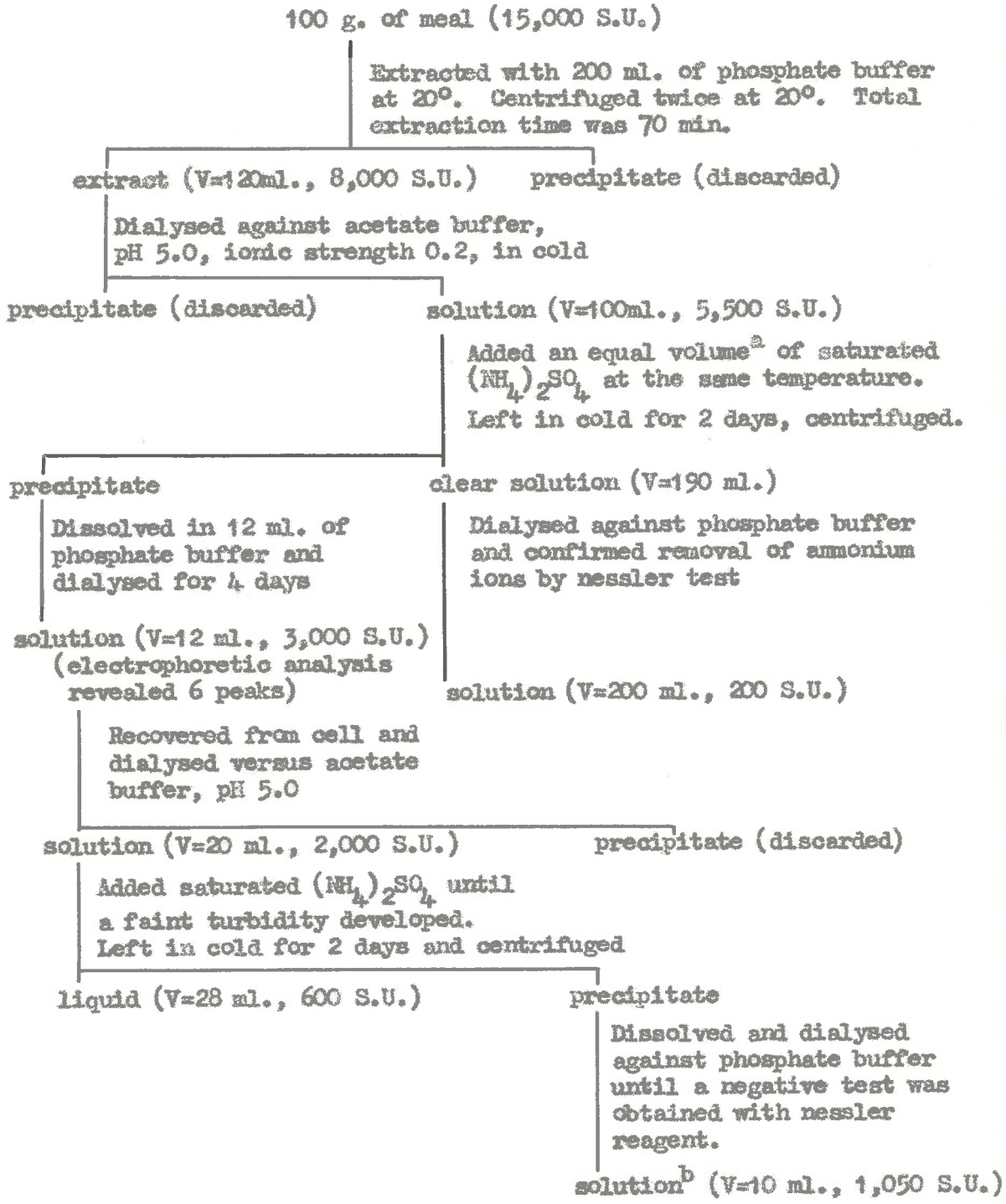
(c) Acetone fractionation

The superior initial buffer extraction was followed by a method exactly analogous to that of Sumner.[1] Thus, flow sheet (II-1) provides an outline of the widely used preparation and an indication of the relative efficiency of each of the steps. In spite of the superior initial extraction, however, the yield was again comparable to those obtained previously.

As each of the methods attempted did not significantly increase the efficiency or the yield of the preparation and as extensive

Flow sheet (II-1)

Preparation of urease by ammonium sulphate fractionation



[a] Other experiments confirmed that precipitation commenced when the solution was 40% saturated with ammonium sulphate and that most of the enzyme had precipitated at the 50% saturation stage.

[b] This represents a 7% yield. The solution was sedimented and the results are reported in the text.

Flow sheet (II-2)

Preparation of urease by a modified acetone fractionation

50 g. of meal (7,500 S.U.)

Extracted with 150 ml. of phosphate buffer at 20°. Filtered through 2 thicknesses of Whatman No.1 filter paper. Refiltered. Extraction time 48 hr.

- solution (V=105 ml., 6,000 S.U.)
  - Dialysed for 4 days against cold water to remove buffer salts. Centrifuged.
    - precipitate (discarded)
    - solution (V=100 ml., 6,000 S.U.)
      - Slowly added acetone at the same temperature until the solution was 32% in acetone by volume. Left stand in cold. Centrifuged.
        - precipitate
          - Drained and dissolved in 4 ml. of water at room temperature
            - solution (V=3 ml., 1,000 S.U.)
              - Added 0.7 ml. of cold acetone. Centrifuged.
                - precipitate
                  - Dissolved in 3 ml. of phosphate buffer
                    - solution[b] (V=3 ml., 900 S.U.)
                - liquid (V=2 ml., 14 S.U.)
            - precipitate (discarded)
        - clear solution[a] (V=130 ml., 4,000 S.U.)
          - Added 12 ml. of cold acetone
            - precipitate
              - Dissolved in 3 ml. of cold phosphate buffer
                - liquid (V=3 ml., 350 S.U.)
                - precipitate (discarded)
            - clear liquid (V=130 ml., 400 S.U.)
- precipitate (discarded)

[a] This is in essence the mother liquor of the Sumner preparation and although highly active, contains large quantities of other material. The considerable loss in activity, which occurred when subsequent fractionation steps were attempted was presumably due to the high concentration of acetone used.

[b] This represents a 12% yield. The solution was not analysed by other physico-chemical techniques.

experimentation would be required to discover improvements, all samples used in this study were prepared by the accepted preparation. The electrophoretic and sedimentation velocity results obtained with these samples, together with the demonstration of several sedimenting boundaries in the salt fractionation procedure, provide strong presumptive evidence for an interaction between urease molecules in solution.

(3) Electrophoresis of urease solutions.

A series of electrophoresis experiments was performed in a Spinco Model H Electrophoresis-Diffusion apparatus, employing simultaneously or separately Rayleigh and schlieren optics. The former system yields a record of the refractive index versus vertical cell coordinate in terms of interference fringes: the number, J, of such fringes between successive schlieren minima is accurately determinable and forms a convenient comparative unit to specify concentrations in electrophoresis experiments.[26] (In other aspects of the work to be described, the concentration of urease was also determined by counting the fringes between the dialysed solution and its dialysate; for this reason all concentrations are quoted in terms of J.) If the specific refractive increment of all components has the value 0.00180 dl. g^{-1},[27,28] then the relation $C = 5.8 \times 10^{-3} J$ (where the concentration C is in g./100 ml.) may be applied to all J values quoted.

All relevant information on the mobility experiments is summarized in Table (II-2), while the pH-mobility curve is shown in Fig. (II-2). The inclusion of this figure, which is a plot of the

Table (II-2)

Electrophoretic mobility data on urease in buffers of ionic strength 0.10 and at 1.0_0°

Sample Number	Buffer composition* molarities	Protein concentration J	Potential gradient volt cm.$^{-1}$	pH	Descending mobility x 10^5 cm.^{2}sec.$^{-1}$volt^{-1}
3	0.08 NaCl, 0.02 NaAc, 0.18 HAc	5	2.95	3.6_9	6.0_8
1	0.08 NaCl, 0.02 NaAc, 0.08 HAc	3	1.98	3.8_9	6.1_3
3	0.08 NaCl, 0.02 NaAc, 0.05 HAc	5	2.93	4.2_3	5.0_8
2	0.06 NaCl, 0.04 NaAc, 0.03 HAc	3	3.22	4.7_0	2.1_6
1	0.03 NaCl, 0.07 NaAc, 0.03 HAc	5	2.48	5.0_1	-0.4
1	0.03 NaCl, 0.07 NaAc, 0.01 HAc	12	2.45	5.4_6	-2.3_6
2	0.08 NaCl, 0.02 NaCac, 0.02 HCac	18	3.04	6.0_1	-3.2_5
1	0.05 NaCl, 0.05 NaCac, 0.01 HCac	12	2.31	6.6_1	-5.5_6
1	0.09 NaCl, 0.01 NaV, 0.02 HV	19	1.91	7.4_5	-6.8_5
1	0.08 NaCl, 0.02 NaV, 0.01 HV	14	2.02	7.9_5	-7.1_5
1	0.07 NaCl, 0.01 NaB, 0.01 HB	9	2.20	8.9_6	-8.0_8

* Ac = acetate, Cac = cacodylate, V = diethylbarbiturate and B = borate.

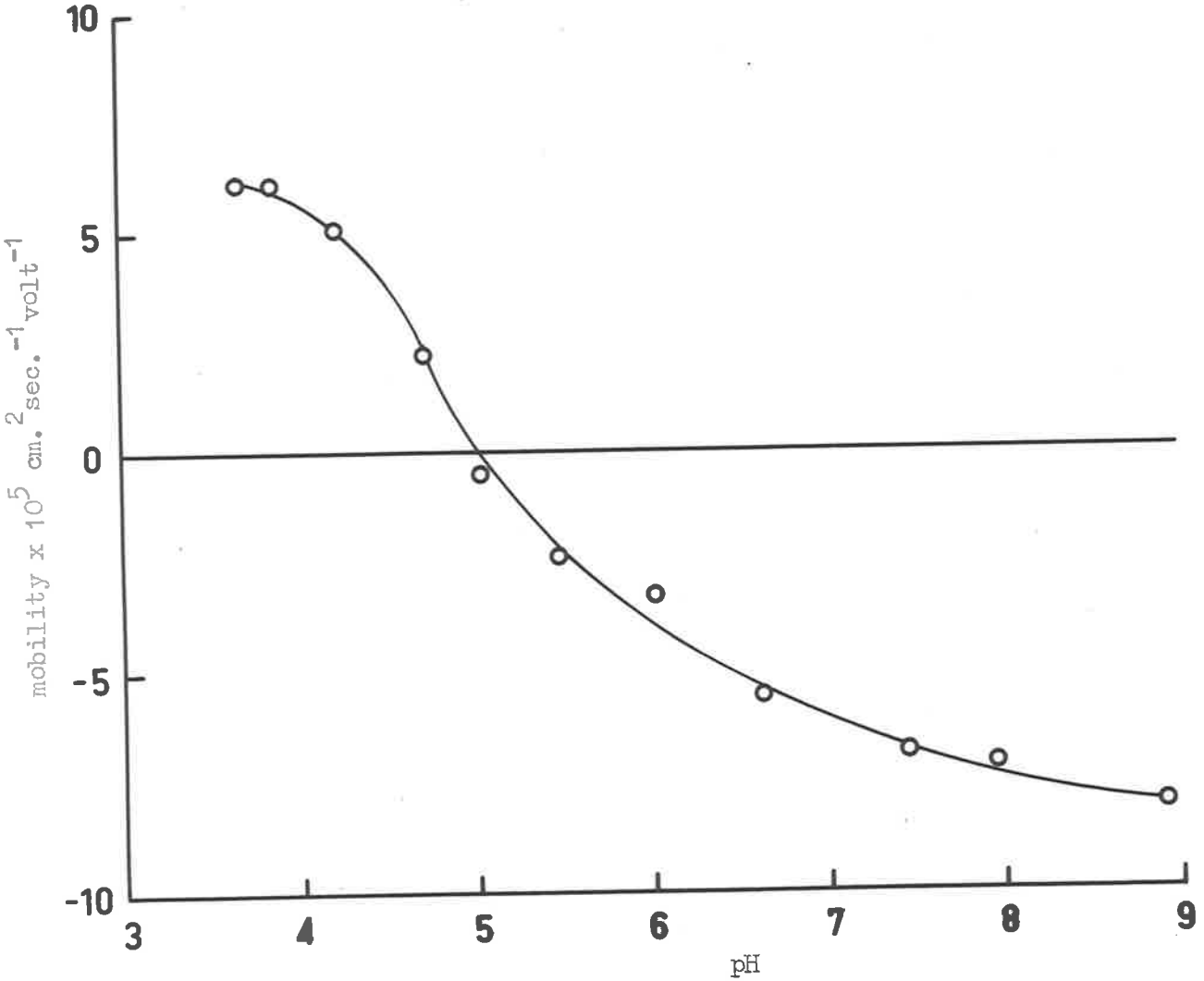


Fig. (II-2). pH-mobility curve for urease, where the circles represent experimentally determined values.

last two columns of Table (II-2), will facilitate interpolations referred to later. The circles represent experimental points and the solid line is an attempt to correlate data found in different buffers and therefore assumes no specific ion binding effects. In fact, the apparent scatter of some points about this line indicates that this assumption is not strictly fulfilled, as the experimental technique precludes large errors in the mobility determinations.

In these experiments the 11 ml. cell was used, the ascending limb being sealed from the atmosphere to prevent flow through the cell due to the continually changing pressure head. Moreover, after a 4 hr. electrophoretic migration in one direction, the current was reversed for an equal period of time. At least five exposures were taken at suitable time intervals to record the progress of the boundary in each direction and the average of the two descending mobilities (determined from the regression of the observed boundary position upon time) was taken as the mobility of the species. This technique (which is applicable only to very dilute solutions) precludes errors due to flow caused by volume changes within the electrode vessels and provides a sensitive means for detecting minor cell leaks. The maximum of the schlieren peak was used to measure the rate of movement of the boundary. Since the peaks were nearly symmetrical (see below), and the concentrations low, it was considered that determination of the first moments[29] would not have led to any useful increase in accuracy. In spite of the relatively large scatter of mobility results, interpolation between points, found within a small pH range and in buffers of the same type, is probably justified. Therefore, the isoelectric point in 0.10 ionic

strength acetate buffer, found by graphical interpolation from Fig. (II-2), can be given as 5.0 ± 0.05 and is therefore in close agreement with other findings.[10-12]

Although no direct study was made on the solubility of urease over this pH range, the fringe counts in Table (II-2) are a reasonably reliable guide to the limitations. Thus, either direct attempts to dissolve the solid urease in buffers of pH 5.0 or less, or to dialyse more concentrated solutions, obtained at higher pH values, against these buffers resulted in solutions of less than 10 fringes. This limited solubility of urease near the isoelectric point unfortunately necessitates the study of other macroscopic properties (e.g., sedimentation and diffusion) using species in the charged state. The complications arising from this will be discussed later.

In order to compare the electrophoretic mobility values reported in Table (II-2) with the two values found by Alberty and Anderson[13] at the lower ionic strength of 0.01, it is necessary to use the procedure outlined by Abramson, Moyer and Gorin,[30] which is based on an imperfect model of electric migration. Using the published diffusion coefficient[9] (which, as will be shown, was confirmed in this study) to calculate the radius, r, of a hydrated sphere, one obtains $r = 61.3 \times 10^{-8}$ cm., and, for example, the value -4.4×10^{-5} cm.^{2}sec.$^{-1}$ volt^{-1} for the corrected electrophoretic mobility at ionic strength 0.10, pH 7.4, from Anderson and Alberty's figure -9.5×10^{-5} for the descending mobility at this pH and ionic strength 0.01. By interpolation from Fig. (II-2), the corresponding value obtained in this work was -6.6×10^{-5} cm.^{2}sec.$^{-1}$ volt^{-1}. Although these calculations are rather

approximate, the discrepancy is large, and if real probably indicative of greater ion binding at the higher ionic strength.

The most interesting feature of these electrophoresis experiments was the appearance of a single relatively symmetrical schlieren peak at all pH values used. The schlieren pattern shown in Fig. (II-3) is quite typical of the results obtained, but in this case the protein concentration (J = 120) was much higher than that used for the mobility determinations. The limited solubility of urease at pH values near the isoelectric point, referred to earlier, prevents similar analytical electrophoresis experiments being performed; but from the Rayleigh interferograms, obtained under these conditions, it may be concluded that not more than 10% of another resovable component can possibly be present. The appearance in an electrophoresis experiment of a single peak which approximates to Gaussian form cannot, of course, be interpreted as convincing evidence for electrophoretic homogeneity. Boundary spreading experiments at the isoelectric point[13] would be desirable, so that the extent of the heterogeneity could be specified: these, however, are prohibited in this case because of the low solubility. While the experiments described indicate that only one peak is evident over a wide pH range, they were performed at only one ionic strength and, therefore, do not absolutely preclude the possibility of the occurrence of components with slightly differing mobilities (see Chapter V). Nevertheless, these patterns when contrasted with sedimentation data provide significant information on the possibility of chemical interaction in solutions of urease.

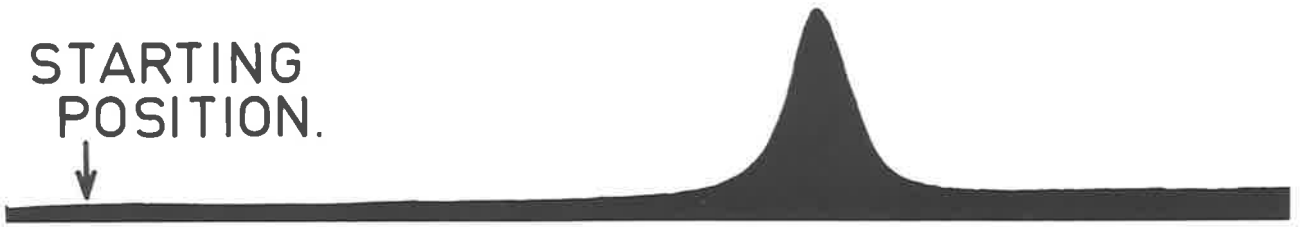


Fig. (II-3). Electrophoretic pattern (ascending limb) of urease at pH 7.4_0 in diethylbarbiturate buffer, ionic strength 0.10, after 8 hr. at a potential gradient of 2.9 volt $cm.^{-1}$

(4) Sedimentation velocity analyses.

Each sample of urease was sedimented and typical patterns are shown in Fig. (II-4). In Fig. (II-4a), which is typical of most preparations, at least four peaks are clearly visible. The S of the slowest moving boundary could only be estimated to lie within the range 4-6 as it was diffuse and never completely resolved in an experiment. Further discussion of this component will be left until Chapter V and attention given here only to the faster sedimenting peaks. The variation of the sedimentation coefficients and relative amounts of the three major peaks completely resolved in Fig. (II-4a), with protein concentration is tabulated in Table (II-3), where the effect of buffer type, pH and sample is also illustrated. Inspection of this table reveals the following points:

(a) the relative areas under the separate peaks varied considerably with different preparations: indeed with sample 4 the fastest sedimenting boundary was completely absent, as is illustrated in Fig. (II-4b), while with sample 5 (which unlike the other preparations was prepared and stored in the complete absence of any cysteine-hydrochloride) the relative amounts of the faster sedimenting materials was greater than usual (Fig. (II-4c);

(b) three major peaks were present, when observed, over the pH range 6-9 and buffer type had little effect on the S values. It is unfortunate that this analysis could not be extended over the complete pH range employed for electrophoretic analysis, but again the solubility limitation at low pH values prohibited this;

(c) the accuracy of the determination of the rate of sedimentation

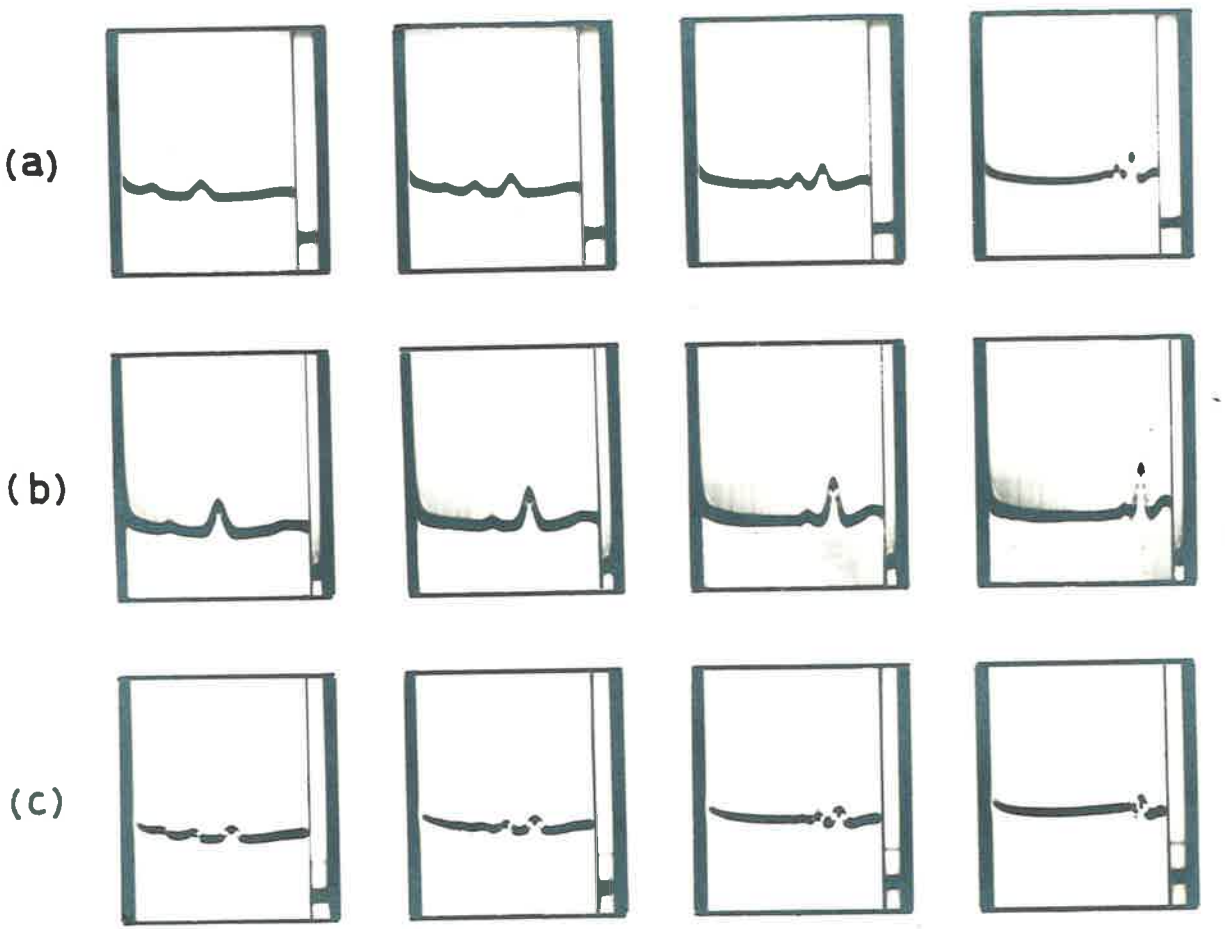


Fig. (II-4). Sedimentation diagrams of different urease preparations in diethylbarbiturate buffer, ionic strength 0.10, pH 7.45 (sedimentation from right to left).

(a) Sample 2; (b) sample 4; (c) sample 5.

Table (II-3)

Sedimentation data on urease solutions

Sample Number	Buffer composition[a] molarities	pH	$S_{20,w}$[b] 1	2	3	% of total area[b] 1	2	3	Concentration of l. J
7	0.07 NaCl, 0.01 NaB, 0.01 HB	8.9	19.2	28	35	73	22	5	-
2	0.09 NaCl, 0.01 NaV, 0.02 HV	7.5	18.9	28	36	66	24	10	46
2			18.9	28	36	63	25	12	44
4			18.8	28	-	90	10	0	40
2			19.0	28	35	61	28	11	39
2			19.3	28	34	61	26	13	29
2			19.2	28	36	61	27	12	16
5			18.8	28	34	55	31	14	-
6	0.05 Na_2HPO_4, 0.02 KH_2PO_4	7.0	19.7	29	36	72	21	7	-
7			19.2	28	35	85	12	3	-
7[c]			21	31	40	83	13	4	-
2	0.08 NaCl, 0.02 NaCac, 0.02 HCac	6.1	19.3	28	36	60	28	12	13
2			19.6	29	-	-	-	-	10

[a] Notation as in Table (II-2).

[b] The resolved boundaries in Fig. (II-4a) are numbered (1, 2 and 3) in order of increasing sedimentation coefficient.

[c] This experiment was performed at 3° (all others were at ~ 20°): the discrepancies evident in the S_{20} values probably arise at least partly from the application of a large temperature correction factor.

for the individual species is limited, in theory, by several factors. First, the rate of movement of the maximum ordinate was used, rather than the velocity of a point corresponding to the position of the square root of the second moment of the concentration gradient curves.[31] However, as the boundaries are reasonably symmetrical, the errors due to this would be small. Secondly, any effects due to the charge on the sedimenting macromolecules has been ignored: in this respect, it should be noted that the use of a neutral supporting electrolyte markedly reduces the primary charge effect.[32] Thirdly, effects due to the concentration dependence of the sedimentation coefficients[33] have been assumed negligible. Certainly, the results over a four-fold concentration range indicate that the concentration dependence, in each case, is small. Thus, in turn, the Johnston-Ogston effect[34] is probably negligible and the J values cited in the last column of Table (II-3), correspondingly reliable (see experimental section). However, in view of the complexity of treating a multi-component charged system, such as this, the extrapolated value $S^{o}_{20,w}$ for boundary 1, in the 0.10 ionic strength, pH 7.5 'veronal' buffer, can only be given as 20 ± 0.5 and less reliance can be placed on the data for the faster sedimenting peaks. Comparison with other available data shows that the values of 18.6 and 28 found by McLaren et al.[14] agree within 1% with values estimated at a comparable protein concentration, and the data of Sumner et al.[9] is in qualitative agreement, but cannot be corrected for quantitative comparison;

(d) no marked change in the relative areas under the peaks was observed on dilution: in this connection, the insensitivity of these area determinations should be strongly emphasized.

All of these points require comment, but of first importance is the observation that over the pH range 6-9 marked heterogeneity of the samples, especially 2 and 5, was apparent in the ultracentrifuge. These patterns, therefore, provided a striking comparison with those obtained in electrophoresis, where, as has been shown, the absence of appreciable amounts of components with different mobilities has been demonstrated. It has been emphasized that the appearance of a single peak, as in Fig. (II-3), obtained with sample 5, does not preclude the possibility of components with slightly differing mobilities. Nevertheless, the marked heterogeneity of some samples in the ultracentrifuge cannot be directly related to the electrophoresis patterns, in terms of individual components. In addition, the contrast cannot be attributed to the different temperatures used for the two sets of experiments (electrophoresis at 1° and sedimentation velocity at 20°) as sedimentation of sample 7 at 3° revealed no alteration in the relative amounts of components present in the ultracentrifuge, compared with a control experiment at 20° (Table (II-3)).

(5) Correlation of electrophoretic and sedimentation velocity data.

To correlate these observations only two likely possibilities exist:

(a) impurities of different S but with essentially the same electrophoretic mobilities as the enzyme are always present in the preparations, and, moreover, the same impurities occur when urease is prepared by salt fractionation;

(b) urease molecules interact in solution to give a series of

stable polymers, the rate of electrophoretic migration being independent of the degree of association.

While the first possibility is highly unlikely, the evidence to date cannot be used to discount it. Indeed, it is the main purpose of the next Chapter to present experimental evidence for the interaction of urease in solution, thus establishing case (b). Nevertheless, it is important at this stage to determine whether it is possible that polymers of a species, apparent as discrete boundaries in the ultracentrifuge, can move with essentially the same electrophoretic mobility. If this could not be done, the hypothesis expressed in (b) would be quite incompatible with the experimental findings. However, using the data already available, it is possible not only to illustrate this point, but also to formulate the additional hypothesis that, provided case (b) operates, then urease tends to dimers, trimers, etc. This hypothesis will be examined in the light of available evidence.

(a) The $S_{20,w}$ values

These lie approximately in the ratio 1:1·4:1·8. Two interpretations of these figures may be made on the basis of models representing extremes in shape. (This type of approximate numerical calculation has been employed by other workers[35,36] to interpret similar systems.) First, the urease 'monomer' (the $S^{o}_{20,w}$ 20 component) was considered as a sphere and the dimer and trimer as cylinders of axial ratio 2 and 3 respectively. The expected ratio for the sedimentation coefficients may then be obtained in the following way. Svedberg[37] has shown (equation (12) of ref. 37), that for

infinitely dilute solutions,

$$f = \frac{M(1 - \bar{V}\rho)}{S} \qquad (1\text{-II})$$

where f is the molar frictional coefficient, M the molecular weight, $\bar{V}$ the partial specific volume, ρ the density of the solution and S the sedimentation coefficient of the macromolecule. Provided $\bar{V}$ is assumed to be the same for all species, it follows that

$$S_{monomer}/S_{dimer} = f_{dimer}/2f_{monomer} \qquad (2\text{-II})$$

and similarly,

$$S_{monomer}/S_{trimer} = f_{trimer}/3f_{monomer} \qquad (3\text{-II})$$

From the tables of Perrin[38] (tabulated by Svedberg and Pedersen[37]) the frictional ratio, f/f_o, may be found for each species, if the axial ratio is assumed. In addition, application of the equation for spherical particles (equation (13) of ref. 37),

$$f_o = 6\pi\eta N \left(\frac{3M\bar{V}}{4\pi N}\right)^{1/3} \qquad (4\text{-II})$$

enables the required ratio of the frictional coefficients, and, hence, of the sedimentation coefficients to be calculated directly. In this case, the three sedimentation coefficients were found to lie in the ratio 1:1·5:1·8$_7$. (These differ little from the values for spheres quoted by Kegeles and Rao.[39]) The alternative interpretation is based on very similar reasoning but correlates the observed frictional coefficient ratio of 1.19,[37] with asymmetry alone. By this means the axial ratio of the 'monomer' was found to be 4. If the polymers

are taken to have axial ratios of 8 and 12, the corresponding values of the sedimentation coefficients lie in the ratio $1:1\cdot3_1:1\cdot5_4$. While both of these calculations must be considered as very approximate, the similarities in the predicted ratios with those observed lends considerable support to the initial hypothesis. (On some models $S_{20,w}$ 36 component could correspond to a tetrameric unit.)

(b) The electrophoretic mobility values

Calculations here are somewhat more uncertain owing to the approximations involved in the theory, but applying the equations referred to by Abramson et al.[30] to each model, it is possible to express the charge on the protein in terms of electrophoretic mobility values. The basic equation used was

$$Q = \frac{6\pi\eta r\,(1 + \kappa r + \kappa r_1)}{f(\kappa r)\,(1 + \kappa r_1)}\,u \qquad (5\text{-II})$$

where Q is the net charge in coulombs/molecule,

η is the viscosity of water at 0°,

r is the radius of the urease molecule in cm.,

r_1 is the average radius of the electrolyte ions in cm.,

κ is the Debye-Huckel parameter defined by equation (19) of ref. 30,

$f(\kappa r)$ is a function of κr formulated by Henry and defined in equation (44) of ref. 30,

and u is the measured electrophoretic mobility in $cm.^2\ sec.^{-1}\ volt^{-1}$.

It is more convenient to express the charge on the protein as the number of excess positive or negative charges per molecule in electronic units. This is defined as the valence of the protein, v.[30] Accordingly the appropriate conversion factor was employed to obtain the results below. The value of κ at 0° and at an ionic strength of 0.1 was calculated to be 1.081×10^{7} cm.$^{-1}$ and the value of r_i was taken as 2.5×10^{-8} cm. Table (II-4) includes values for the other variables. In the last column are presented data which allow an approximate value of the valence of the species to be calculated at different pH values, when correlated with the mobility data in Table (II-2).

If the further assumption is made that the total charge is conserved, mobility values for the first model (spherical monomer and cylindrical dimers and trimers) lie in the ratio $1:1{\cdot}0_9:1{\cdot}1_2$. The second model described (cylinders of axial ratios 4, 8 and 12) gave lower mobilities but these lay in the <u>same</u> ratio as for the first model. This result is partly coincidence and does not imply that the mobility ratios are independent of the geometry of the model; thus a change from spherical monomer to dimer of axial ratio 4 produces mobilities in the ratio $1:0{\cdot}8_9$. In short, the theory predicts relatively small changes in mobility as a consequence of polymerisation and that these could either increase or decrease the mobility relative to that of the monomer, depending on the model used.

To sum up, it is clear that provided the aggregation process (postulated in (b) above), is visualized as taking place between nearly spherical monomer units and that the essential structural

Table (II-4)

The relation between electrophoretic mobility and valence

Axial ratio	r cm. x 10^8	$6/\xi(\kappa r)$*	valence, v
First model			
1	61.33	4.96	6.61u
2	77.28	4.90	12.15u
3	88.44	4.86	18.04u
Second model			
4	51.53	5.11	7.52u
8	64.93	4.98	14.09u
12	74.31	4.92	20.35u

* This value was obtained by interpolation from a graph of the tabulated values.[30]

integrity of the monomer is largely or wholly preserved in the dimers and trimers (or tetramers), then the hypothesis is compatible with both the ultracentrifuge and electrophoresis results. (It might be thought that the mobility calculations indicate a minor degree of unfolding consequent on dimerization, but in fact the calculations are too tentative for this to be justified.) It is pertinent to note that quite analogous behaviour has been recorded for several other systems, for example, l-amino acid oxidase,[40] haemocyanin,[41] ground nut globulins,[42] α-keratose[43] and soy bean proteins.[44] In several of these cases, independent evidence showed that the components evident on ultracentrifugation, which had the same electrophoretic mobilities, were, in fact, aggregates of the same species. This together with the theoretical considerations above suggests that comparison of electrophoretic and sedimentation velocity analyses could provide strong <u>presumptive</u> evidence for a possible interaction.

It remains to establish a direct relationship between the peaks evident on sedimenting urease preparations before the system can be conclusively demonstrated to be interacting. This problem is discussed in the next Chapter.

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CHAPTER III

DIRECT EVIDENCE FOR THE INTERACTION OF UREASE IN SOLUTION AND THE GROUPS INVOLVED

(1) The application of chemical modification to the study of interacting protein systems.

(2) The sulphydryl groups of urease
   (a) those likely to be involved in disulphide bond formation,
   (b) the active site.

(3) The cleavage of the disulphide bond, with particular reference to the effect of S-nucleophilic bases, such as sulphite ions.

(4) The effect of chemical modifying reagents on the properties of solutions of urease. Experimental and results.
   (a) the reaction with sodium sulphite,
   (b) the reaction with H_2S and KCN,
   (c) the reaction with potassium ferricyanide,
   (d) the effect of cysteine-hydrochloride.

(5) The effect of sulphite ions on the enzymic activity of urease. Discussion.

(1) The application of chemical modification to the study of interacting protein systems.

In view of the indication of a possible relation between the discrete boundaries observed in the ultracentrifuge with solutions of urease, it is necessary at this stage to determine a suitable method, which will confirm or deny this basic hypothesis. The use of transport experiments, in particular sedimentation velocity runs, has in some cases, for example with α-chymotrypsin,[1,2] provided direct evidence for a polymerization. The inferences drawn from the appearance of one or two peaks and the variation of S with protein concentration have been outlined in Chapter I. If the possible urease interaction were of the same type, the conclusions made by Gilbert[3] and Kegeles and Rao[2] would imply that the discrete boundaries apparent in the schlieren ultracentrifuge patterns (Fig. (II-4)) must indicate the existence of monomers and polymers greater than the dimer and trimer postulated. Furthermore, dilution would be expected to favour a higher relative proportion of the slower sedimenting peak: in fact, Gilbert[3] has shown that the area under this peak would remain constant, as the concentration dropped, until the faster sedimenting peak had disappeared.

While the data in Table (II-3) are limited by the accuracy of the determination of areas, they show conclusively that this predicted behaviour is not fulfilled. It therefore appears that if an equilibrium or a relation exists at all between the components the kinetic constants regulating the interconversion are small and the system is of the second type discussed in the Introduction.

In short, as the concentration gradient falls to zero between the peaks, implying independence of flow of each component, the system exhibits the ultracentrifugal properties of an heterogeneous solute rather than a reversibly interacting system, such as α-chymotrypsin. This does not imply that the urease system cannot be reversibly interacting but shows that transport experiments are not amenable to the elegant interpretation possible with a system in rapid reversible equilibrium. Indeed, only by a careful study of the effect of factors, such as temperature, on the relative amounts of the separate peaks could ultracentrifugal analysis reveal the existence of an equilibrium between the related components. However, it is possible that these changes may be small and difficult to detect (depending on the magnitudes of the relevant thermodynamic functions), and the design of the experimental procedure in relation to time might be somewhat difficult as there is no information on the rate of the reaction. Consequently, while worthy of future consideration, this approach is unsuitable in providing evidence for a direct relation between the peaks.

The use of reagents which will react specifically with a particular type of chemical grouping in the molecule[4-6] provides an alternative approach to this question. Provided the nature of the possible intermolecular cross linkage can be deduced, it may be possible to cleave this linkage with a chemical modifying reagent and thereby convert the higher polymers to the monomeric form. Thus, in the presence of an excess of the reagent, the sedimentation velocity pattern would show a single peak, whose area, in this ideal situation, would be the sum of the areas of the individual peaks observed in a

control experiment without the cleaving agent. This elegant approach, which has been applied to other systems such as the soy bean proteins,[7] relies entirely on the ability to predetermine the nature of the cross links. These bonds would have been formed originally by reaction of chemical groupings situated in a suitable structural position on the molecule. Information on such reactive groups is therefore a pre-requisite in an attempt to deduce the nature of the intermolecular bonds. Little is known about the amino acid composition, structure or potentially reactive groups of urease.[8] However, a survey of the literature shows that sulphydryl groups in urease may be extremely important in this connection.

(2) The sulphydryl groups of urease.

Crystalline undenatured urease preparations respond positively to the nitroprusside test.[9,10] This, together with numerous reactions involving modifying reagents, which were claimed to react specifically with sulphydryl groups, showed conclusively that urease possessed easily detectable and very reactive thiol groups in the native state.[10-13] Thus, undenatured urease solutions have been shown to react with oxidizing agents, such as iodine and iodosobenzoate,[10] alkylating agents (iodoacetamide and iodoacetate[10]) and mercaptide forming reagents, for example p-chloromercuribenzoate and mercuric chloride.[14] Other proteins known to possess sulphydryl groups (for example, ovalbumin) react in the native state with only a few of these group-specific reagents and it is only after denaturation that ovalbumin solutions give a colour with nitroprusside.[10] It is apparent that the thiol

groups of urease, in comparison, are much more readily 'available'[6] for reaction. Moreover, the ability of sulphydryl groups to form disulphide linkages either intermolecularly or intramolecularly is well known:[6,15,16] indeed, the distinct possibility of disulphide bond formation from sulphydryl groups in urease has been suggested theoretically, but not proven.[11,12,17] The possibility of intermolecular disulphide bond formation becomes even more important when it is realized that inhibition studies, using both metal ions and group-specific reagents, have shown that sulphydryl groups in all probability form part of the active site of urease.[10-14,18] In view of this and the necessity for postulating a plausible cross linkage for the proposed polymeric species of urease, it is essential to examine in detail the nature of the thiol groups.

Hellerman, Chinard and Dietz[10] titrated solutions of recrystallized urease with p-chloromercuribenzoate and correlated the amount of reagent added with the overall enzymic activity at various stages of the titration. This procedure (and a study of additional reactions) enabled the authors to deduce the following conclusions. (For clarity, the figures cited below refer to a mole of urease, the molecular weight being taken as 480,000,[19] rather than to the hypothetical 'equivalent weight' used by Hellerman et al.)

(i) 22 -SH groups reacted with p-chloromercuribenzoate without loss in enzymic activity and with dilute solutions of porphyrindin. These extremely reactive groups, referred to as type (a), were presumably responsible for the colour with nitroprusside;

(ii) 22 -SH groups, comprising type (b), did not react at all with

porphyrindin and only with iodosobenzoate and p-chloromercuribenzoate after the type (a) groups had reacted. These appeared directly concerned with the enzymic activity, as complete inactivation resulted when they were effectively removed;

(iii) 60 -SH groups, type (c), were estimated with o-iodoso-benzoate only after denaturation with guanidine hydrochloride. This latter type is clearly in a structural position inaccessible to substrate, and cannot form intermolecular bonds. They are, therefore, not important in this study, which is primarily concerned with the intact molecule.

Desmuelle and Rovery[20] followed an essentially similar procedure, using phenyl isocyanate as the reagent, and confirmed that urease fully maintained its activity when the type (a) groups were reacted: the activity decreased only when an excess of the reagent was added. Two further significant observations were made in this work.

(i) The quantitative aspects of the work of Hellerman et al.[10] were questioned as the evaluations assumed that p-chloromercuri-benzoate reacted stoichiometrically and completely with the thiol groups. Ambrose, Kistiakowsky and Kridl[13] emphasized this criticism by contrasting the results found by Sumner and Myrback,[21] who found that seven silver ions sufficed to inactivate one molecule of urease, with the data reported above.

(ii) It was pointed out that it was unlikely that the reduced reactivity of the type (b) groups toward p-chloromercuribenzoate and phenyl isocyanate could be attributed to their inaccessibility

due to structural position, because this would apparently contravene the necessary ease of formation of the enzyme-substrate complex. An alternative reason suggested was that the groups were involved in labile structural bindings (presumably by hydrogen bonding). In this connection, it is pertinent that a third and more reasonable suggestion has been proposed which postulates that the seemingly 'less reactive' thiol groups do not exist as cysteine residues, but are covalently bonded with adjacent groups.[6,22,23] In particular, Smith[23] has recently proposed the existence of a preformed internal thiol ester, $R - \underset{\underset{O}{\|}}{C} - S - R$, on the basis of thermodynamic, kinetic and chemical information on the 'thiol enzyme' papain.

This hypothesis represents, in many ways, a significant and interesting advance. First, it explains the apparent anomalous 'non reactivity' of the type (b) thiol groups toward certain reagents, such as nitroprusside, even though the same groups react with mercurials.[24] Secondly, if the linkage between the thiol and carboxyl groups represents a thermodynamically unstable and kinetically reactive structure maintained by the folding energy of the protein, it explains not only the ready formation of the enzyme-substrate complex, but also the dependence of activity on the folded tertiary structure of proteins, referred to in Chapter I.

This survey leads to the following pertinent observations:

(i) There are available for intermolecular reaction several sulphydryl groups, the type (a). Although consideration of the

available mechanisms for the formation of the disulphide bond must logically be left until direct evidence for its occurrence in the urease system is presented, it is convenient at this stage to visualize the interaction as,

$$2ESH + \text{oxidant}^{+2} \rightleftharpoons ESSE + \text{reductant} + 2H^{+} \qquad (1\text{-III})$$

(ii) The groupings of the active site, type (b), which may exist as the thiol ester or some similar structure, may or may not interact in this way. In either case, however, the interaction would be expected to affect the overall activity, either by direct removal of the active site, or by structural impedance of the substrate to the active site inherent on polymer formation.[25]

(3) The cleavage of the disulphide bond.

In studies such as this it is desirable that the reagents and the environmental conditions chosen to cleave the disulphide linkage be mild, specific and efficient.[6] Thus, the reaction should involve only the possible intermolecular disulphide links and not others, which as in insulin[26] and ribonuclease[27] are essential in maintaining the structural integrity of the protein molecule: indeed, if the protein is denatured in any way, any attempt at subsequent sedimentation velocity analysis would prove futile. Consequently, the use of denaturing agents or severe conditions such as those involved in direct reductive cleavage[28] is prohibited. Several reagents, $SO_3^{=}$, CN^{-}, and $S^{=}$, are all known to cleave the disulphide linkage,[6,29] almost certainly by a nucleophilic attack and the conditions (pH, temperature, etc.) are in many cases mild.[5] The use of these

S-nucleophilic bases,[29] while desirable in this sense, is nevertheless complicated by other factors[6] (pH, steric effects and side reactions). This will be illustrated by an outline of the reaction of the disulphide group with sulphite ions.

Clark[30] showed that cystine reacted with sulphite ions in an addition reaction, which resulted in mercaptide ions and S-sulphocysteine. This reaction may be represented generally by,

$$RSSR + SO_3^{=} \rightleftharpoons RS^{-} + RSSO_3^{-} \quad ^{a} \qquad (2\text{-III})$$

The reaction is both mild and specific. However, a study of the nature of the equilibrium[32] and kinetics[33] of the reaction has shown that the efficiency, both with respect to rate and equilibrium position, depended strongly on pH for the following reasons:

(a) At low pH values the thiol formed is present as RSH. As the reaction is reversible to RS^{-}, an optimum pH, which depends on the pK value and concentration of thiol as well as the equilibrium constant for the reaction, may theoretically be chosen for the maximum formation of the thiol: in practice only an empirical approach is feasible.

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[a] It is preferable to refer to this ion as a sulphenyl sulphite rather than as a S-sulpho derivative.[29] Alternatively, $-SSO_3^{-}$ is termed the thiosulphate group and a molecule containing this group is called the thiosulphate form (for example, an S-alkyl thiosulphate would be one product of the reaction of sulphite with an alkyl disulphide).[31] The latter nomenclature has been adopted in this study.

(b) In turn, the amount of HSO_3^- will increase with decreasing pH (the second pK of H_2SO_3 being approximately 7[33,34]) and as the rate of the reaction with the bisulphite ions is apparently virtually negligible compared with that of the sulphite ions, it appears undesirable to choose a pH value below 6.5;

(c) the rate of reaction with $SO_3^=$ is much greater with a protein (or simple disulphide compound) bearing a zero or net positive charge (particularly in the vicinity of the disulphide bond) than with that bearing a net negative charge, where electrostatic repulsion hinders or possibly prevents the nucleophilic attack.

In this study a choice of a pH optimum for maximum formation of the thiol and thiosulphate form (both of which will be evident as the 'monomer' in the ultracentrifuge) is required rather than for a maximum rate of reaction. Buffered sulphite solutions of pH 7 were chosen, although in Chapter VI the effect of lowering the pH is discussed. At this pH and with an <u>excess</u> of $SO_3^=$, almost complete conversion could be expected. Moreover, the effect of atmospheric oxygen[35] and traces of metal ions[36] may in fact operate to aid the complete shift of the equilibrium to the apparent 'monomeric form'. Thus, while the mechanism of the reaction involving molecular oxygen and traces of metal ions is unknown, the overall reaction may be represented as,[6]

$$\underset{\uparrow\text{molecular oxygen}\downarrow}{RSSR} + SO_3^= \rightleftharpoons RS^- + RSSO_3^- \qquad (3\text{-III})$$

The net effect is complete conversion of the disulphide compound to the thiosulphate form. It is apparent that, if intermolecular

disulphide linkages bind urease units to form polymers, addition of excess $SO_3^=$ should have a pronounced effect, providing the disulphide linkages are accessible both with respect to structural position and charge environment. Essentially similar considerations should apply to the effect of CN^- and $S^=$, which act on the disulphide bond according to the following equations:[29]

$$RSSR + CN^- \rightleftharpoons RSCN + RS^- \qquad (4\text{-III})$$

$$RSSR + S^= \rightleftharpoons RSS^- + RS^- \qquad (5\text{-III})$$

(4) The effect of modifying reagents on the properties of solutions of urease.

(a) The reaction with sodium sulphite

The solutions used in the experiments performed in the presence of sulphite ions had the following composition: 0.048M Na_2HPO_4, 0.021M KH_2PO_4, 0.031M Na_2SO_3, 0.013M $NaHSO_3$ and were accordingly identical with those employed earlier.[19,37] No special precautions were taken to prevent atmospheric oxidation in this series of experiments. The oxidation-reduction potential of the solutions was not measured, and consequently, no quantitative significance was attached to the total sulphite concentration. When buffered urease solutions, pH 7.0 (samples 4, 5, 6 and 7) were dialysed against this buffered sulphite solution and the resulting protein solution sedimented, the faster sedimenting boundaries (Fig. II-4) were no longer evident and the amount of the $S_{20,w}$ 20 boundary increased markedly. This is illustrated by Fig. (III-1). With sample 4, this increase in area equalled, within the limits of accuracy of the area determinations,

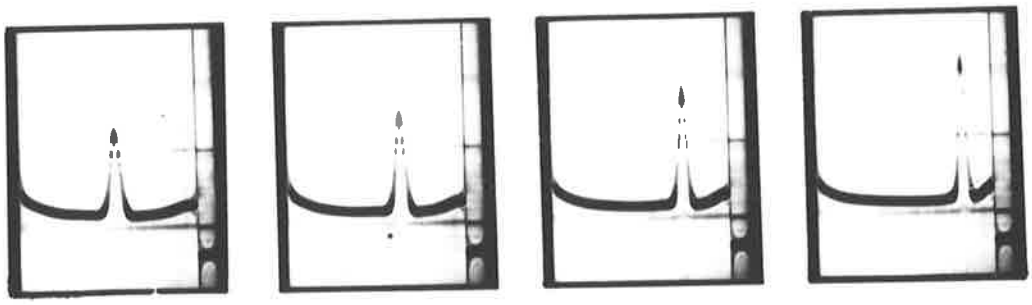

Fig. (III-1). Sedimentation patterns of a urease solution, sample 5, in a buffered sulphite solution, pH 7.0_9 (see text). Sedimentation is from right to left.

the area previously under the $S_{20,w}$ 28 peak, which was the only other peak visible in this sample (see Fig. II-4b). However, with sample 5, the increase was apparently greater than could be accounted for by the disappearance of the peaks visible in the sulphite-free solutions during photography, Fig. (II-4c). Thus, although the increase in area of the $S_{20,w}$ 20 component could not always be correlated quantitatively with the disappearance of the faster components (possibly because of the neglect of very large molecular weight material, evident at the bottom of the cell, which may also be affected by sulphite) it is highly improbable that sulphite would convert either impurities or differently shaped urease molecules to a modified form, which sediments together with the enzyme as a single boundary. Consequently, the only apparent hypothesis is that polymers of urease exist in solution and that sulphite reverses the polymerization.

(b) The reaction with H_2S and KCN

The action of both sulphide and cyanide ions (also S-nucleophilic bases) confirmed this significant finding. In the former case, a phosphate buffer was 50% saturated with H_2S, previously cleaned by bubbling through water, and a buffered urease solution dialysed against it. The ultracentrifuge pattern, which was quite analogous to Fig. (III-1), again revealed the absence of any peaks other than that with an $S_{20,w}$ of 20.

Dialysis of a urease solution against a phosphate buffer containing 0.05M KCN resulted in the removal of the $S_{20,w}$ 36 peak and a reduction in the amount of $S_{20,w}$ 28 peak, both originally present in a control ultracentrifuge analysis. Several side reactions are

possible with KCN[38-40] and as solutions are likely to be strongly alkaline (thus introducing the complication of a large net negative charge on the urease molecule) extended investigations with this reagent were not considered desirable at this stage. Nevertheless, these observations, together with the effect of sulphite, indicate strongly that intermolecular disulphide cross linkages are the cause of the polymeric species present in solutions of urease. This is a plausible theory in view of the discussion above on the readily available nature of the sulphydryl groups on the molecule and of the known reactions of these groups, expressed in equation (1-III).

(c) The reaction with potassium ferricyanide

The equation (1-III) shows that deliberate addition of an oxidising agent should promote the formation of the disulphide bonds. Experimentally it was found that addition of 0.01M $K_3Fe(CN)_6$ caused immediate and complete precipitation of the protein. The product was insoluble in buffer at pH 7.0 and in this respect resembled the precipitate obtained on dialysing a urease solution to pH 5.0. It is tentatively suggested, therefore, that the reduced solubility of urease near the isoelectric point, where electrostatic repulsive forces are at a minimum, may be partly due to the formation of very high molecular weight polymers involving disulphide cross linkages. One further observation of a qualitative nature may be made.

(d) The effect of cysteine-hydrochloride

This reagent was intentionally used to prevent inhibition by metal ions, which could accidentally be present (see experimental section). Accordingly, no fixed amount was added during the prepar-

ation of most of the samples. As has been described, the apparent proportions of the components observable in the ultracentrifuge varied widely between different samples (see Table (II-3)). It is possible that this variability was the direct result of the varying content of cysteine, as reduction of disulphide bonds by thiols is a distinct possibility.[6]

$$RSSR + 2R'SH \rightleftharpoons 2RSH + R'SSR' \qquad (6\text{-III})$$

To test this, cysteine was not added at any stage of the preparation of sample 5. The resulting preparation had a low activity (see later) and relatively greater amounts of the faster sedimenting components (also apparent in Table (II-3)). While not conclusive, this result supports the contention that cysteine does have a similar action on urease as $SO_3^=$, CN^- and $S^=$, in respect of reducing or reversing aggregation.

To sum up, the results so far have shown that urease tends to polymers (possibly dimers, trimers, etc.) by the reaction of available sulphydryl groups on the molecule to form intermolecular disulphide bonds. The polymerization is apparently completely reversed by sulphite ions, under the conditions chosen, to form a modified derivative. The nature of this form of urease, both with respect to enzymic activity and identity to the $S_{20,w}$ 20 component in sulphite-free solutions, remains to be established. Both are extremely important points. Thus other disulphide bonds which may be fundamental to the structural integrity of the protein, and hence to the enzymic activity, may also have been cleaved. Bovine plasma albumin is not reactive with $SO_3^=$ until the protein has been denatured, but some of the disulphide bonds of insulin[41] are cleaved. This is

particularly relevant to the urease study as the reliability of the existing diffusion data,[19] as well as certain kinetic studies[17] performed in the presence of sulphite, relies entirely on the assumption that the species studied have not been denatured. Indeed, most kinetic studies have been performed on solutions containing as 'protecting' or 'activating' reagents the S-nucleophilic bases described: these were intentionally added to prevent the inhibition of urease by metal ions.[42]

(5) The effect of sulphite ions on the enzymic activity of urease.

The choice of sulphite as a modifying reagent in this and all other aspects of the work to be presented was governed by the following factors:

(a) this S-nucleophilic base is specific for the disulphide bond cleavage, unlike cyanide ions for example;

(b) in contrast with the use of H_2S, the experimental technique is simple, and interpretation of the results is greatly facilitated by the possibility of accurately and easily estimating the concentration of the ions present;

(c) the form of urease obtained in the presence of sulphite ions was used by Sumner et al.[19] in diffusion experiments performed to obtain the molecular weight.

A comparison between the overall enzymic activity between solutions of urease in the presence and absence of sulphite ions is given in Table (III-1), where, also, the effect of the ions on the relative amounts of the separate sedimenting peaks is illustrated. It is apparent that an increase in activity accompanies an increase

Table (III-1)

The effect of sulphite on the enzymic activity and sedimentation velocity patterns of urease[a]

Sample history	% of total area[b]			Enzymic activity of 1 ml. of solution S.U.
	1	2	3	
Control: Urease dissolved in pH 7.5 'veronal' buffer, ionic strength 0.1, stored in cold for 1 week and diluted with phosphate buffer for assay.	55	31	14	25
Sulphite treated sample: An aliquot of above solution of urease in 'veronal' buffer, dialysed for 1 week against a sulphite solution and diluted with phosphate buffer for assay, performed within 2 hr. from above.	100	0	0	50

[a] Data reported were found with sample 5, which had a low specific activity (10,000 S.U./g.). The effect was reproduced with sample 7.

[b] Notation as in Table (II-3). Approximately 0.7% solution of protein was used.

in the amount of the $S_{20,w}$ 20 component.

This finding has several important implications. First, the polymeric forms must have reduced activities, as had been previously suspected.[43] This could arise from the direct participation of the type (b) 'sulphydryl groups' postulated by Hellerman et al.[10] in disulphide bond formation. However, as we have seen, the reversal of the polymerization results in a modified form, in which at least some, if not all, the original thiol groups are converted to thiosulphate groups. It is unlikely that these too could function as part of the active site. Consequently, it is more reasonable to presume that the type (a) groups are directly involved and that the reduction in activity is due to steric hindrance to the approach of the substrate, inherent on polymer formation. Certainly, if the active site involved structures of the thiol ester type, direct disulphide bond formation would be unlikely. In this respect it should be emphasized that the inhibiting effects of the various sulphydryl blocking agents do not establish conclusively the existence of thiol groups, as such, in the active site. While these results support the contention that thiol groups, not part of the active site, are the cause of the polymerization and that the active site involves a structure of the thiol ester type, they are in no way conclusive. Nevertheless, the discussion illustrates the extremely tentative nature of recent attempts[44] to define the nature of the enzyme-substrate complex.

Secondly, the data in Table (III-1) and the observations of the effect of H_2S and KCN throw direct light on the function of such reagents as 'activating' or 'protecting' agents. It was generally accepted that the reagents removed metal ions and thereby prevented

inhibition of the enzyme.[42] Their value in this respect is not denied, but their function in reversing or preventing the polymerization explains satisfactorily, for the first time, the decrease in activity of unprotected solutions with time and their subsequent reactivation.[45] The existence of two forms of urease in unprotected solutions, suggested by the results of Kistiakowsky et al.,[17] is also possibly explained. However, as Kistiakowsky and coworkers employed H_2S in most kinetic experiments, as was pointed out in Chapter I, the possibility of two types of active sites cannot be explained, at the moment, by the existence of polymeric forms with reduced activities.

Finally, the results to date indicate that the modified or stabilized form existent in the presence of sulphite ions, which retains enzymic activity, has not undergone any marked structural change: at least it may be inferred that sulphite ions do not have a pronounced effect on the tertiary structure of the protein. This conclusion, if substantiated, has the consequence that either there can be no structurally important disulphide linkages in urease, or that if they are present, they are considerably less 'accessible' than the intermolecular linkages in the environment chosen. Moreover, if the modified form has remained intact, the assumption inherent in the molecular weight determination[19] that this species was identical in size and shape to the $S^{\circ}_{20,w}$ 20 component observed in unprotected solutions would be justified.

The next chapter is devoted to an examination of the sedimentation and electrophoretic properties of this stabilized species. When correlated with observations made in this and the preceding chapter, the results prove valuable in elucidating the nature and

mechanism of the polymerization. This point has been deliberately omitted until the groups involved and the nature of the cross linkage had been established.

References to Chapter III

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CHAPTER IV

THE MECHANISMS OF THE POLYMERIZATION REACTION AND OF ITS REVERSAL BY SULPHITE IONS

(1) The statement of the problem.

(2) The properties of the sulphite modified form of urease

- (a) The sedimentation properties,
  - (i) the dependence of sedimentation coefficient on concentration
  - (ii) the effect of the removal of sulphite ions by dialysis.
- (b) Equilibrium considerations of the reaction of sulphite ions with disulphide bonds. A theoretical discussion in relation to the urease reaction.
- (c) Electrophoresis of urease solutions in the presence of sulphite ions.

(3) The mechanism of the formation of a disulphide bond from -SH groups.

- (a) The mercaptan-disulphide interchange.
- (b) Direct oxidation.
- (c) The participation of molecular oxygen in the polymerization reaction.

(4) Further studies. The estimation of the heterogeneity of the sulphite modified form.

(1) The statement of the problem.

The demonstration of the existence of a polymerization reaction in urease solutions involving disulphide bond formation, and of the reversal of this interaction to form an active form of urease opens out two possible fields of study. In the first place, attention could be given to the polymerization reaction, where several problems of interest remain. Thus, the intermolecular disulphide bridges could arise, either by direct oxidation of sulphydryl groups on separate molecules, illustrated by equation (1-III), or by the mercaptan-disulphide interchange, originally proposed by Huggins, Tapley and Jensen[1] to account for the gelation phenomena encountered with plasma albumin in urea solutions. Moreover, the related question of whether it is an equilibrium reaction (in the sense that comparable proportions of e.g. monomer and dimer can exist at equilibrium at a realizable concentration) could be investigated and thermodynamic parameters pertinent to the equilibrium studied. This latter point might prove of prime importance in correctly interpreting kinetic experiments performed with urease solutions free of any 'protecting' agent[2,3] (containing, as a consequence, polymeric forms of the enzyme).

On the other hand, the physico-chemical characterization of the modified form of urease, free of higher polymers, is of equal importance. Many kinetic experiments, particularly those of Kistiakowsky and coworkers, have been performed in an environment, which in all probability reversed and prevented polymerization giving a similar 'monomeric' species. Certainly a comparison between the $S^{o}_{20,w}$ 20 component in the polymeric mixture and this form would be most informative. Equation (2-III) suggests that the heterolytic cleavage of

the disulphide bond will result in minor chemical differences in the two forms, and these, together with any marked change in size and shape inherent on chemical modification, should be carefully examined.

A study of some properties of urease solutions containing sulphite provided information of importance in the latter connection: it also suggested suitable experimental approaches to the questions posed in connection with the mechanism and possible equilibrium nature of the polymerization reaction. The latter point will be discussed later in the Chapter.

(2) The properties of the sulphite modified form of urease.

(a) The sedimentation properties

(i) The dependence of sedimentation coefficient upon concentration

The effect of protein concentration on the S of the boundary observed in the presence of sulphite ions is shown graphically in Fig. (IV-1). Sample 6 in a buffered sulphite solution,[4] pH 7.0, was used and, accordingly, the protein bore a net negative charge. The highest concentration was determined refractometrically and subsequent dilutions were made by weight. The determination of concentration was therefore more accurate than that in Table (II-3), and thus the extrapolated $S^{o}_{20,w}$ value can be given as 20.3 ± 0.1. It is, therefore, the same within experimental error as the corresponding value obtained in sulphite-free solutions. While the identity of the two values cannot be taken as decisive evidence that no marked change in the size and shape of the macromolecule has occurred on

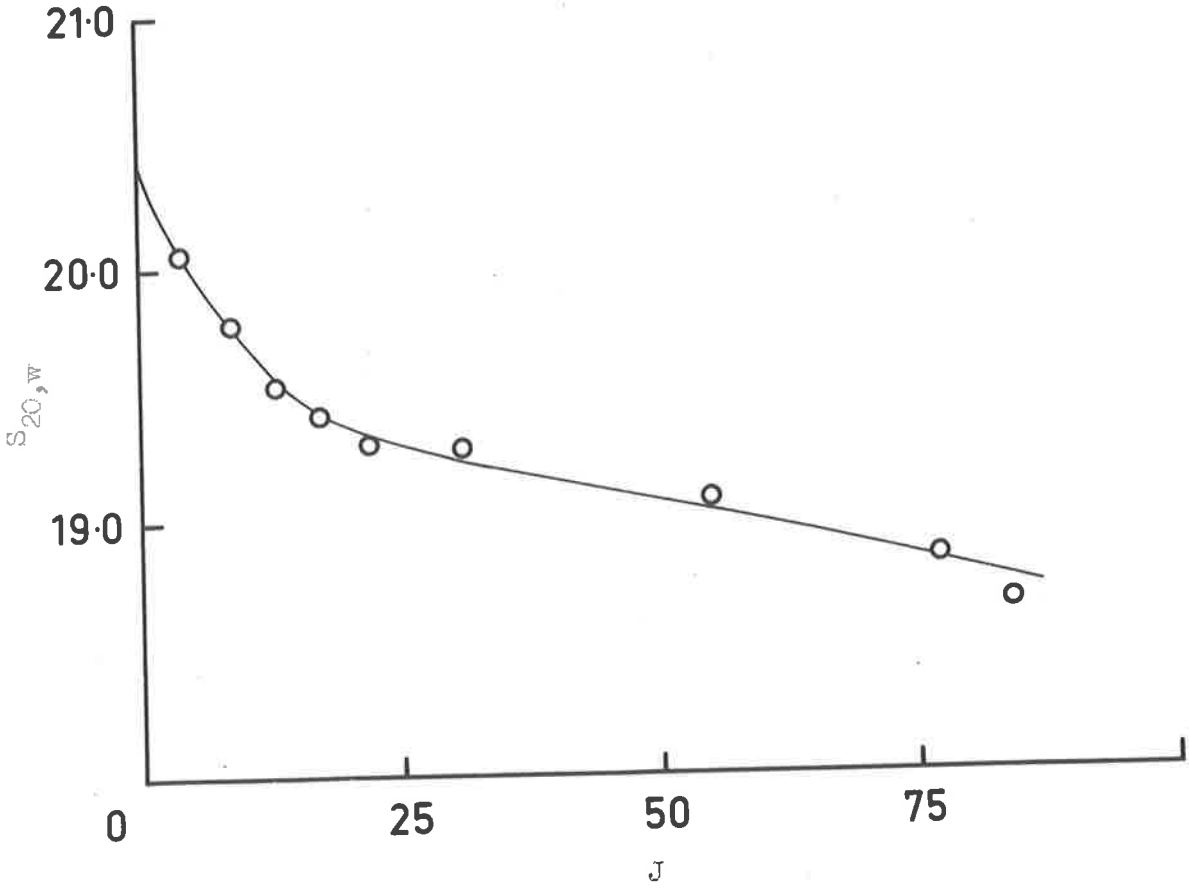


Fig. (IV-1). The variation of sedimentation coefficient with concentration (expressed as fringes, J) of the species present in a buffered sulphite solution of urease, sample 6.

treatment with sulphite, it is strongly suggestive of this, especially when considered with the activity data presented in Chapter III.

The curvature of the solid line in Fig. (IV-1), which is an attempt to average the data, was unexpected but apparently outside experimental error. Provided the term $(1 - \bar{V}\rho)$ has relatively little effect, it follows from equation (1-II) that the concentration dependence of S is a direct measure of the concentration dependence of f, the frictional coefficient. However, the information summarized in Fig. (IV-1) is insufficient to decide between a possible structural transition and other factors affecting the apparent frictional coefficient. It is pertinent to note, however, that as S increases with decreasing protein concentration, the possibility is excluded of a rapidly reversible equilibrium between polymeric forms, of the type outlined in Chapters I and III. Consequently, there is some justification in assigning the term monomeric unit to this form of urease. Further consideration of possible heterogeneity in the samples obtained, and analytical procedures to estimate the extent of the heterogeneity will be deferred until later.

(ii) The effect of the removal of sulphite ions

The sedimentation velocity analysis of sample 5 in the presence of sulphite ions was presented in Fig. (III-1). This solution was dialysed against phosphate buffer, at constant pH, until free of sulphite; the sedimentation behaviour then observed is illustrated in Fig. (IV-2). The pattern shows clearly the presence of three components with $S_{20,w}$ values of 18.8, 28 and 35. The apparent reversal of the sulphite reaction was repeated with sample 8,

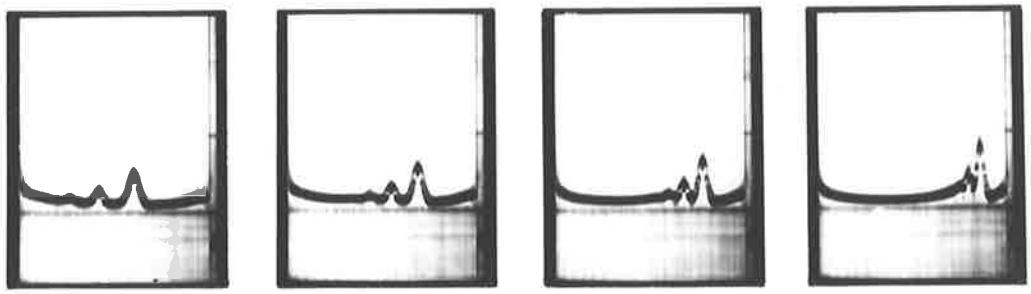
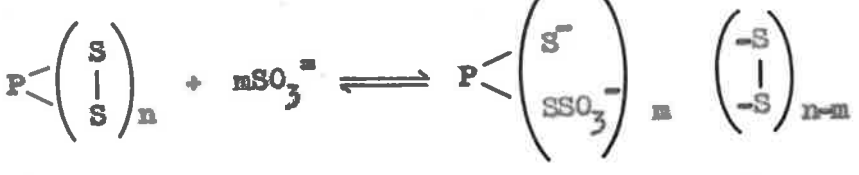


Fig. (IV-2). Sedimentation patterns of urease, sample 5, in 0.9% phosphate buffer, pH 7.0, after the sample had been dialysed free of sulphite ions (see text). Sedimentation from right to left.

where again repolymerization occurred. These observations led to the following conclusions. First, they support the contention that sulphite does not alter the molecular weight of the $S^{o}_{20,w}$ 20 component in the sulphite free solutions, as it is unlikely that a markedly changed molecule could polymerize to give S values identical to those obtained prior to sulphite treatment. Secondly, it is apparent that even after 7 days (the total dialysis time) in the presence of sulphite ions, residual sulphydryl groups exist, which are capable of reacting to form disulphide linkages. The thiosulphate groups, $-SSO_3^-$, cannot reoxidize. To appreciate the possible implications of this finding, it is necessary to inspect more closely the reaction of sulphite ions with disulphide bonds in protein systems. The simple equations presented in Chapter III, while valid for simple disulphide compounds, represent an oversimplification of the possibilities pertinent to the complex case under consideration.

(b) Equilibrium considerations of the reaction of sulphite ions with disulphide bonds

Kolthoff and coworkers[5] have formulated the reaction of the cleavage of intramolecular disulphide bonds in serum albumin. The same general considerations as to the position of the equilibrium should apply in this case.



(1-IV)

The equilibrium constant, K, was formulated for the case where m = 1,

$$K = \frac{[\Sigma - SH]^2}{[P(S - S)][\Sigma SO_3^{=}]} \qquad (2\text{-IV})$$

where [Σ - SH] denotes the total molar concentration of this group in the equilibrium mixture, [P(S - S)] that of the unreacted disulphide and [$\Sigma SO_3^{=}$] the equilibrium concentration of total sulphite. It was implicit in this relation that only -SH groups formed by the cleavage reaction (1-IV) were present and that [Σ - SH] = [Σ- SSO_3^{-}]. As the pK values of -SH groups in proteins appear to have a minimum value of approximately 8,[6-8] it is reasonable to assume that undissociated groups are formed rather than mercaptide ions at the pH values used in this study ($\sim$ 7). While the equilibrium position and the rate of the reaction would be governed by pH (see Chapter III), Kolthoff et al.[5] found that even at pH 5 about 80% of the disulphide bonds available in bovine serum albumin were cleaved in the presence of denaturing reagents.

In these studies with urease, the concentration of the protein employed ($\sim 1 \times 10^{-5}$ molar) was of the same order as that of the albumin, while the initial concentration of the sulphite was larger by a factor of 10. Consequently, it was not surprising that an apparently complete conversion occurred to the monomeric form (illustrated by Fig. (III-1)). This analogy is based, of course, on the bold assumption that the K values in each case are of the same magnitude. Therefore the conclusions support only in a qualitative way the contention that urease, in the presence of sulphite, exists almost completely as molecules of the same size, which differ only in the ratio of sulphydryl and thiosulphate groups present. The ratio could be expected to vary widely on different molecules: the molecules present originally as the monomer would possess only -SH

groups, while the cleavage of several disulphide linkages in, for example, a trimeric unit, would result in a monomeric form with several thiosulphate groups, the actual number depending on the number of intermolecular cross links binding the monomeric units in the polymer and on the 'direction' of the unsymmetrical scission. In short, the apparently homogeneous samples sedimented in the presence of sulphite might be expected to exhibit considerable micro-heterogeneity,[9] when subjected to electrophoresis at pH 7, due to the charge differences (-SH and $-SSO_3^-$).

The tentative nature of this discussion, which is deficient in quantitative data on the equilibrium and complicating effects, such as the presence of an equilibrium concentration of HSO_3^-,[10] is illustrative of the general inability to treat adequately protein systems in which the number, type, state of ionization and structural position of the groups involved directly or indirectly in a particular reaction are unknown. In this case, however, it is possible that a more satisfactory conclusion regarding the state of the final product in the reaction mixture can be reached by invoking the logical extension formulated in equation (3-III). This suggests that mercaptide ions (or more correctly -SH groups), which are present either as unreacted groups on the monomer or as a result of the disulphide bond cleavage, will combine to form a disulphide linkage. Regardless of whether this involves a direct oxidation or a mercaptan-disulphide interchange or is an equilibrium reaction, the ultimate result will be a quantitative conversion to the thiosulphate form of all those -SH groups structurally capable of reacting.

If this mechanism operates, the quantitative conversion to the monomeric form is explained without having to assume that the equilibrium (equation (1-IV)) lies completely to the right. Furthermore, should the chain of reactions proceed to completion, the end-product would have all its -SH groups converted to $-SSO_3^-$ and might therefore be expected to have a higher (negative) mobility, at all pH values, than the untreated urease (cf. Table (II-2)). One may note at this stage that this is extremely unlikely, for if only thiosulphate groups were present removal of sulphite by dialysis would not result in repolymerization by disulphide bridging. An investigation of the effect of sulphite upon the electrophoretic properties of urease was therefore made; the results are reported in the next section.

(c) Electrophoresis of urease solutions in the presence of sulphite

Two sets of electrophoresis experiments were performed on samples previously analysed in sedimentation velocity experiments, one at ionic strength 0.10 to correlate the data with that previously determined in Table (II-2), and the other at lower ionic strengths in an attempt to obtain better resolution of the components present.[11] The data relevant to this discussion are tabulated in Table (IV-1) and the electrophoretic patterns obtained in the presence of sulphite ions shown in Fig. (IV-3). (The patterns reveal the presence of components with lower electrophoretic mobilities than those tabulated, and these correspond to the boundary with an S $\sim$ 4-6, which was present in detectable amounts in several samples. The identity was shown by preparing the leading components (boundaries 1, 2 and 3 in

Table (IV-1)

Relevant electrophoretic data on urease in the presence and absence of sulphite ions at pH 7.4_5 and at 1.0°

Expt. no.	Sample no.	Buffer[a] composition (molarities)	Ionic strength	Potential gradient volt cm.$^{-1}$	Descending[b] mobilities cm.^{2}sec.$^{-1}$volt^{-1} x 10^5			Proportions % electrophoretic[c]			sedimentation[d]		
					1	2	3	1	2	3	1	2	3
1	8	0.03 NaCl, 0.01 NaV, 0.02 HV	0.04	4.8	-8.9						82	13	5
2	8	0.013 Na_2SO_3, 0.01 NaV, 0.02 HV	0.05	5.6	-8.4	-9.8		25	75		100	0	0
3	9	0.09 NaCl, 0.01 NaV, 0.02 HV	0.10	2.9	-6.8						47	27	18
4	9	0.03 Na_2SO_3, 0.01 NaV, 0.02 HV	0.10	2.5	-7.1	-9.3	-9.8	20	(80)		100	0	0

[a] Notation as in Table (II-2). [b] The mobility values refer to the boundaries labelled in Fig. (IV-3).

[c] The bracketed figure in expt. no. 4 represents the total for boundaries 2 and 3: reliable resolution was not possible.

[d] The boundary numbering in this case is that of Table (II-3). For expt. no. 3 a fourth boundary with an $S_{20,w}$ of 41 was present to the extent of 8% of the total.

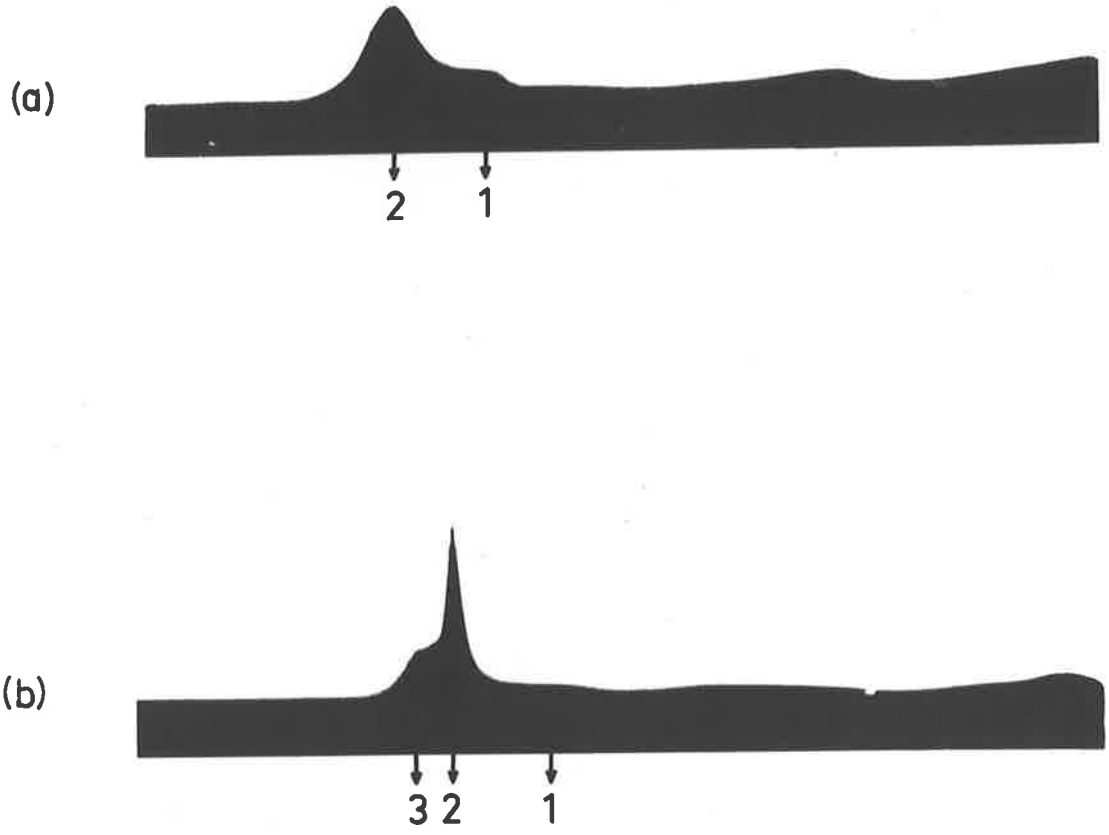


Fig. (IV-3). Electrophoretic patterns of urease solutions (descending limb) in the presence of sulphite ions at pH 7.4_5.

(a) Sample 8 in a diethylbarbiturate buffer containing sulphite ions, ionic strength 0.05 after 155 min. at a potential gradient of 5.6 volt $cm.^{-1}$

(b) Sample 9 in a diethylbarbiturate buffer containing sulphite ions, ionic strength 0.10, after 400 min. at a potential gradient of 2.5 volt $cm.^{-1}$

Fig. (IV-3)) in expt. no. 1 and 2 by electrophoretic fractionation and sedimenting: in each case the S 4-6 component was absent and the material sedimented as expected, cf. Fig. (II-4) and Fig. (III-1)). The following points in relation to the table require comment:

(i) the mobility values for the control experiments 1 and 3 were in excellent agreement with those previously reported. The value for expt. no. 3 could be compared directly and the value for expt. no. 1, when corrected by the method of Abramson, Moyer and Gorin, mentioned in Chapter II, became at ionic strength 0.10, -6.6×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} (i.e. within 3% of the value at ionic strength 0.10). The latter experiment will be discussed more fully in Chapter V;

(ii) the mobility values were generally less accurate than those reported earlier as the location of the maximum ordinate proved difficult in some cases. However, it was quite definite that in the presence of sulphite, electrophoretic components with a higher mobility existed, as well as components with a mobility essentially identical with that in the control experiment. (For expt. no. 2 the mobilities, corrected to an ionic strength 0.04 for comparison with expt. no. 1, became -9.0 and -10.5×10^{-5} cm.^{2}sec.$^{-1}$ volt^{-1}). The implication was that some molecules bore an increased charge due to thiosulphate groups, in agreement with the discussion above and that a smaller proportion existed in the original monomeric form with only -SH groups present. The diffuse nature of boundary 1 in Fig. (IV-3) suggested that the agreement of the mobility values with those in the control experiments may have been fortuitous. Therefore, conclusive proof of the existence of unmodified monomer cannot be claimed. Nevertheless, as all the electrophoretic components

represented by boundaries 1, 2 and 3 (prepared by electrophoretic fractionation) sedimented as one boundary, quite analogous to that in Fig. (III-1), the experiments represented a convincing demonstration of the micro-heterogeneity anticipated. The repolymerization evident on removal of the sulphite ions by dialysis was also in accord with the finding that not all the -SH groups had been converted to thiosulphate groups;

(iii) in view of this, it appeared at first sight that the mechanism suggested by equation (3-III) and discussed above was inapplicable, as it implied complete conversion to the thiosulphate form. However, closer inspection of the proportions (albeit approximate[12]) apparent from the two analyses revealed that the mechanism was still a possibility. For example, in expt. no. 1 only 20% of the urease was present as the polymeric form. Provided thiosulphate groups arose only by intermolecular disulphide bond cleavage, this represented the maximum percentage of urease with a higher mobility obtainable on treatment with sulphite. However expt. no. 2 showed that 75% of the protein moved with a higher electrophoretic mobility. This could be explained by cleavage of intramolecular disulphide bonds in urease, but only by assuming the existence of such groups and by further assuming that they were not important in determining the structural integrity of the molecule (which on the basis of sedimentation and activity measurements appears to remain intact). A more reasonable hypothesis is that the mechanism involving oxidation of all -SH groups is operative, but has not reached completion at the time of these experiments. Critical appraisal of this hypothesis requires more information on

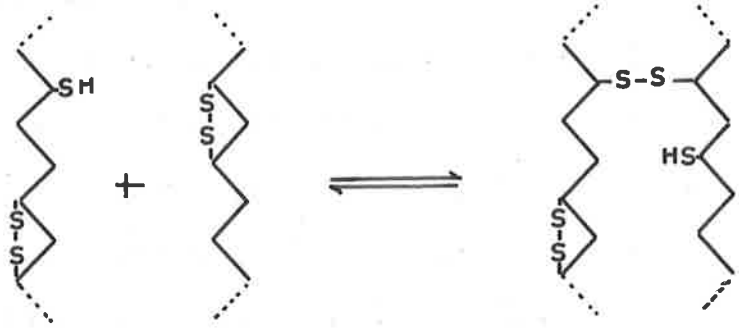
the reaction of sulphydryl groups in urease to form disulphide bonds, i.e. on the problem outlined at the beginning of this Chapter. Before this aspect is treated, one further observation can be made;

(iv) insertion of the mobility data in Table (IV-1) into the relations between valence and mobility given in Table (II-4) enabled the valence difference due to the apparent conversion of -SH to thiosulphate groups to be estimated. For example, at ionic strength 0.10 the valences of the molecules constituting boundaries 1, 2 and 3 of Fig. (IV-3) were -47, -62 and -65, respectively, on the basis of the first model selected and with the assumptions implicit in considering the second model were -53, -70 and -73. The maximum valence difference, presumably due to $-SSO_3^-$ groups, was therefore approximately -20 and represented the minimum number of -SH groups, originally present, capable of forming a disulphide linkage. The calculation was of course very approximate, but agreed with the estimation of Hellerman et al.[13] that 22 -SH groups of the type (a) were present on each molecule. At ionic strength 0.05, the valence difference was -7, and suggested that if complete conversion of all -SH groups to thiosulphate groups was possible, the experimental conditions in this case were less effective than in the former case. In short, the results suggest that numerous disulphide linkages were possible, and that the electrophoretic analyses of samples in which these bonds were cleaved would only be reproducible if all available -SH groups had been reacted. In this respect it was surprising that the micro-heterogeneity, evident especially in Fig. (IV-3b), was not more marked. However, it was entirely possible that each of the boundaries, and in particular boundary 1, was composed of species with small charge differences.

(3) The mechanism of the formation of a disulphide bond from sulphydryl groups.

(a) The mercaptan-disulphide interchange

The mechanism proposed by Huggins et al.[1] may be written schematically as,



(3-IV)

If the above formulation of an equilibrium reaction is correct, application of a 'pseudo-thermodynamic' reasoning leads to some interesting tentative conclusions. As the number and type of bonds are conserved, ΔH^{o} is probably approximately zero, and the equilibrium is essentially 'entropically' controlled. It follows that no variation in the relative proportions of the polymers should be observable on alteration of the temperature: this is in accordance with the results found with sample 7 in Table (II-3). However, changing the concentration of the protein should have an effect, and as we have seen in Chapter II, this does not appear to be the case. Moreover, if ΔS^{o} is large and negative (a reasonable assumption), the equilibrium constant would be correspondingly small, which suggests again in contrast to the results obtained that a very low proportion of the dimer, etc. would exist at the concentrations used. These results may simply reflect that the interchange is not an equilibrium reaction. Thus, although this type of interchange has been studied quantitatively for

simple molecules,[14-16] where a two step equilibrium mechanism is proposed, there appears to be no justification to extend the reasoning to protein systems, where in some instances (β-lactoglobulin and serum albumin) analogous behaviour is not observed.[17] The strongest argument against the operation of the interchange in these experiments is that the reaction almost certainly involves mercaptide ions rather than undissociated -SH groups,[15,16,18,19] the latter groups being present in this study.

(b) Direct oxidation

The formation of polymeric species by this mechanism may be represented as,

$$2P\text{-}(SH)_n \rightleftharpoons (HS)_{n-1}\text{-}P\text{-}S\text{-}S\text{-}P\text{-}(SH)_{n-1} + 2H$$

$$(HS)_{n-1}\text{-}P\text{-}S\text{-}S\text{-}P\text{-}(SH)_{n-1} + P(SH)_n \rightleftharpoons (HS)_{n-1}\text{-}P\text{-}S\text{-}S\text{-}P(S\text{-}S\text{-}P(SH)_{n-1})\text{-}(SH)_{n-2} + 2H \qquad (4\text{-IV})$$

where n represents the number of -SH groups per molecule and the presence of H on the right hand side of each equation emphasizes the fact that no oxidizing agent has been designated. In this case the most plausible oxidizing agent to suspect is atmospheric oxygen.[8,20] Considerable confusion exists as to the mechanism of the molecular oxidation,[8] but it seems clear that the reaction is strongly catalyzed by minute traces of metal ions, such as iron and copper. As the experimental technique employed in the preparation and handling of the samples did not exclude atmospheric oxygen and

possible traces of metal ions, the environment appears suitable for the operation of this reaction. In this connection, it is noteworthy that urease solutions containing copper ions are inactivated by contact with air: activity is restored on the addition of H_2S or KCN.[21,22] Moreover the objection levelled at the mercaptan-disulphide interchange regarding the state of ionization of the sulphydryl groups does not seem to apply to this mechanism (although there is some controversy[8]). Oxidation of several simple thiol compounds by atmospheric oxygen has been observed at pH values where few, if any, mercaptide ions exist.[6,23]

With regard to the equilibrium nature of the reaction, it is pertinent that, while concentration changes may not alter the relative amounts of the polymers present, a variation should be detectable on altering the temperature as ΔH^o would be finite. Therefore, while the operation of direct oxidation involving molecular oxygen appears to be a reasonable hypothesis it seems unlikely that the reaction is in equilibrium. If this conclusion proves correct, it is clear from the indecisive literature on the oxidation of simple thiols that extensions of the mechanism in more detailed terms (e.g. as steps involving free radicles[24]) would prove futile, until the simpler cases are more intensively investigated.

(c) The participation of molecular oxygen in the polymerization reaction

Three observations were made which indicated that the oxidation of -SH groups by molecular oxygen was important in disulphide bond formation in urease solutions, both in the presence and absence of

sulphite ions;

(i) with sample 4 the relative proportions of the components were unaffected by standing in solution for periods which ranged from 2 hr. to 30 days after the final recrystallization and initial solution. Thus, six sedimentation velocity analyses over this period revealed the amounts 90%, 10%, 0% for the components referred to in Table (II-3). The result did not appear compatible with the hypothesis above, which implied that provided molecular oxygen and traces of metal ions were available, the relative amounts of the higher polymers would increase with time. The disparity could be attributed to the presence of cysteine-hydrochloride, which was shown in Chapter III to affect the apparent relative proportions in an unpredictable manner (quantitative information on the equilibrium reaction (6-III) being unavailable).

It became of considerable interest therefore to repeat these experiments with a sample prepared in the complete absence of any cysteine-hydrochloride. The relative proportions of the components in sample 9, prepared in this way, were reported in Table (IV-1). The results were obtained two days after the final recrystallization. Oxygen was bubbled slowly and carefully through an aliquot of the solution which was then left to stand at room temperature for a further 24 hr. The subsequent sedimentation velocity analysis revealed the following percentages of the components, 37%, 27%, 21% and 15%. The decrease in the relative amount of the slowest sedimenting species and the increase in the relative proportions of some of the higher polymers, while small, supports the hypothesis under consideration. In these experiments a double sector cell was employed, thus eliminating errors in locating the position of the

base line in area determinations, and a phase plate replaced the usual inclined wire in the schlieren optical system, thereby improving the definition. Accordingly, the areas under the peaks (corrected for radial dilution) were more accurately determinable and hence the differences observed are significant. The oxygenated sample after a further three days had precipitated and sedimentation velocity analysis revealed the absence of the components under consideration. It was a general finding in this work that those solutions which were not directly oxygenated developed a faint turbidity and lost half their activity after about two months;

(ii) the complete exclusion of oxygen during the preparation of urease would provide a direct and elegant test of the hypothesis; but the experimental difficulties with the facilities available were considered prohibitive. (The alternative removal of metal ions by chelating agents would be less conclusive and in view of the recent findings of the effect of ethylenediaminetetra-acetic acid on disulphide bonds, perhaps undesirable[25]). However, a simple and analogous experimental approach follows from the observation that, even after 7 days in the presence of sulphite, urease was capable of repolymerization on removal of the sulphite by dialysis. The effect of the removal of sulphite ions from sample 8 has already been reported and this experiment served as a control to the one in which an aliquot of the solution was dialysed for the same time against the same phosphate buffer, which had been freed from traces of dissolved oxygen by bubbling oxygen free nitrogen through the boiling buffer. The dialysis, including several changes of buffer, was conducted in a dry box under nitrogen and the ultracentrifuge cell filled in the same environment.

Traces of oxygen in the commercial nitrogen used were removed by passing the gas through four consecutive bubbling towers containing chromous chloride. Sedimentation velocity analysis revealed, in contrast to the control experiment, only the $S_{20,w}$ 20 peak evident in the presence of sulphite;

(iii) the implication from the findings at this stage was that molecular oxygen could effect the complete oxidation of all sulphydryl groups to disulphide linkages in the presence or absence of sulphite ions, provided sufficient oxygen were available and enough time allowed. It follows that under these conditions complete conversion of the -SH groups to the thiosulphate form should be apparent on electrophoresis in the presence of sulphite ions. To test this hypothesis sample 9 (cf. expt. no. 4 of Table (IV-1)) was recovered from the electrophoresis cell and dialysed against the same buffer, which was periodically oxygenated, for a further two weeks and subjected to electrophoresis. The leading boundary was a single symmetrical peak (instead of boundaries 2 and 3 in Fig. (IV-3b)) with a descending mobility of -9.8×10^{-5} cm.^{2}sec.$^{-1}$volt^{-1}, with no evidence for a boundary with a mobility corresponding to boundary 2. This supported the general hypothesis of ultimate conversion to the thiosulphate form. However, the diffuse boundary 1 was apparently unaltered both with respect to proportion and approximate mobility, and, therefore, represented an anomalous finding, if the simple hypothesis were correct. The persistence of this boundary, which, as has been emphasized could not unequivocally be attributed to the enzyme existing in the -SH form, might have been due to electrostatic binding of small traces of metal ions to negatively charged $-SSO_3^-$, resulting

in species with a range of mobilities less than -9.8×10^{-5} cm.^{2} sec.$^{-1}$volt^{-1}; but this explanation is extremely tentative.

In spite of the anomaly, the evidence in Table (IV-1) and the experiments above, directly employing molecular oxygen, appear overwhelmingly in favour of a direct oxidation of the sulphydryl groups of urease to form disulphide bonds, rather than a mercaptan-disulphide interchange mechanism. In the presence of sulphite, it appears that the same mechanism operates and cleavage of the bonds formed results largely in a single species possessing $-SSO_3^-$ groups rather than -SH groups.

(4) Further studies.

Considerable emphasis has been given to the single peak observed in sedimentation velocity studies in the presence of sulphite ions. While the identification of the species with that in the sulphite free solutions has been partially justified, no evidence regarding the possible heterogeneity of the species, other than that due to charge differences has been adduced. Since such information on the extent of the heterogeneity would be very desirable, two further series of studies have been made on urease. The first of these consisted of an examination of its behaviour in free-diffusion experiments, and the second of a detailed analysis of the boundary-spreading in sedimentation velocity experiments. Both of these fields have somewhat complicated theoretical backgrounds, a fact which leads to the use of rather elaborate symbolism in expressing the results. Accordingly, the next chapter (on the diffusion studies) and Chapter VI (on boundary spreading) each commence with a brief

summary of basic theory relevant to the topic considered.

In conclusion a third general method of attacking the problem must be mentioned. This is the starch-gel electrophoresis procedure of Smithies,[26-28] which has been so successful in resolving the minor components of serum. Its application to the urease system has not yet been explored.

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CHAPTER V

THE APPLICATION OF THE DIFFUSION METHOD TO UREASE

SECTION A Essential Theory

(1) General outline. The choice of the Rayleigh optical system.

(2) Analysis of Rayleigh interferograms for three component systems.

(a) The estimation of heterogeneity. Definition of the Ω_R function and the deviation plot.

(b) The determination of the "height area" diffusion coefficient average, D_A.

(c) The determination of the concentration dependence of the diffusion coefficient.

SECTION B The Diffusion of Urease

(1) Introduction

(2) The diffusion of the polymeric mixture. Consideration of the size of the polymers.

(3) The diffusion of the sulphite modified form of urease.

(4) The molecular weight of the modified form of urease.

SECTION A Essential Theory

(1) General Outline.

When a sharp boundary is formed in a cell between solutions of two different concentrations, the macroscopic flow of components which results due to differences in chemical potential is termed diffusion.[1] In modern diffusion experiments the progress of this mass transport with time is followed by using either Gouy or Rayleigh optical systems:[2-12] the former gives essentially the refractive index gradient distribution, and the latter, the refractive index versus vertical cell coordinate in terms of interference fringes. In this study, the Rayleigh optical system was employed exclusively and accordingly further discussion will be restricted to the analytical procedures applicable to this type of interferogram.

Creeth and Gosting[11] have formulated equations, which allow analysis of Rayleigh interferograms to be made in terms of two solutes, estimates being obtained of the ratio of the diffusion coefficients and the relative proportions on a refractive index basis. The potential value of this procedure to protein diffusion experiments is immediately apparent. Provided the salts, constituents of the necessary buffering medium, are assumed to be part of the solvent rather than additional solutes, the protein solution may be considered as a three component system, consisting of the solvent, the main protein component and a single impurity. The Creeth and Gosting treatment permits an evaluation of a "height area" diffusion coefficient average and of the extent of the heterogeneity in terms of a fringe deviation diagram. Moreover, curve fitting procedures

have been described,[11,12] which enable the diffusion coefficient of the main protein component at the mean concentration of the experiment to be estimated. A correct and meaningful interpretation of the data in terms of these quantities relies on the following assumptions:

(a) each solute component diffuses independently: i.e. no interaction of flow between the components present;

(b) the diffusion coefficient of each component, D_i, is either constant or a linear function of the concentration of the component of the form,

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

C_i is the concentration of the i'th component and $\overline{C}_i$ the mean concentration, given by $\overline{C}_i = \frac{1}{2}[(C_i)_A + (C_i)_B]$, where the subscripts A and B refer to the concentration of the component i in the two phases between which the boundary is formed;

(c) the refractive increment, $\Delta n/\Delta C$, of each solute component is either constant or depends linearly on its concentration and is independent of other components. This means that the refractive index, n, of the solution will be given by the expression,

$$n = n(\overline{C}_1,\ldots.\overline{C}_q,) + \sum_{i=1}^{i=q} R_i(C_i - \overline{C}_i)[1 + a_i(C_i - \overline{C}_i)] \qquad (2\text{-V})$$

where $n(\overline{C}_1,\ldots.\overline{C}_q,)$ is the refractive index of a solution in which all solutes have a concentration $\overline{C}_i$ equal to their mean concentration. R_i and a_i are coefficients characteristic of each of the q solute components (the solvent being excluded).

The applicability of these assumptions will be carefully examined later. Before this, however, it is necessary to outline briefly the Creeth and Gosting treatment of three-component systems:[11]

only those equations and functions are discussed which are directly applicable to the experimental sections.

(2) Analysis of Rayleigh interferograms for three-component systems.

(a) The estimation of heterogeneity:

A typical Rayleigh interferogram is shown in Fig. (V-1). Each minimum between the fringes is assigned a number, j, from j = 0, in the region of homogeneous solvent to j = J, in the region of homogeneous solution: J, then, is the total number of fringes and is therefore a measure of the total refractive index change across the boundary. The distance between minima with particular j values is measured on a series of photographs taken at suitable times. The data so obtained are used to define two functions, $H(z^*)$ and $H(v_j)$, which are identical only in the case of a single solute. The origin of the two functions will now be briefly outlined.

(i) $H(z^*)$

This is defined by the relation,

$$f(j) = \frac{2j - J}{J} = H(z^*) \qquad (3\text{-V})$$

where z^* represents the ideal normalized displacement for the fringe numbered j. The symbol H is used throughout this section for the probability integral.

$$H(z) = \frac{2}{\sqrt{\pi}} \int_0^z e^{-\beta} \, d\beta \qquad (4\text{-V})$$

The $H(z^*)$ value for each fringe is readily obtained from a count of the fringes in the interferogram.

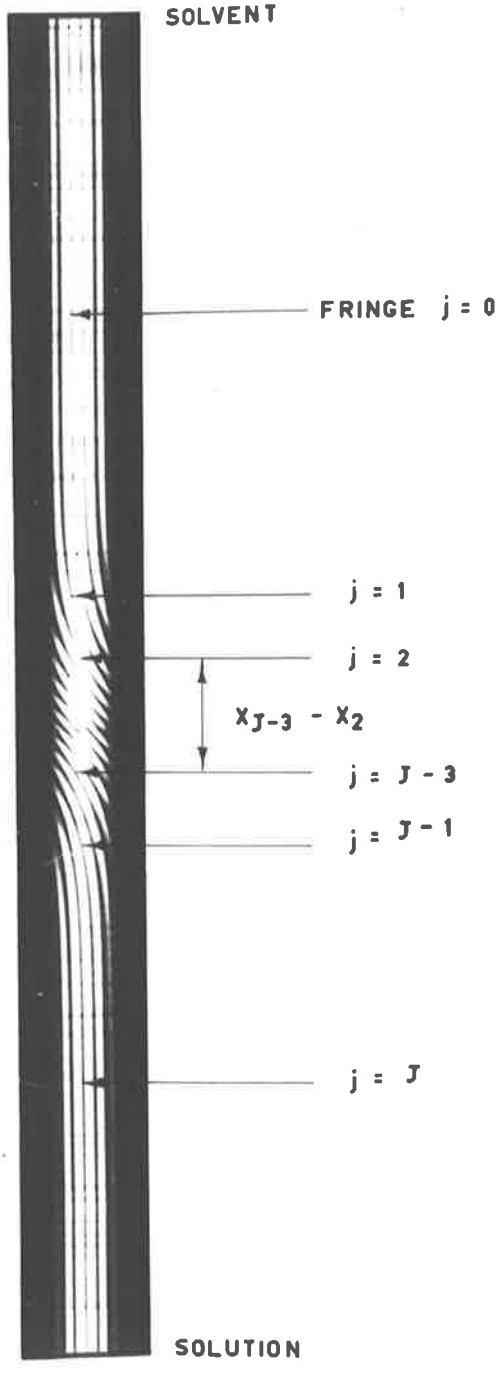

Fig. (V-1). A Rayleigh interferogram of a diffusing boundary: the method of fringe numbering is illustrated.

(ii) $H(v_j)$

It is convenient to mention first the function $H(z_A)$. This is the probability integral for z_A, the experimentally observed reduced displacement for the fringe j, in terms of the "height area" diffusion coefficient average D_A:

$$z_A = \frac{x}{2\sqrt{D_A t}} \qquad (5\text{-V})$$

where x is the 'actual' displacement of fringe j at time t from the initially sharp boundary at t = 0, and D_A is defined by the relation,

$$D_A^{-\frac{1}{2}} = \sum_{i=1}^{i=q} a_i \left(D_i^{-\frac{1}{2}}\right) \qquad (6\text{-V})$$

where a_i is the refractive index proportion of the i'th solute, whose diffusion coefficient is D_i.

$$a_i = \frac{\Delta n_i}{\sum_{i=1}^{i=q} \Delta n_i} \qquad (7\text{-V})$$

In contrast to $H(z^*)$, the function $H(z_A)$ cannot be obtained directly, because x (see equation (5-V)) cannot be measured. This difficulty is overcome by measuring the separations between fringes symmetrically placed about the J/2 position: in this way first order concentration-dependence effects are eliminated.[10] However, in a concentration-dependent system, it cannot be assumed that the distance between two symmetrical fringes is equal to twice the distance of both from the initially sharp boundary position. Consequently, it is only possible to obtain an estimate of z_A, termed v_j.

$$\nu_j = \frac{X_{J-j} - X_j}{4M\sqrt{D_A t}} = \frac{x_{J-j} - x_j}{4\sqrt{D_A t}} \qquad (8\text{-V})$$

where X is an arbitrary comparator reading. Accordingly, the difference function, Ω_R, may be evaluated from the quantities actually measured.

$$\Omega_R = H(\nu_j) - H(z^*) \qquad (9\text{-V})$$

In the rigorous derivation of Ω_R, values obtained in this way have been shown to be free of first order concentration-dependence effects.[11]

Values of Ω_R are plotted against the corresponding $[H(z^*)]^3$ values (chosen so that the limiting slope of the resultant curve is finite as $H(z^*) \longrightarrow 0$), to give a deviation plot. These diagrams, illustrated by Fig. (V-2), give values of Ω_R, the relative fringe deviation in terms of a probability integral, over the whole boundary. Curve fitting procedures have been devised to analyse this curve in terms of the relative concentrations and the ratio of the two diffusion coefficients of the two solute components. The relevant equation for the case of a slightly impure solute is

$$\Omega_R \sim a_2 F(z^*, \sqrt{r_2}) \qquad (10\text{-V})$$

where $r_2 = D_1/D_2$. (a_1 and D_1 denote the refractive index proportion and diffusion coefficient of the main component, respectively, and a_2 and D_2 the corresponding quantities for the impurity.) $F(z^*, \sqrt{r_2})$ is a complicated function and has been tabulated for values of z^* and r_2.[11] It is implicitly assumed in equation (10-V) that only a small amount of impurity is present and therefore any terms involving a_2^2

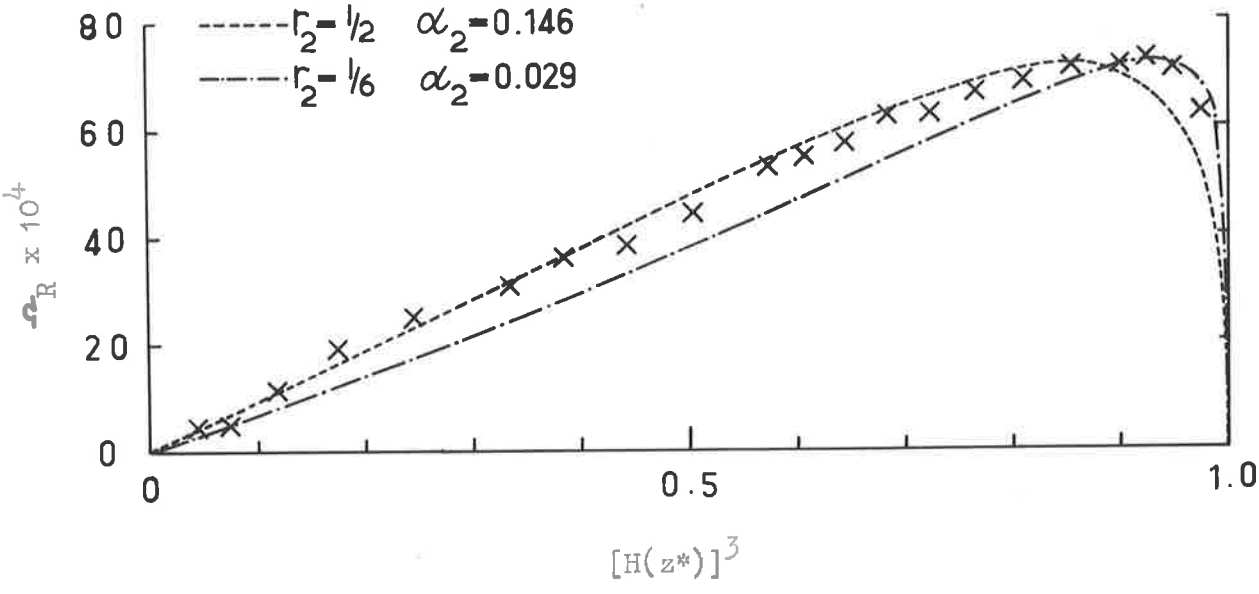

Fig. (V-2). A typical 'deviation plot', the crosses representing the mean observed deviations, whilst the lines are attempts at curve fitting.

are omitted. If this is the case, the abscissa position of the maximum of the deviation plot is determined essentially by r_2 and, consequently, an approximate value of r_2 can be found by inspection of the table of the $F(z^*,\sqrt{r_2})$ values. A corresponding value of a_2 can be estimated directly from equation (10-V) using the maximum value of Ω_R. As the position of the maximum is not very sensitive to variation in r_2, it is not possible to fit the observed deviations to a unique pair of values of a_2 and r_2. Instead the curve fitting procedure is used to define an upper and lower limit for r_2: this is illustrated in Fig. (V-2), where the corresponding values of a_2 are also given. In addition, by combining equations (5-V) and (6-V) and employing the relation $a_1 = 1 - a_2$ it follows that

$$D_1 = D_A \left[1 + a_2 \left(\sqrt{r_2} - 1\right)\right]^2 \qquad (11\text{-}V)$$

and thus the diffusion coefficient of the main component can be given within the limits defined for r_2.

(b) <u>The determination of D_A</u>

The evaluation of v_j and hence Ω_R requires one other quantity D_A. To obtain D_A, in practice, use is made of the function Y_t, defined as,

$$Y_t = \frac{X_{J-j} - X_j}{-2\, z_j^*} \qquad (12\text{-}V)$$

where X_{J-j} and X_j are arbitrary comparator readings for the symmetrical fringes J-j and j. The quantity $Y_t/\sqrt{t}$ is time independent and when plotted against $(z^*)^2$ gives an extrapolated value as $z^* \longrightarrow 0$, which can be directly employed to evaluate D_A.

$$D_A = 1/4M^2 \left[\left(Y_t/\sqrt{t}\right)_{g^* \to 0}\right]^2 \qquad (13\text{-V})$$

M is the magnification from cell to photographic plate. The extrapolation of $Y_t/\sqrt{t}$ in principle is unsatisfactory as it uses progressively smaller fringe separations: accordingly, an extensive averaging procedure using photographs taken at different times is employed. (Similarly an average value of v_j is also obtained.) The experimentally calculated value of the "height area" diffusion coefficient average is commonly referred to as $\mathcal{D}_A$[13] to emphasize that it will only equal D_A[14] in the absence of flow interaction effects and second order concentration-dependence effects.[10,15]

Any variation in $Y_t/\sqrt{t}$ or v_j with time is a clear indication that the diffusing solute is changing with time and, in this respect, provides a useful check on the validity of diffusion experiments involving labile materials.

(c) <u>The determination of the concentration-dependence of the diffusion coefficient</u>

As has been mentioned, measurements for the determination of Δn_R and D_A are made between fringes symmetrical about the J/2 position because it can be shown that such measurements are independent of first order concentration-dependence effects. If unsymmetrical fringe separations are taken, these effects and others due to higher terms are observed. Provided second and higher order effects are absent and the refraction increment of the main solute component is independent of the concentration, the skewness of the boundary due to the concentration dependence of the diffusion coefficient can be

calculated using the relation,

$$\Delta z_A - \Delta z^* - \Delta\left(\frac{\Omega_R}{H'(z^*)}\right) = -k_1\left(\frac{\Delta C_1}{2}\right)\Delta R(z^*) + \ldots \qquad (14\text{-V})$$

where Δz_A is obtained by pairing any two unsymmetrical fringes, k and l, i.e.

$$\Delta z_A = (x_k - x_l)/2\sqrt{D_A t} \qquad (15\text{-V})$$

$\Delta\left(\frac{\Omega_R}{H'(z^*)}\right)$ is obtained by pairing the corresponding values of $\frac{\Omega_R}{H'(z^*)}$, where H' denotes the derivative of the error integral. $R(z^*)$ is a function tabulated by Creeth.[10] A plot of the function on the left hand side of equation (14-V) against $\Delta R(z^*)$ gives a straight line, the slope of which enables k_1 to be determined. It is, thereby, theoretically possible to determine k_1 from a single experiment. Thus a value for the diffusion coefficient at zero protein concentration, D_o, may be obtained.

D_o may be related directly to the molar frictional coefficient, f. Thus, if one considers the diffusion of a single solute, of concentration C, in a two-component system, an expression for the diffusion coefficient, D, may be derived by the methods of thermodynamics of irreversible processes.[16]

$$D = RT/f\ [1 + C d\ln\gamma/dC] \qquad (16\text{-V})$$

where γ is the activity coefficient of the solute. At zero solute concentration this equation reduces to

$$D_o = RT/f \qquad (17\text{-V})$$

Nevertheless the skewness results in this study were not used to

evaluate D_o; but were interpreted only in a qualitative manner (see experimental section).

SECTION B The Diffusion of Urease

(1) Introduction

A necessary preliminary to the application of the diffusion method to urease was a study of a 'model' protein system, where the major assumptions could be tested. Earlier work[12] has given plausible analyses for several proteins, but in each case, the experiments were done at the isoelectric point. However, the diffusion of urease (or more particularly, the modified derivative present at pH 7) involves species bearing a net negative charge. In order to check the effect on the results of using charged species, a series of experiments have been made on ovalbumin: these are summarized (together with some earlier work[17] on the diffusion of ovalbumin at the isoelectric point) in Appendix 1 as they do not form part of the main thesis. Reference to this Appendix will, however, be made frequently in the following sections.

(2) The diffusion of the polymeric mixture

Some attempts were made to obtain information on an average diffusion coefficient and the extent of heterogeneity (in terms of species with differing diffusion coefficients) of samples of urease in the absence of any reducing agent. The outline of one experiment will suffice to illustrate the type and limitations of the results obtained. Sample 2 was dialysed against a 'Veronal' buffer, pH 7.5, ionic strength 0.10 and diffused in a Rayleigh diffusiometer. In

this experiment, where J = 64.66, the sharpness of the initial boundary was judged satisfactory as the zero time, Δt, was 334 sec., a value comparable in magnitude to those cited in Table (A-1). The experimentally observed diffusion coefficient ($\mathcal{D}_A = 1.8 \times 10^{-7}$ cm.^{2}sec.$^{-1}$ at 20.00°, corrected to water) was much lower than the value of 3.46×10^{-7} cm.^{2}sec.$^{-1}$ found by Sumner et al.,[18] who diffused urease in the presence of sulphite ions. This discrepancy is to be expected if the species with sedimentation coefficients greater than 20 correspond to larger molecular weight units with smaller diffusion coefficients.

The nearly symmetrical deviation plot, shown in Fig. (V-3a), was very much greater in magnitude ($\Omega_{R\ max.} = 680 \times 10^{-4}$) than any previously reported using this analysis procedure, including all those presented in Appendix 1. In this case, any attempt at curve fitting would undoubtedly be quite misleading. In particular, it should be emphasized that the experimental conditions do not approximate to the diffusion of a <u>slightly</u> impure solute in an otherwise two-component system, and, therefore, equation (10-V) would be inapplicable even if higher order terms were considered.

In short the results qualitatively agree with the premise, founded on sedimentation velocity analyses, that urease samples in the environment chosen are a mixture of macromolecular components of different molecular weight; but no estimate can be given of the relative proportions on a refractive index basis nor of the relative diffusion coefficients. It is pertinent to note, therefore, that all transport experiments, with the exception of electrophoresis,

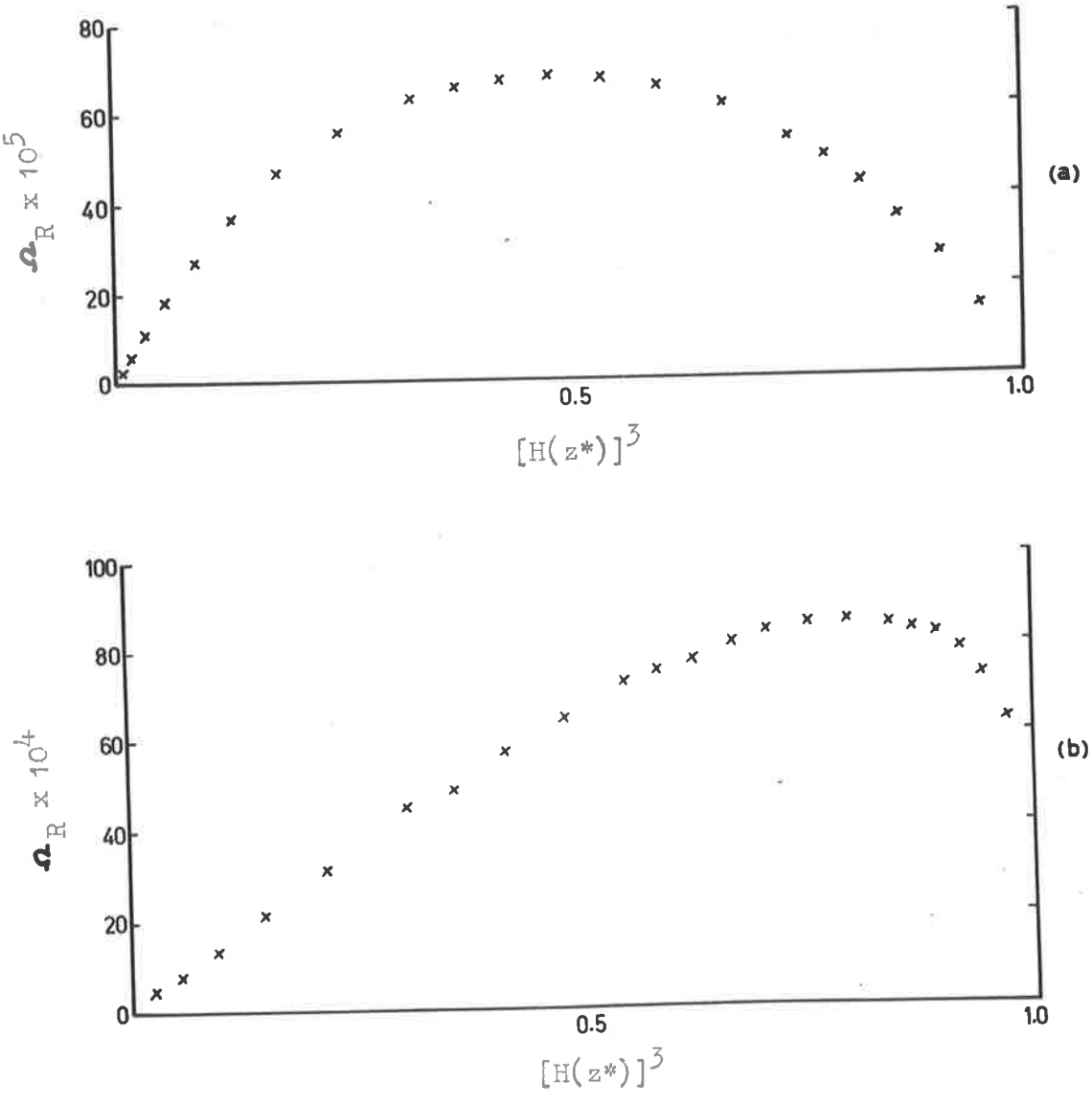

Fig. (V-3). Fringe deviation diagrams for urease, the crosses representing mean observed deviations.

(a) Sample 2 in 'Veronal' buffer, pH 7.4_5, ionic strength 0.10,

(b) sample 6 in a phosphate buffer containing sulphite ions (pH 7.0_9, 0.048M Na_2HPO_4, 0.021M KH_2PO_4, 0.031M Na_2SO_3, 0.013M $NaHSO_3$).

have indicated the complexity of the composition of samples of urease.

It was of interest as a consequence to study electrophoretically a similar sample employing conditions where better resolution might be achieved. In Table (IV-1), expt. no. 1, analytical and electrophoretic data were presented for sample 8: Fig. (V-4) shows the electrophoretic pattern obtained. When compared with Fig. (II-3), obtained at a higher ionic strength, the slight resolution of the leading boundary in Fig. (V-4) is apparent. The resolution into three peaks (labelled 1, 2 and 3 in order to descending negative mobility) did not permit any quantitative estimation of the relative amounts of the components; but at least in a qualitative manner the proportions indicate that boundaries 2 and 3 could correspond to the dimer and 'trimer' postulated previously. On the basis of the reasoning given in Chapter II, the resolution would be expected to be poor even under favourable conditions, if the models used approximated to the actual case. In fact the ratio of the descending mobility values for boundaries 1, 2 and 3 was approximately 1:0·9:0·8. It was, therefore, compatible with the hypothesis of a monomer-dimer-etc. system (for example, a change from a spherical monomer to a dimer of axial ratio 4 would give a mobility ratio of $1:0{\cdot}8_9$). The possibility of impurities with relatively low mobilities constituting boundaries 2 and 3 was eliminated by electrophoretically fractionating the three leading boundaries, 1, 2 and 3, and sedimenting. The centrifuge pattern showed the usual three fast sedimenting peaks in the ratio 80%:15%:5% and no trace of any slower moving boundary.

Further confirmation is required before the hypothesis concerning the size of the polymers resultant on disulphide cross-

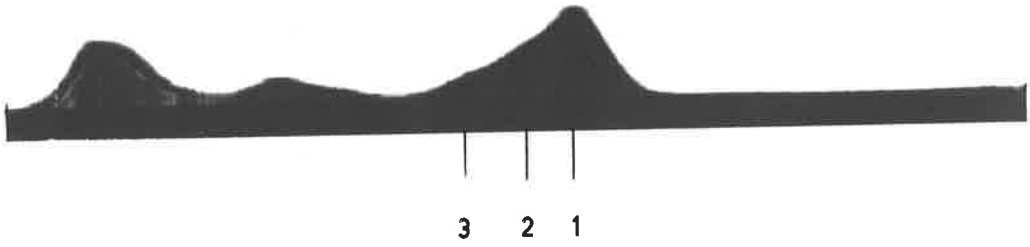

Fig. (V-4). Electrophoretic pattern (ascending limb) of urease, sample 8 in 'Veronal' buffer, pH 7.4_5, ionic strength 0.04 after 166 min. at a potential gradient of 4.8 volt $cm.^{-1}$

linking can be accepted. Fundamentally this would involve the evaluation of the molecular weight of each of the species, and in this respect the determination of a diffusion coefficient for each would be invaluable. The diffusion experiment outlined in this section, together with the conclusions presented in Appendix 1, serves to emphasize the inability to treat the data with existing theories to obtain this information directly. An alternative approach would be to employ the Archibald method[19] to obtain a weight average value for the ratio of sedimentation coefficient to diffusion coefficient, and hence a weight average molecular weight. Provided the molecular weight of the monomer could be assessed, ultracentrifugal analysis of the sample and a refractometric determination of the initial concentration would provide the additional information required. It remains, however, to establish the molecular weight of the monomer before the procedure could be applied. The addition of sulphite has been shown to produce a species of urease which appears to be identical, except for minor chemical differences, to the monomer in the polymeric mixture. Therefore, it is logical to focus attention on this species, to assess the molecular weight, to test the homogeneity and to confirm that it represents the monomeric form of urease. (In view of a few unsupported suggestions in the literature[20,21] concerning the possible existence of low molecular weight units of urease ($\sim$ 10,000), the latter point requires careful examination before the term monomer can be categorically assigned to the $S^{o}_{20,w}$ 20 component). The diffusion of the sulphite modified form of urease will now be discussed.

(3) The diffusion of the sulphite modified form of urease

A diffusion experiment on sample 6 ($J = 110.84$) in a buffered sulphite solution (0.048 Na_2HPO_4, 0.021M KH_2PO_4, 0.031M Na_2SO_3, 0.013M $NaHSO_3$), pH 7.0$_9$, gave the observed diffusion coefficient, $\mathcal{D}_A$ of 3.28×10^{-7} cm.^{2}sec.$^{-1}$ at 20.00°, corrected to water. The sharpness of the initial boundary was again judged satisfactory as the Δt value was 165 sec. There appeared to be no variation in the diffusing solute with time: $Y_b/\sqrt{t}$ and v_j were time independent.

The deviation graph, Fig. (V-3b), was markedly smaller in magnitude than that shown in Fig. (V-3a): it should be noted that the scales chosen for the vertical axes are different. The striking contrast is illustrative of the pronounced effect of sulphite on the system. Nevertheless in comparison with other results, for example those presented on ovalbumin in Appendix 1, the $\Omega_{R\ max.}$ value is relatively large. Consequently, the following factors should be carefully considered.

(a) As the diffusion experiment is conducted with dialysed solutions, and the protein bears a net negative charge, a negative increment of salt must be superimposed on the positive concentration increment of protein at the start of the experiment. Provided that the flow of salt (due to this Donnan concentration gradient) proceeds in the manner described in Appendix 1, consideration of its effect would give a larger value for $\Omega_{R\ max.}$. Moreover the shape of the resultant curve would be altered: the maximum would presumably be shifted to the right. In this case, however, no attempt was made to assess the magnitude of the buffer gradient inherent on dialysis

as the assumptions involved in assigning a charge to the urease species present were considered prohibitive.

(b) The results on ovalbumin provided evidence indicative of a flow of buffer salts resultant on the flow of protein. This effect was reflected as a positive deviation in the fringe deviation diagrams. In addition, it appeared that larger deviations could be expected if the charge on the protein were increased. It is likely that similar electrostatic coupling of ion flows occurs in the urease experiments. Consequently it may be implied by analogy that the observed deviations could be attributed, in part, to flow interaction effects.

(c) A distinct possibility exists that a small amount of impurity unrelated to urease was present in the sample. In several ultracentrifuge patterns, particularly those found using samples 4, 8 and 9, the refractive index gradient observed near the meniscus after 30 min. was too large to be accounted for by the slight redistribution of buffer salts evident in control experiments. Accordingly it was attributed to a macromolecular impurity of S 4-6. In these samples the presence of electrophoretic components with low mobilities (cf. Fig. (IV-3)) was associated with this impurity. In all probability it was largely concanavalin B, a protein with an S of ~ 4,[22] which crystallizes slowly from Jack Bean Meal extracts.[23] The identification was supported by the examination, using a microscope, of the precipitate obtained by standing solutions of these samples at 0° for several days. Long needle-like crystals were observed and ultracentrifugal analysis showed that the supernatant

solution contained a reduced amount of the S 4-6 component. The persistent appearance of concanavalin B in certain samples even after two recrystallizations must, it appears, be attributed to the well known variable nature of the meal, as several samples prepared from different batches of meal appeared virtually free of this contamination. This was the case with sample 6 which was used for the diffusion study.

In view of these considerations it is clear that little can be deduced concerning the homogeneity of the sulphite modified form of urease from the diffusion studies. Certainly, the observed deviations cannot be taken as decisive evidence that urease species exist with different diffusion coefficients.

(4) The molecular weight of the sulphite modified form of urease

It also follows from the above considerations that the value of $\mathcal{D}_A$ (which agrees quite closely with that found by Sumner et al.[18] under identical conditions) is not the diffusion coefficient appropriate to the Svedberg molecular weight expression (cf. the exact form of this expression given by Baldwin[24]). Therefore, the results do not allow a reliable value of the molecular weight to be derived. If the extrapolated sedimentation coefficient in sulphite, $S^{o}_{20,w}$, is combined with the observed $\mathcal{D}_A$ (also in sulphite) the apparent value of the molecular weight is 550,000 ($\bar{V}$ being taken as 0.73[18]). This differs from the presently accepted value of 480,000 mainly because Sumner et al.[18] did not attempt to extrapolate their sedimentation data. However, the value of 550,000 is almost certainly incorrect and the uncertainty emphasizes the inadvisability

of placing undue reliance in calculations involving the molecular weight.

A better procedure to evaluate the molecular weight would be to combine $S^{o}_{20,w}$ with a similarly extrapolated $\mathcal{D}_A$, if such a value were available. The limitations of the diffusiometer available in these laboratories prohibited the direct application of equation (14-V) (see experimental section). A series of diffusion experiments at different protein concentrations would thus be required to obtain the extrapolated value. In addition, the Svedberg expression in its simplest form applies strictly to two-component systems and will thus be valid only so far as the system urease-buffer-sulphite-water approximates to such a system. However it could be applied with some confidence provided the protein (free of any impurity) was electrically neutral and the concentration of the buffer did not exceed 0.1M.[25,26] A preliminary investigation on the effect of lowering both the ionic strength and the pH of the solutions is therefore desirable. The latter point is of particular interest as possible flow interaction effects would be reduced as the charge on the protein decreased, and the value of $\mathcal{D}_A$ would correspondingly approximate more closely to the value required.

The survey on some of the complications involved in the determination of a reliable molecular weight from diffusion and sedimentation measurements shows clearly the need for more diffusion studies and subsidiary investigations on the effect of variation of environmental conditions. The interpretation of both of these studies would be materially facilitated if the homogeneity of the sulphite

modified form of urease could be demonstrated by some other means. This aspect is considered in the next chapter.

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CHAPTER VI

THE HOMOGENEITY OF THE SULPHITE MODIFIED FORM OF UREASE

(1) A theoretical outline of boundary analysis.
   (a) The definition of the apparent sedimentation coefficient distribution, $g^*(s)$.
   (b) Extrapolation procedures to infinite time.

(2) The distribution of sedimentation coefficients in samples of urease in the presence of sulphite. Experimental and results.

(3) The effect of lowering the pH and ionic strength.
   (a) The effect of pH. Results and discussion.
   (b) The effect of ionic strength.

(1) A theoretical outline of boundary analysis.

Analysis of the boundary gradient curves observed in sedimentation velocity experiments is in principle capable of giving a distribution of the sedimentation coefficients of the molecular species present in terms of the proportion of each species in the solute.[1] In practice the determination of such a distribution is complicated by several factors.

First, radial dilution and the increase of gravitational field with distance from the centre of rotation affect the shape of the experimental schlieren pattern. Signer and Gross[2] have formulated an equation which relates any observed refractive index gradient boundary curve to an apparent sedimentation coefficient distribution, $g^*(\delta)$: the distribution obtained is free of these effects. The function $g^*(\delta)$ is defined as follows:

$$g^*(\delta) = \frac{dn}{dx} \frac{\omega^2 t\, x^3}{\Delta n\, x_o^2} \qquad (1\text{-VI})$$

where dn/dx = the refractive index gradient at x

ω = the angular velocity

t = the time from the commencement of sedimentation

Δn = the total refraction increment of the solute at $t = 0$

x_o = the position of the meniscus

x = distance of a point in the boundary from the centre of rotation.

The value of δ (a reduced coordinate with units of sedimentation coefficient) may be determined for each point in the

boundary by employing the integrated form of the definition of a sedimentation coefficient.[3]

$$\delta = \frac{\ln(x/x_o)}{\omega^2 t} \qquad \text{(2-VI)}$$

A plot of δ values versus the corresponding $g^*(\delta)$ values gives a normalised distribution, which represents the apparent distribution of the sedimentation coefficients of the sedimenting species on a refractive index concentration scale. However the apparent distribution will reflect the actual heterogeneity of the sample only if diffusion and concentration-dependence effects are negligible and the specific refractive increment of each species is constant. More elaborate procedures must be employed when any of these effects are pronounced.

The effect of diffusion, when significant, may be eliminated from the apparent distribution by extrapolation of $g^*(\delta)$ values for a number of δ values at different times during a sedimentation experiment to infinite time.[4-6] This procedure utilizes the fact that the spreading of the boundary due to diffusion is proportional to the square root of t, whereas the spreading due to the difference in sedimentation coefficients of the species present is directly proportional to xt. Thus where diffusion is a significant effect, one has the apparent paradox of the <u>sharpening</u> of the $g^*(\delta)$ - δ curves with time: this occurs because the effects of diffusional spreading become <u>relatively</u> less important as time increases, until they disappear altogether at infinite time. The distribution obtained will be the distribution of sedimentation coefficients

with the effects of concentration dependence superimposed. The latter effects may in turn be eliminated by procedures which assume linear, and known, dependence of sedimentation coefficient on concentration.[7-9] Alternatively sedimentation experiments at a series of different concentrations can be performed and the diffusion and dilution independent distributions extrapolated to zero concentration.[10,11]

The possibility of the existence of little or no heterogeneity in the modified samples of urease is very strong. Moreover, the reasonably large value of $\mathcal{D}_A$, reported in the preceding chapter, suggests that the diffusion effect may be large. In such cases considerable care is required in selecting a suitable extrapolation procedure to eliminate the diffusion effect.[6] Baldwin[6] has shown that the calculation of the standard deviation, p, of the sedimentation coefficient distribution from 6^2, the second moment about the mean $\bar{x}$ of the boundary gradient curve, would be more meaningful under these circumstances. A detailed equation has been obtained expressing the time dependence of the second moment of the gradient curve about the mean, which applies to materials showing a linear dependence of S on C,[9] and reduces to a simpler form if the dependence is negligible.[10] The expression has been applied in a more approximate form[12] and provided a suitable test for the homogeneity of the sample; but the application relies on the estimation of the linear dependence of S on C. In view of the results plotted in Fig. (IV-1) this procedure is not strictly applicable to the case of urease. As an alternative Baldwin[13] has devised an extrapolation procedure to infinite time, which applies particularly to cases where the effects of diffusion are

large and the heterogeneity of the sample is small. The method is essentially empirical, but was successfully applied to sedimentation patterns obtained with hog thyroglobulin (which has similar macroscopic properties to urease). A brief outline will now be presented of the data required for the extrapolation.

From each boundary gradient curve values of the function $(\delta - \bar{s})^2$ are found for particular values of $g^*(\delta)/g^*(\delta)_{max.}$ (e.g. 0.2, 0.4, 0.6 and 0.8). In essence this involves the choice of a suitable value of dn/dx from the experimental record and the evaluation of δ from the corresponding value of x: in practice a graphical interpolation procedure[13] is employed. Sets of values may be obtained from both the trailing and leading edges of the boundary, and, in this respect, the method is superior to other approaches, where equations must be applied to the whole boundary curve. The value of $\bar{s}$ is found from the rate of movement of the position corresponding to the square root of the second moment of the boundary curve. The procedure is applied to several exposures in the one experiment, taken as sedimentation proceeds. Baldwin suggested that a plot of the $(\delta - \bar{s})^2$ values against $1/te^{\bar{s}\,\omega^2 t}$ should extrapolate to the origin (the point 0,0), if the material were homogeneous and other effects were negligible. Consideration of these latter complications will be deferred until the extrapolation procedure has been applied to urease.

(2) <u>The distribution of sedimentation coefficients in samples of urease in the presence of sulphite.</u>

The refractive index gradient boundary curves chosen for analysis were obtained by sedimenting different concentrations of

sample 6 in a buffered sulphite solution (0.048 Na_2HPO_4, 0.021M KH_2PO_4, 0.031M Na_2SO_3 and 0.013M $NaHSO_3$). All experiments were performed at 47,660 r.p.m., at a temperature within 0.2° of 20°.

Values of $g^*(\delta)$ and $\delta_{20,b}$ were found from each of four exposures, taken at regular time intervals in the one experiment: three separate experiments, performed with different protein concentrations, were analysed in this way. (The symbol $\delta_{20,b}$ is used to emphasize that the values of δ, found at 20°, have not been corrected to water). Fig. (VI-1) shows a typical result of plotting $g^*(\delta)$ against $\delta_{20,b}$ for four exposures of the one experiment. Examination of the normalized distributions reveals that they are nearly symmetrical. Also, it is apparent that the range of $\delta_{20,b}$ values progressively decreases from 12.6 Svedbergs at the earliest time (dashed curve) to 7 Svedbergs at the longest time (solid curve). As we have seen, the distributions are only <u>apparent</u> because diffusion and the dependence of S on C have been ignored. The reasonably large value of $\mathcal{D}_A$ and the progressive decrease in the range of $\delta_{20,b}$ values with increasing time suggests that the spreading of the boundary due to diffusion is the most important of these factors (see p. 107). Accordingly, the data were subjected to the method outlined by Baldwin.[13]

Values of the function $(\delta - \bar{s})^2$ were found from every exposure at the fixed values of $g^*(\delta)/g^*(\delta)_{max.}$ of 0.2, 0.4, 0.6 and 0.8. Although both sides of the boundary were analysed in this way, the corresponding values of $(\delta - \bar{s})^2$ proved to be essentially identical. The position corresponding to the square root of the

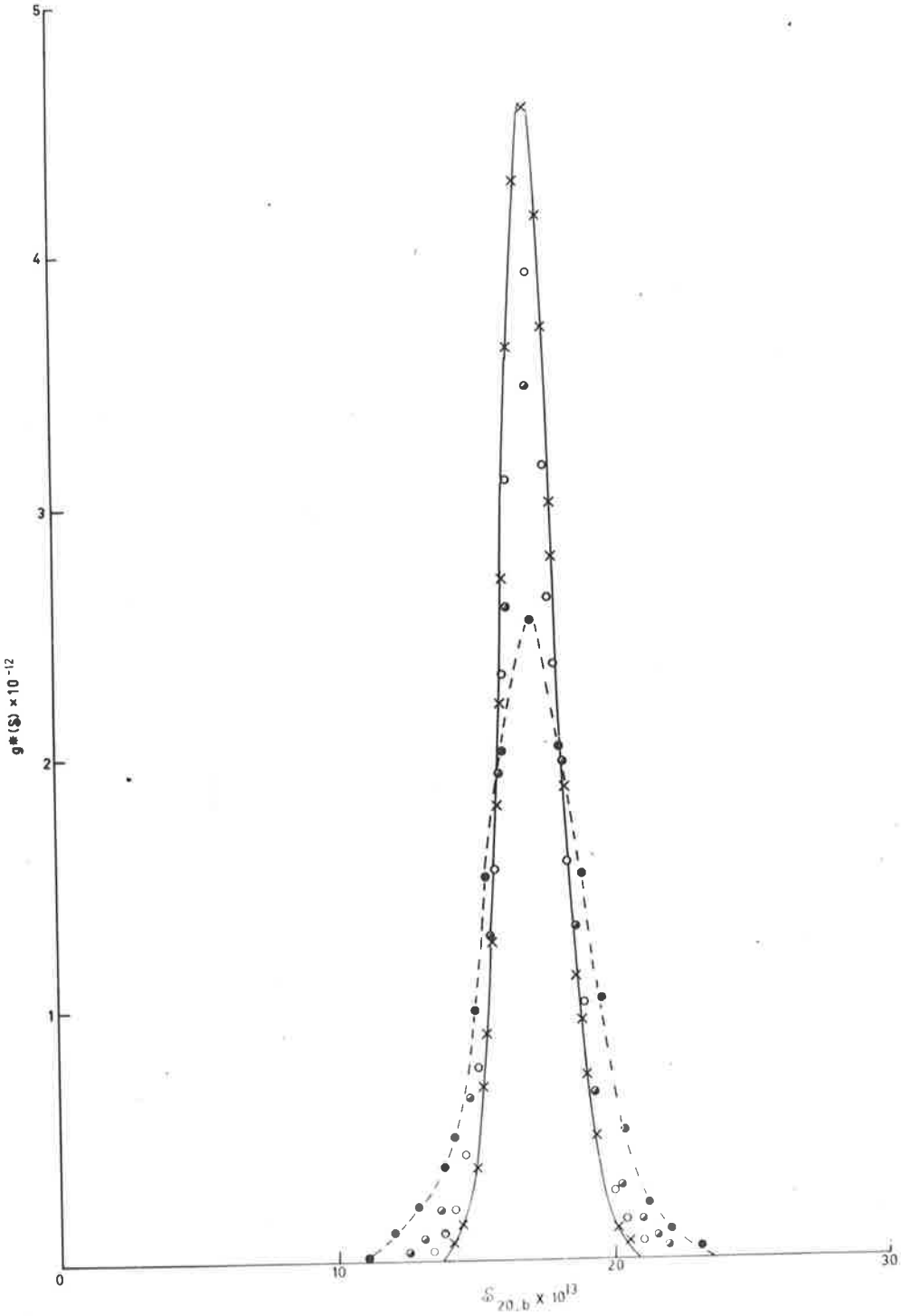

Fig. (VI-1). Apparent normalized sedimentation coefficient distributions of urease in phosphate buffer containing sulphite ions. Protein concentration was 80 fringes (~ 0.47%). The distributions were found at different times in the same experiment: crosses, t = 2,381 sec.; open circles, t = 1,901 sec.; half filled circles, t = 1,421 sec.; filled circles, t = 941 sec. The lines (solid and dashed) represent attempts to average the data for the two extreme times.

second moment of the boundary gradient curves was used to define $\bar{s}$ for each experiment. Only a 0.5% difference existed between these values and those found either by averaging δ values (calculated at several positions across the boundary) or by employing the rate of movement of the maximum ordinate. A plot of $(\delta - \bar{s})^2$ against $1/te^{\bar{s}\omega^2 t}$ for an experiment where J = 80 is shown in Fig. (VI-2a). Other results found with lower protein concentrations were of the same type. The attempted extrapolation (indicated by the dashed line) is somewhat unsatisfactory as the experimental points lie on a curve. However it shows clearly that the values of $(\delta - \bar{s})^2$ closely approximate to zero at infinite time. Thus it may be concluded that the sulphite modified form of urease does not show any measurable heterogeneity when subjected to this test. This conclusion, however, is subject to two important limitations.

First, it must be stressed that the concentration dependence of the sedimentation coefficient has not been considered: this effect may tend to mask any slight heterogeneity. However, experiments were chosen where the sedimentation coefficient was within 10% of its value at infinite dilution.[13] Moreover the extrapolation procedure provided additional evidence that this complication would not markedly affect the validity of the conclusion. Fig. (VI-2b) shows the effect of varying the protein concentration and is typical of other results of the same type. The curves are displaced downward with increasing protein concentration, in accordance with expectation. The curvature is not markedly different, and the curves extrapolate to the origin.

Secondly, the apparent homogeneity of the sulphite modified

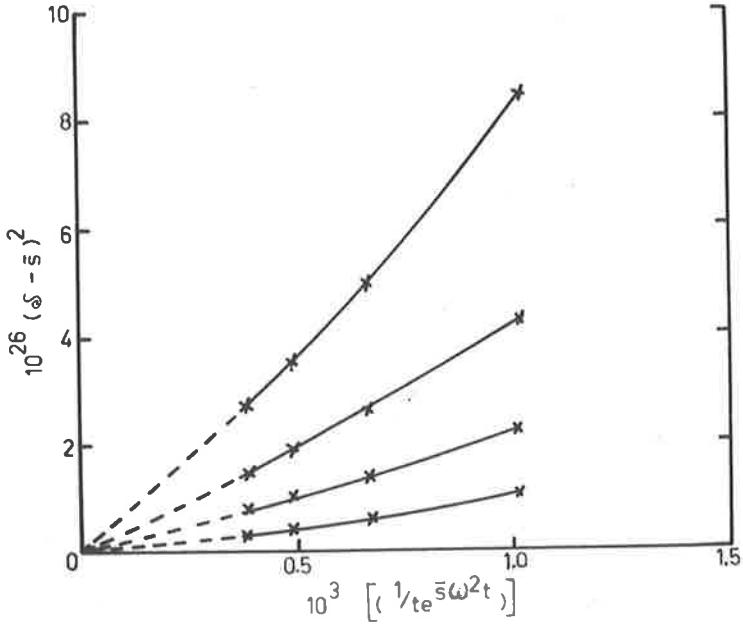

Fig. (VI-2a). The extrapolation procedure to infinite time, employed in studying urease. The crosses show $(\delta - \bar{s})^2$ as a function of $1/te^{\bar{s}\ \omega^2 t}$, at fixed values of $g^*(\delta)/g^*(\delta)_{max.}$ (0.2, 0.4, 0.6, 0.8) on the trailing side of the boundary. The protein concentration in this experiment was 80 fringes.

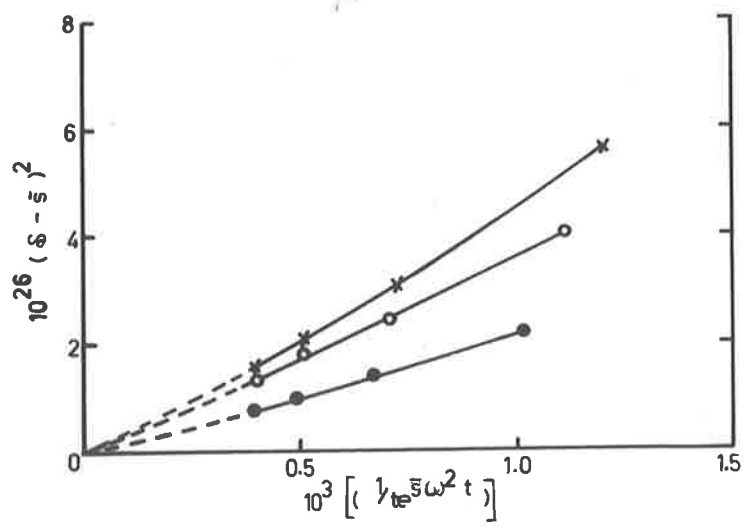

Fig. (VI-2b). The extrapolation procedure at varying concentrations of urease; crosses, J = 17; open circles, J = 31; filled circles, J = 80. The value of $g^*(\delta)/g^*(\delta)_{max.}$ is 0.6.

form of urease has been demonstrated only under one set of experimental conditions. An investigation of the effect of altering the environmental conditions led to some unexpected results, which are pertinent to this point.

(3) The effect of lowering the pH and the ionic strength of solutions of urease in the presence of sulphite.

(a) The effect of pH

Sample 5 was dialysed against an acetate buffer, pH 5.6_2, ionic strength 0.16 (0.07M sodium acetate, 0.03M acetic acid, 0.03M sodium sulphite). The solution (which had precipitated slightly) was sedimented: the result is shown in Fig. (VI-3a). Two peaks with $S_{20,w}$ values of 12.1 and 18.7 Svedbergs are evident. The effect was repeated with sample 8, both in the presence and absence of the S 4-6 impurity. The solution for the latter experiment was prepared by the electrophoretic fractionation described. Moreover in each case the $S_{20,w}$ 12.1 component remained on re-dialysis to pH 7.

The implication of these findings is that a portion of the urease, in the presence of sulphite, does not remain intact at pH values in the acid range, but is irreversibly changed. The presence of impurities does not prevent this alteration. It seems probably that the appearance of the slow sedimenting component at pH 5.6 is associated with the nucleophilic cleavage of an intramolecular disulphide bond, which is not 'available' for reaction at higher pH values. The recent work of Cecil and Loening[14] on the action of sulphite on the disulphide bonds in insulin provides valuable material for comparison. In the proposed structure for insulin[15] three disulphide

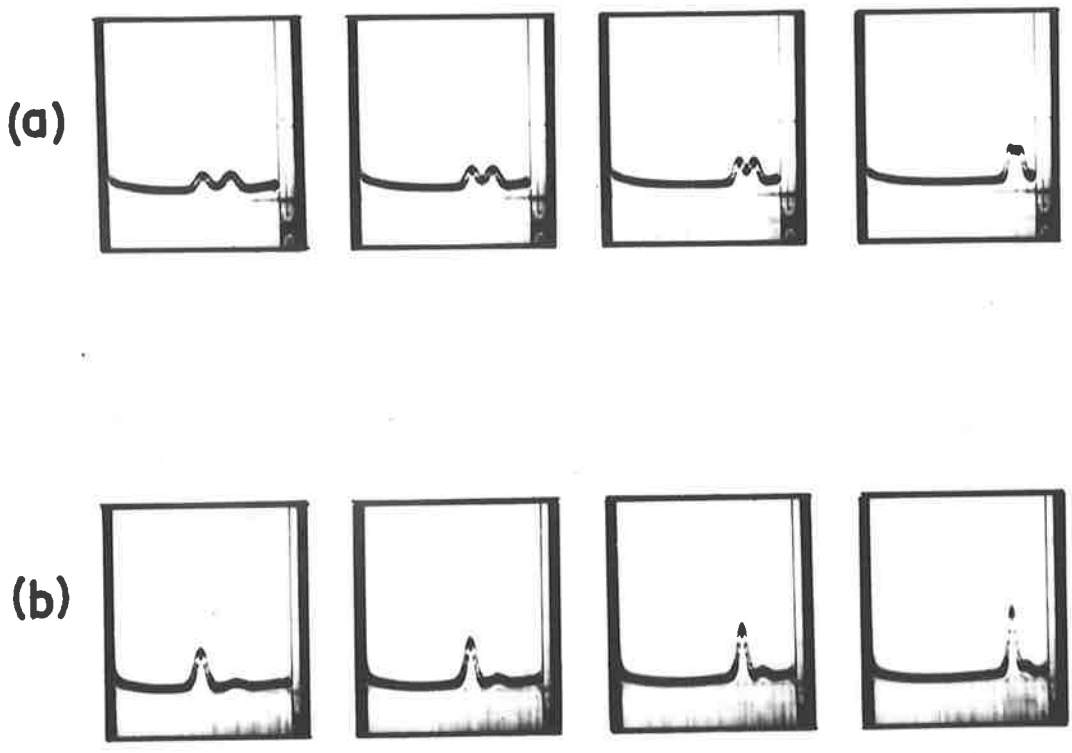

Fig. (VI-3). Sedimentation diagrams of urease in buffered sulphite solutions (sedimentation from right to left).

(a) Sample 5 in acetate buffer (ionic strength 0.16, pH 5.6_2, 0.07M sodium acetate, 0.03M acetic acid, 0.03M Na_2SO_3),

(b) sample 6 in phosphate buffer (pH 7.0, 0.003M Na_2HPO_4, 0.001M KH_2PO_4, 0.03M Na_2SO_3).

bonds exist: two join the A and B chains and the third forms an internal ring between residues 6 and 11 in the A chain. Each of these bonds could be cleaved by sulphite provided the experimental conditions were carefully selected: marked differences were also observed in the reactivity of these bonds. In brief it was found that the major factor governing the effectiveness of the nucleophilic attack by sulphite (or possibly, bisulphite[16] at low pH values) was the net charge in the vicinity of the bond. On this analogy, therefore, it is proposed that one or more intramolecular disulphide bonds exist in the $S^{o}_{20,w}$ 20 component of urease in a negatively charged environment at pH 7. When the pH is lowered, the net charge approaches zero or a positive value and the approach and attack of sulphite is facilitated. The reason why only some of these bonds are apparently cleaved under these conditions is obscure. Similar observations[14,17] have not been interpreted: for example, Cecil and Loening found that 2.5 disulphide bonds in insulin were cleaved by sulphite in the pH range 3-6 in the presence of phenyl mercuric hydroxide, but could not formulate an explanation.

The structural nature of an intramolecular disulphide bond in urease is of considerable interest. It appears unlikely that it constitutes part of an intra-chain ring structure similar to the 6-11 ring in insulin. This configuration appears particularly stable, the stability being attributed to hydrogen bonding.[14] Thus the intra-chain disulphide linkage in insulin (which finds analogues in bovine serum albumin[17] and ribonuclease[19]) was only cleaved by sulphite in the presence of reagents such as urea and guanidine.[14,18] In view of these results, it appears more probable that the bond is of the

inter-chain type. If this were the case, cleavage could result in actual dissociation into sub units. Alternatively the marked internal structural change could permit internal rotation of fragments of the molecule without actual dissociation taking place.[20] The molecular weight would remain constant but the frictional ratio could be increased, causing a reduction in the observed sedimentation coefficient. Sedimentation studies on fumarase[21] in the presence of thiocyanate have been interpreted, in part, by this theory. However this explanation must be considered with caution in this case, where there is a pronounced reduction in the value of the sedimentation coefficient for part of the material.

The evidence available does not permit a definite choice to be made between the three possibilities; but the cleavage of an inter-chain bond resulting in dissociation into sub units appears favoured. If the hypothesis proves correct, the apparently anomalous value of the molecular weight for urease found by Setlow[22] using a deuteron bombardment technique may find an explanation. The cleavage of disulphide bonds by ionizing radiation is currently being investigated[23] and a possible connection between these studies forms a question of considerable interest.

(b) <u>The effect of ionic strength</u>

Sample 6 was dialysed against a series of phosphate buffers containing sulphite of progressively decreasing ionic strength: the phosphate and sulphite concentrations were either lowered independently or together. The result of sedimenting solutions of

lower ionic strengths than previously reported is illustrated by Fig. (VI-3b). The similarity of the pattern with Fig. (VI-3a) is apparent: both show a component with an $S_{20,w}$ of 12. It is unlikely that a structural change in urease occurs at low ionic strengths exposing an inter-chain bond, which is subsequently cleaved by sulphite, as the S value of the main component remains unaltered. Moreover the $S_{20,w}$ 12 component was not observed when 'Veronal' buffer containing sulphite, pH 7.45, ionic strength 0.05 was used as the solvent. It is possible that electrostatic interactions and specific ion binding (both important in kinetic studies on urease[24,25]) should be invoked to explain the observation. However the results available are too inconclusive for this to be justified.

(c) Discussion

The observation of the appearance of an $S_{20,w}$ 12 component under certain conditions has two important implications.

(i) Further studies on the homogeneity of the sulphite modified form of urease are limited by the restricted choice of environmental conditions. Certainly the samples could not be considered homogeneous if either the total salt concentration or the pH of the solutions were lowered. Accordingly diffusion studies are not possible under conditions which are theoretically more favourable to obtain data suitable for a molecular weight determination of the monomeric form. Therefore the evaluation of a more reliable molecular weight (and the solution of the related problem concerning the size of the polymers resultant on disulphide cross linking) would be better approached by methods insensitive to a charge on the macromolecule and to reasonably high salt concentration.

Furthermore, it should be noted that even if a reliable value were available considerable caution would be required in utilizing it in kinetic calculations. These frequently rely on other data found over wide ranges of pH and ionic strength.

(ii) It follows that the nature of the $S_{20,w}$ 12 component must be investigated before full use can be made of the available kinetic data. The fundamental point to be established is whether it constitutes a 'sub-unit' of urease possessing enzymic activity different from the parent material. The work of Kistiakowsky and Thompson[25] appears particularly relevant to this point as it involves a comparison between the activity of urease (in the presence of the S-nucleophilic base, H_2S) stored at pH 7 and at pH 5.3. They found (cf. Fig. 1 or ref. 25) that over a period of 24 hr. the activity of solutions stored at pH 7 was always less when the assay was performed at pH 4.3 rather than at pH 7. This decrease in activity in the acid range agreed with previous findings.[24,26,27] Kistiakowsky and Thompson emphasized, however, that it could not entirely be attributed to equilibria between the active neutral and inactive acidic forms of the enzyme. It appears plausible that the effect could partly be explained by the nucleophilic cleavage of an inter-chain bond at pH 4.3, resulting in the formation of an $S_{20,w}$ 12 component: in view of the results in Chapter III it seems reasonable to assume that sulphide and sulphite act on the disulphide linkage in a similar manner at low pH values. It is implicit in this explanation that the $S_{20,w}$ 12 component must possess a reduced activity (or be completely inactive). If this were the case, the enzyme stored at pH 5.3 should exhibit a lower activity when assayed at pH 7 than a control sample stored and assayed

at pH 7, since the formation of the $S_{20,w}$ 12 material was shown to be irreversible. The findings of Kistiakowsky and Thompson[25] support this conclusion: for example, after 24 hr. the sample stored at pH 5.3 possessed only 45% of the activity of a control sample stored at pH 7, when tested under identical conditions.

The explanation of these kinetic observations on the basis of a low molecular weight 'inactive' form of urease must remain tentative at this stage. However it clearly indicates the necessity of a thorough investigation of the $S_{20,w}$ 12 material. In many ways this problem is similar to that encountered at the beginning of this work: both involve a mixture of species of urease with different activities. Again the most suitable approach would be to isolate a single species (in this case the $S_{20,w}$ 12 material) and characterise it. However, it was felt that such a study would form a separate topic since the primary objective in this study was to examine the $S_{20,w}$ 20 component, referred to as urease, and the faster sedimenting material commonly associated with it.

A short summary will now be given of the general findings of this study and their relation to other data concerning the mechanism of the urease action.

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CHAPTER VII

GENERAL CONCLUSIONS

It has been shown that urease molecules interact in solution to form polymeric species. The values of the sedimentation coefficients of the polymers were compatible with those expected for association to dimers and possibly trimers: electrophoretic evidence was in accord with this hypothesis. The polymerization reaction involved the oxidation of readily available sulphydryl groups to form intermolecular disulphide bonds. It has been inferred that the sulphydryl groups oxidized were not those directly concerned with the active site. Nevertheless the polymers exhibited a reduced enzymic activity which has been attributed to steric hindrance effects.

Consequently, kinetic results found with such polymeric mixtures cannot readily be interpreted. This is particularly true as the polymerization reaction appeared to involve molecular oxygen: the relative amounts of species with different activities would be expected to alter if solutions were either let stand or diluted with buffer containing dissolved oxygen and traces of metal ions. The anomalous and non-linear dependence of enzymic activity on concentration observed by some workers is illustrative of the complications which ensue when such factors are not controlled. It is well known that considerable care is required in interpreting kinetic results obtained with a system in reversible equilibrium. Sedimentation data indicate that the urease interaction is not of this type; but similar caution is necessary in treating data found with this variable system.

The formation of an enzymically active monomeric form is therefore especially significant. This was achieved by the nucleophilic scission of the intermolecular disulphide bonds with reagents

such as sulphite and sulphide. The physico-chemical properties of the sulphite modified form of urease were examined.

Three separate items of evidence (identity of the $S^{o}_{20,w}$ values, activity measurements and the repolymerization resultant on removal of the sulphite by dialysis) indicated that this form was essentially identical to the monomer in the polymeric mixture. Electrophoresis showed, in fact, that minor chemical differences existed: the presence of thiosulphate groups, $-SSO_3^-$, in the modified form was supported and micro-heterogeneity was observed. However, the material appeared homogeneous when subjected to a test designed essentially to determine the sedimentation coefficient distribution in the sample. This result, therefore, contrasted with diffusion results where a finite deviation graph was observed. However, the latter finding was shown to be of doubtful significance as at least flow interaction effects and the buffer gradient inherent on the dialysis of the charged species were not considered. An approximate value of the molecular weight was calculated and comment made on its accuracy.

It appeared that this stable form of urease was suitable for use in kinetic studies provided the environmental conditions were carefully chosen. In the author's opinion, therefore, the extensive work of Kistiakowsky and coworkers is particularly valuable. The consistent reproducibility of their results and the observation that the enzymic activity varied linearly with concentration emphasize the advisability of employing an S-nucleophilic base in solutions of the enzyme. Nevertheless, their final conclusion that two types of

active site exist with different Michaelis-Menten parameters requires careful scrutiny. The conclusion was drawn, in part, from results obtained at low pH values. It has been shown that a marked structural change (which may result in actual dissociation) occurs under these conditions.

CHAPTER VIII

EXPERIMENTAL SECTION

SECTION A Materials

(1) Urease preparations.

(2) Ovalbumin preparations
   (a) the crystalline complex,
   (b) the fractionated A_1 component.

(3) Buffers
   (a) pH values,
   (b) conductivity values,
   (c) relative viscosity and density data.

SECTION B Methods and Apparatus

(1) Enzymic activity measurements.

(2) Electrophoresis.

(3) Sedimentation,
   (a) the determination of sedimentation coefficients,
   (b) the determination of relative areas and concentrations in terms of J values.

(4) Diffusion,
   (a) techniques,
   (b) the modification and testing of the diffusiometer.

SECTION A Materials

(1) Urease preparations.

The nine samples of urease used in this study were prepared by the acetone fractionation method of Sumner[1] from defatted Jack Bean Meal, obtained from the Sigma Chemical Co., St. Louis, Missouri. In each case 1,000 g. of meal were stirred for 10 min. with 5 litres of a 32% acetone water mixture (by volume) at 28°[2] and filtered twice through one thickness of Whatman No. 1 filter paper. The filtrate was cooled to approximately 3° and 100 ml. of redistilled acetone (analytical reagent quality) at the same temperature were added slowly and carefully in an attempt to readjust the acetone concentration, after the evaporation losses incurred during the 12 hr. filtration at room temperature.[3] After 3 days in the cold the filtrate was treated according to the procedure outlined in flow sheet (II-2), with the exception that the final recrystallization[4] was repeated.

Glass distilled water previously passed through both anionic and cationic exchange columns was used throughout the experiments to avoid accidental inhibition of the enzyme by traces of metal ions. All glassware was cleaned using a nitric and sulphuric acid bath at 80°, rinsed thoroughly and steamed.[5,6] However these precautions could not be claimed to be absolutely effective in eliminating all traces of metal ions. Indeed the specific activity of the samples varied from less than 10,000 to 80,000 S.U./g., this being quite typical of other findings.[3,7,8] Moreover the variability of the specific activity with sample

apparently could not be attributed entirely to the different degree of polymerization, as reversal of the polymerization (see Table (III-1)) did not provide a sample with the apparent[9] maximum specific activity of 100,000 S.U./g.[10] Therefore the distinct possibility of metal ion impurities remained.

The uncertain results of the measurement of the enzymic activity emphasized the necessity of an alternative analysis procedure to identify the species present and to estimate concentrations; accordingly all samples were sedimented in the ultracentrifuge, while the total protein concentration was found refractometrically (see below).

With the exception of nos. 5 and 9, all samples were stored in solution in the presence of cysteine-hydrochloride. After two months, these solutions developed a faint turbidity and the activity in some cases had decreased by 50%.[8] Consequently, the sample was discarded after one month (during which time the solutions remained clear and maintained their activity reasonably well). The urease solutions in the presence of sulphite, on the other hand, were colourless and remained active for longer periods of time.

(2) Ovalbumin preparations.

(a) The crystalline complex

Crystalline ovalbumin, sample 1, was obtained from fresh hens' eggs by the ammonium sulphate fractionation technique outlined by LaRosa.[11] The twice recrystallized sample was analysed electrophoretically in a 'Veronal' buffer (10.30 g. of sodium diethyl-

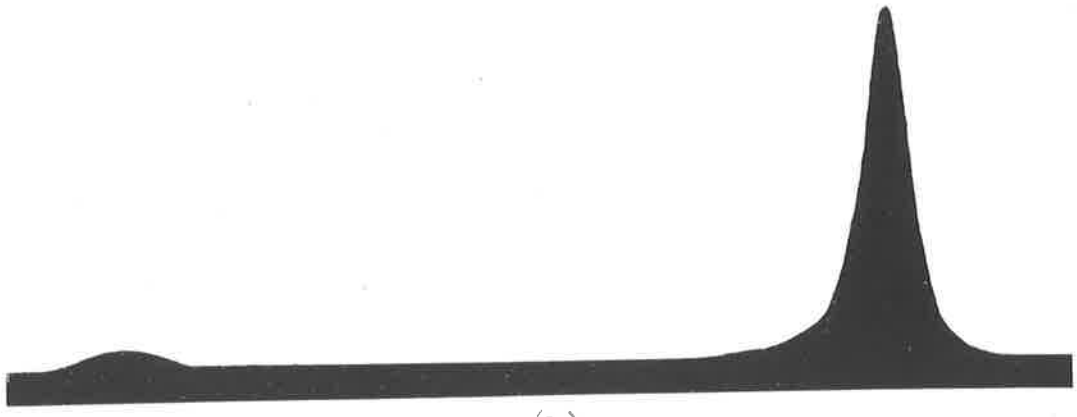
barbiturate, 1.85 g. of diethylbarbituric acid per litre, ionic strength 0.05, pH 8.7$_0$). The resulting pattern is shown in Fig. (VIII-1a): the apparent proportions of the components resolved in this photograph were approximately 83% of A_1 and 17% of A_2 (including presumably the small amount of A_3). Electrophoretic analysis of ovalbumin samples 2 and 3, prepared by an identical procedure, revealed essentially the same relative proportions of A_1 and (A_2 + A_3), but in addition approximately 1% of a component with a lower electrophoretic mobility (presumably representing a globulin impurity). As further recrystallization did not remove this impurity, preparative electrophoresis (see below) was employed and the resulting solutions shown to be free of the trace of globulin by electrophoresis. The relative proportions were in good agreement with the literature values,[12] but the separation of the components under the analytical conditions is not good and this proportion cannot be considered to be accurately determined.

Each sample was stored as a paste containing much ammonium sulphate at -15°. Even after 18 months, electrophoretic analysis of sample 3 showed no change and the method of storage was accordingly judged satisfactory.

(b) The fractionated A_1 component

The leading electrophoretic component of ovalbumin, designated A_1, was obtained by a preparative electrophoretic technique. Perlmann[13] reported the preparation of A_1 by an electrophoretic fractionation but no details of the method or yield were given. The method used in this study can be conveniently divided into two

(a)



(b)

Fig. (VIII-1). Electrophoretic patterns (ascending limb) of the ovalbumin complex and fractionated A_1 at pH 8.7_0 in diethylbarbiturate buffer ionic strength 0.05 after 3 hr. at a potential gradient of 6.2 volt $cm.^{-1}$ (a) ovalbumin complex, (b) fractionated A_1.

stages.

(i) The removal of the δ boundary

To obtain A_1, electrophoresis must be continued for long times and this involved a compensation technique which pushed the δ boundary into the bottom section of the cell and ultimately into the descending limb. To avoid the possibility of convective disturbance in the ascending limb, initial experiments were made following the procedure of Charlwood,[14] in which the δ boundary is completely removed by increasing the buffer salt concentration in the upper solution. It was for this reason, as well as the better resolution,[15] that the value of 0.05 was chosen as the ionic strength of the buffer against which the protein solutions were dialysed. 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 hr. duration of the experiment. This partial removal of the δ boundary required a preliminary electrophoresis experiment on the 2% ovalbumin solution which had been dialysed against the 'Veronal' buffer for three days, using the dialysate as supernatant buffer, a count of the δ boundary fringes and an estimation of the supernatant excess from a previously determined graph. This 2% ovalbumin solution required about a 15% excess concentration as the supernatant buffer, the actual value not being critical.

(ii) The 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 rest of the apparatus was filled with the buffer of the appropriate excess

concentration. Electrophoresis, using a current of 8 ma., was commenced as soon as the boundary was formed at the bottom of the centre section and was continued for 24 hr., with intermittent compensation by the removal of liquid from the descending limb in order to keep the ascending boundary in view. When sufficient separation of A_1 and A_2 had been obtained, compensation was continued until only A_1 remained in the ascending limb. Electrophoresis was stopped and after sectioning off the bottom section, the protein in the ascending limb was removed. The maximum yield for such a preparation was 16%, no more being obtainable due to the convection in the descending limb. To obtain sufficient protein for a diffusion experiment giving the recommended 100 fringes, four such preparative experiments, each yielding approximately 6.5 ml., were performed consecutively. The purity of every sample prepared in this way was checked by electrophoresis, a typical pattern being shown in Fig. (VIII-1b).

(3) Buffers.

The buffer solutions were prepared from analytical reagent grade materials; their compositions have been given in the body of the text. The protein (urease or ovalbumin) was either dissolved directly in the cold buffer and dialysed, or a minimum five day dialysis, in the cold, was employed to exchange different buffers, keeping the protein in solution. Necessary data on the buffer solutions were obtained by the following methods.

(a) pH values

A Doran Universal pH meter was used employing a glass

electrode and a saturated calomel half cell as the reference electrode. The values obtained have been quoted in the text.

(b) Conductivity values

The values needed for electrophoretic mobility calculations were measured at the temperature of the experiment, using a Philips' Conductivity Bridge, PR 9500, and a 2 ml. conductivity cell (Philips PR 9512/01) of cell constant 1.28.

(c) Relative viscosity and density data

These were measured directly, using, respectively, an Ostwald viscometer and a 25 ml. Pyrex pyonometer. The values obtained are summarized in Table (VIII-1). These values were employed in the correction of sedimentation velocity and diffusion data (see later).

SECTION B Methods and Apparatus

(1) Enzymic activity measurements.

The assays were performed using the standard conditions specified by Sumner and Hand:[17] 1 ml. of a 3% urea solution in a phosphate buffer (0.5M Na_2HPO_4, 0.2M KH_2PO_4) was thermostated at 20.0° and 1 ml. of the enzyme solution in the same buffer at the same temperature was added and mixed. The reaction was stopped after 5 min. with 2N hydrochloric acid, and the ammonia formed was determined in accordance with the general method described by Kistiakowsky et al.[8] In this case a Zeocarb 225 ion exchange column (a unifunctional sulphonated polystyrene resin in bead form, the column dimensions being 2 cm. x 1 cm.^{2}) was used to adsorb the ammonium ions, which

Table (VIII-1)

The relative viscosity and density values of buffers employed in this study

Buffer composition[a] (molarities)	pH	η_{rel}[b]	ρ_{rel}[b]	Page reference
0.07 NaCl, 0.01 NaB, 0.01 HB	8.9	1.030	1.006	33
0.12 NaCl, 0.04 NaV, 0.01 HV	8.4	1.040	1.008	xiii
0.15 NaCl, 0.01 NaV, 0.02 HV	7.5	1.022	1.005	xiii
0.09 NaCl, 0.01 NaV, 0.02 HV	7.5	1.022	1.005	33,95
0.048 Na_2HPO_4, 0.02 KH_2PO_4	7.0	1.040	1.009	33,68
0.048 Na_2HPO_4, 0.021 KH_2PO_4, 0.031 Na_2SO_3, 0.013 $NaHSO_3$	7.1	1.055	1.014	54,67, 99,110
0.08 NaCl, 0.02 NaCac, 0.02 HCac	6.1	1.030	1.006	33
0.09 NaCl, 0.07 NaAc, 0.01 HAc	5.4	1.034	1.006	xiii
0.15 NaCl, 0.01 NaAc, 0.01 HAc	4.6	1.011	1.007	xiii
0.15 KCl, 0.01 KAc, 0.01 HAc	4.6	1.004[c]		iv

[a] Notation as in text.

[b] Values relative to water at 20.0°.

[c] This value was obtained by Akeley and Gosting.[16]

were eluted with 0.1M potassium hydroxide and the solution diluted accurately to 250 ml. The quantitative adsorption and elution of ammonium ions in the presence of buffer salts and urea were periodically tested with standard ammonium tartrate solutions. The diluted eluant was nesslerized,[18] left for 15 min. and its optical density measured with a UNICAM SP 500 spectrophotometer. Each assay reported was performed on at least three different dilutions of the enzyme sample (thermostated for approximately 20 min.), chosen such that 1×10^{-5} to 4×10^{-5} moles of ammonia were produced, and each assay was duplicated. The absolute amount of ammonia formed was obtained by interpolation from a graph derived, for each set of measurements, from the nesslerization of solutions containing known quantities of ammonium ions. Fig. (VIII-2) is illustrative of the standardization plot and shows that Beer's Law is obeyed in the range of concentration used. The wavelength of light selected for the determinations was 4,250 Å.[8] Each set of measurements also included blanks, in which the acid was added before the enzyme; since the buffered urea solutions (used as substrate) were freshly prepared for each set of assays, the corrections were negligible.

(2) Electrophoresis.

All electrophoresis experiments were conducted at 1-2° in a Spinco Model H Electrophoresis-Diffusion apparatus, employing simultaneously or separately Rayleigh and schlieren optics. In all experiments (unless stated otherwise in the text) 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

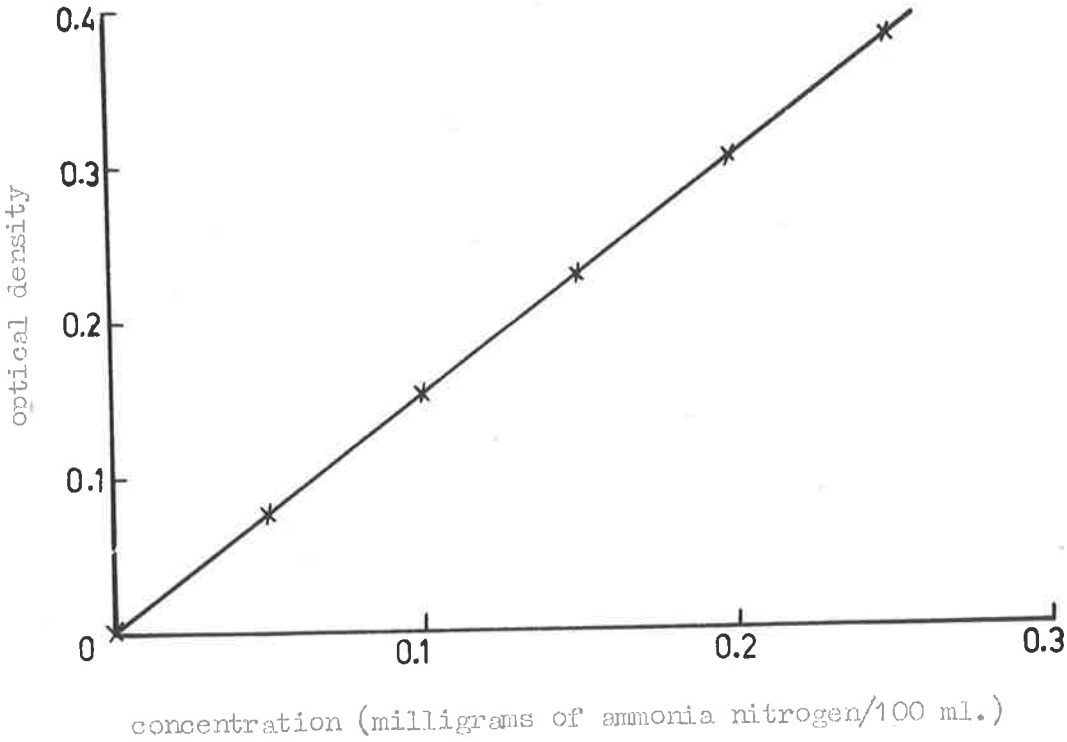


Fig. (VIII-2). Calibration graph for the optical density (1 cm. solution path length) of nesslerized standard ammonia salt solutions. The wavelength was 4,250 Å.

for at least 3 days against the buffer used to fill the rest of the apparatus. In mobility determinations, the temperature of the bath was adjusted to 1.0° and periodically recorded with a previously standardized Beckmann thermometer. The general procedure for the conduct of the experiments has been outlined in Chapter II. The photographic record of the experiment was obtained using "Process Panchromatic" film[19] and the distance traversed by the boundary measured with a two-dimensional Pye comparator.[20] No further comment is necessary on the buffer characteristics required for the mobility determinations except to note that, as the concentration of the protein used in mobility determinations was generally kept at a low value (3-20 fringes), the error is small in assuming that the conductivities of the buffer and protein solution are the same. The magnification factor, M, for the apparatus was obtained for each optical system employed, the procedure being the same. A horizontal graduated scale was placed in the position equivalent to the centre of the solution (the rear of the cell) and a photograph taken of the resultant image. A series of comparator measurements of the scale and corresponding image graduations was made, M being determined by the regression of image distance upon the object distance.

(3) Sedimentation

(a) The determination of sedimentation coefficients

Sedimentation velocity experiments were performed in a Spinco Model E Ultracentrifuge, using the schlieren optical system, which incorporated an inclined wire (bright 33 gauge nichrome). (In a few experiments, specified in the text, a similar ultracentri-

fuge[21] was used employing a phase-plate). Runs were generally carried out at 47,660 r.p.m., but in a few experiments they were performed at 42,040 r.p.m.: no measurable difference was detected in $S_{20,w}$ values of comparable species. Photographs were taken with "Ilford Rapid Process Panchromatic" plates.[22] Generally a Spinco A.N.D. rotor was employed together with a sector shaped cell centre piece made of epoxy-resin. This cell was chosen to avoid possible complicating effects due to prolonged contact of the enzyme solutions with aluminium alloy centre pieces.[23] However, for dilute solutions of urease in the presence of sulphite ions the A.N.B. rotor and a duralumin centre piece were used: the greater optical path through the solution led to a higher resolution without loss in definition.

The temperature was recorded but not controlled using the "Rotor Temperature Indicator and Control Unit" and the average value was taken as the temperature of the run. A variation of no more than 0.1° was observed during the normal 40 min. duration of the experiments when performed near 20° (about 0.3° for the runs at 3°). The temperatures derived from this procedure and the data in Table (VIII-1) were used to correct the observed sedimentation coefficients to 20° in water (i.e. to $S_{20,w}$), using the expression

$$S_{20,w} = S_t \frac{\eta_t^w}{\eta_{20}^w} \eta_{rel} \frac{1 - \bar{v}_{20} \rho_{20}^w}{1 - \bar{v}_t \rho_t} \qquad (1\text{-VIII})$$

where S_t is the sedimentation coefficient at t°,

η_{rel} is the viscosity of the solvent relative to water at 20°,

η_t^w is the viscosity of water at t°,

η^{w}_{20} is the viscosity of water at 20°,

$\bar{V}_{20}$ is the partial specific volume of the solute at 20° in the medium,

$\bar{V}_{t}$ is the partial specific volume of the solute at t° in the same medium

ρ^{w}_{20} is the density of water at 20°,

ρ_{t} is the density of the medium at t°.

The partial specific volume of 0.73 reported by Sumner et al.[10] was used in each case, assuming that it was independent of temperature and buffer composition. The further assumptions were made that the viscosity increment and the absolute density of the medium did not vary markedly with temperature: as most of the runs were performed near 20°, where the values were determined, the errors would be small. Measurements with the Pye comparator[20] of the distances of the maximum of the schlieren peaks from a reference line in a direction at right angles to the reference line enabled S_t to be evaluated. (The distance of the edge generating the reference line to the centre of rotation and the optical magnification factor were determined in separate experiments).

(b) <u>The determination of relative areas and concentrations in terms of J values</u>

Graphs of refractive index gradient versus distance in the cell from the centre of rotation were obtained by tracing an enlarged projected image of the photographic plate onto graph paper. Except in the few cases cited in the text where a double sector cell was used, reference base lines were obtained in separate experiments.

The area under each peak was measured with a planimeter, reading to 0.01 cm.^{2}. Trautman and Schumaker[24] have shown that, for multicomponent non-interacting systems which reveal a series of symmetrical boundaries in the ultracentrifuge, the total initial protein concentration, C_o, may be found from the observed concentrations, $C_{1,t}$, $C_{2,t}$.... $C_{n,t}$, of the different components, 1, 2,....n, at time t.

$$C_o = (x_t/x_m)^2 C_{1,t} + (x_t/x_m)^2 C_{2,t} + ... + (x_t/x_m)^2 C_{n,t} \quad (2\text{-VIII})$$

where x_t and x_m are the distances of the respective boundaries and the meniscus, respectively, from the centre of rotation. The area under each peak is proportional to the refractive index change across the boundary and therefore, presumably, to the concentration in the plateau region below the boundary. Each area was corrected for the radial dilution effect in the manner indicated (equation (2-VIII)) and the ratio of each individual corrected area to the total expressed as a percentage. The assumption was made that the Johnston-Ogston effect[25] was negligible.

In experiments which revealed a single peak, the average of the corrected areas for the several exposures (which exhibited a maximum scatter of 3%), was correlated directly with the fringe count obtained refractometrically in a separate experiment (see Chapter II). Subsequent dilutions were made by weight and the dilution factor applied to the fringe count obtained on the original solution. In this way the data shown in Fig. (IV-1), and the corresponding concentration data associated with the boundary analysis in Chapter VI, were obtained. In associating a J value with the area under a particular peak in a multicomponent system, as in Table (II-3),

the percentage (determined above) of the corrected area to the initial total area was multiplied by the fringe count corresponding to C_o. All attempts to correlate areas with J values would not be valid unless all non-dialysable material was visible (and resolved from the meniscus) in sedimentation experiments during photography. For this reason, only those samples which revealed a small amount of slow sedimenting material were subjected to this procedure, but even in these cases strong reliance could not be placed on the data obtained. It should be noted that the areas used in the determination of $g^*(\delta)$ values in Chapter VI are not subject to this limitation.

(4) Diffusion

All diffusion experiments were performed in the Spinco Model H apparatus referred to above, employing Rayleigh optics alone, and following as closely as possible the general procedures for the conduct of experiments outlined previously by Creeth.[26] It was necessary at first to establish both the absolute accuracy of the apparatus and its suitability for heterogeneity estimations. Wagner and Scheraga[27] have given a comprehensive summary of the use of the Spinco apparatus with Gouy optics, and have included some results on sucrose which allow an estimate of the accuracy obtainable. In what follows, therefore, comment is only made on the differences in procedure arising either from the different optical system, or for other reasons, from that followed by Wagner and Scheraga.

For assessing the reliability of a Rayleigh diffusiometer when used with single solutes, the following three criteria have been suggested;[26] (a) constancy of the reduced fringe separations

Y_t over the whole boundary; (b) reasonable correlation between observed and predicted skewness, with a linear relation between the deviations and the appropriate $AR(z^*)$ function; (c) accuracy of the differential diffusion coefficient $D_{\overline{C}}$.

The results of a diffusion experiment using sucrose, for which reliable D values are available,[28] satisfied only the first of these criteria, the apparent value of $D_{\overline{C}}$ being 1.2% higher than expected, while 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.

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

(b) 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.

(c) The following alterations were made to the optical system: (the sketch of the optical system, shown in Fig. (VIII-3) is essentially the same as that published by Wagner and Scheraga.[27]).

(i) A new source slit, 35 microns in width, was constructed

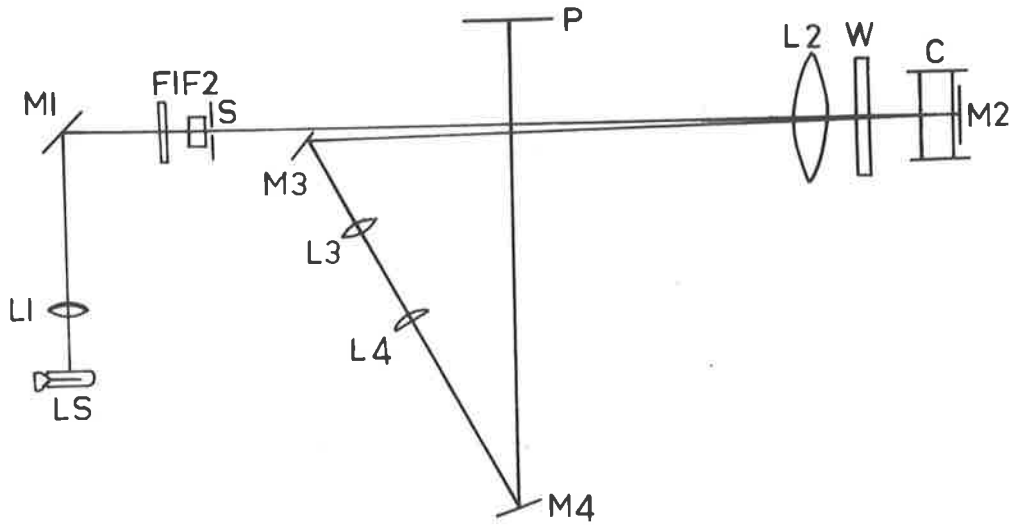


Fig. (VIII-3). Sketch of the optical path in the Spinco Model H diffusion apparatus: LS, light source; L1, condensing lens; M1, M2, M3 and M4, first surface mirrors; F1 and F2, light filters; S, slit assembly; L2, schlieren lens; W, bath mirror; L3, camera lens; L4, cylindrical lens; P, plane of photographic plate (camera). The sketch is not to scale.

from razor blades.

(ii) 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 minimize this vertical displacement of the light rays, the bath mirror, M2, previously tilted downwards, was adjusted until vertical. 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[29] that the necessary divergence of incident and reflected light is much better accomplished by lateral rather than vertical displacement. In consequence to the adjustment of the 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 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.[29]

At the completion of these alterations a further experiment using sucrose (ΔC = 0.7500 g./100 ml., $\bar{C}$ = 0.3750 g./100 ml.) was performed. Y_t values were again constant and $D_{\bar{C}}$ was 2.411 x 10^{-6} cm.^{2} sec.$^{-1}$ at 1.00°, compared with Gosting and Morris's value for

this concentration of 2.408×10^{-6} cm.^{2} sec.$^{-1}$ obtained with the Gouy method.[28] This represented a $0.1_2\%$ discrepancy, an allowable margin. (Results on other substances also have agreed with the literature values within these limits). Skewness results, while showing considerable scatter, were of the correct sign but of smaller magnitude than predicted. Fig. (VIII-4), obtained with ovalbumin, is a plot of the quantities in equation (14-V) and shows that the experimental points lie essentially on a line of zero slope (in agreement with the data in Table (A-1), indicating the lack of significant concentration dependence of $\mathcal{D}_A$ and D_1). Nevertheless, in view of the discrepancies with simple solutes, skewness results could not be considered reliable. 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. However, since this discrepancy has no effect on the determination of differential diffusion coefficients or on the analysis for heterogeneity the apparatus was judged satisfactory.

In order to confirm this conclusion, a diffusion experiment was performed on a synthetic mixture for which a theoretical result could be calculated.[30,31] 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 the "height-area" average diffusion coefficient ($D_{A\ calcd.} = 6.58_3$, $D_{A\ obsd.} = 6.59_3 \times 10^{-6}$ cm.^{2} sec.$^{-1}$) and total number of fringes ($J_{calcd.} = 104.82$, $J_{obsd.} = 104.83$) and also between the predicted and observed

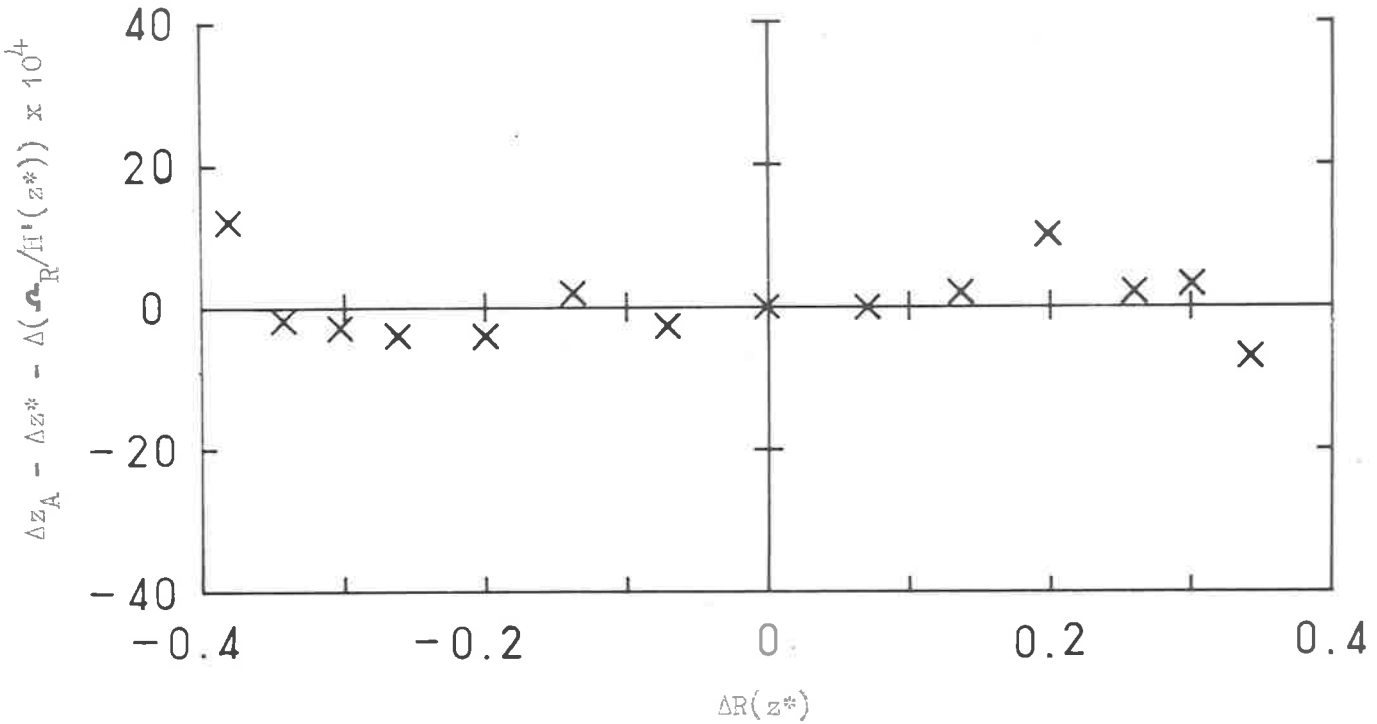


Fig. (VIII-4). Skewness diagram obtained in expt. no. 7 of Table (A-1). The crosses represent the mean of four experimental observations.

deviation plots, shown in Fig. (VIII-5). The error in an individual Ω_R value is approximately $\pm$ 3 x 10^{-4} for $J \sim 100$.

The temperatures of the experiments were periodically recorded, using a previously standardized Beckmann thermometer. The Rayleigh interferograms were recorded on Kodaline C.T.C. plates,[32] the latter being measured up with a two dimensional comparator[33] accurate to 2 x 10^{-4} cm., fitted with a projection screen.

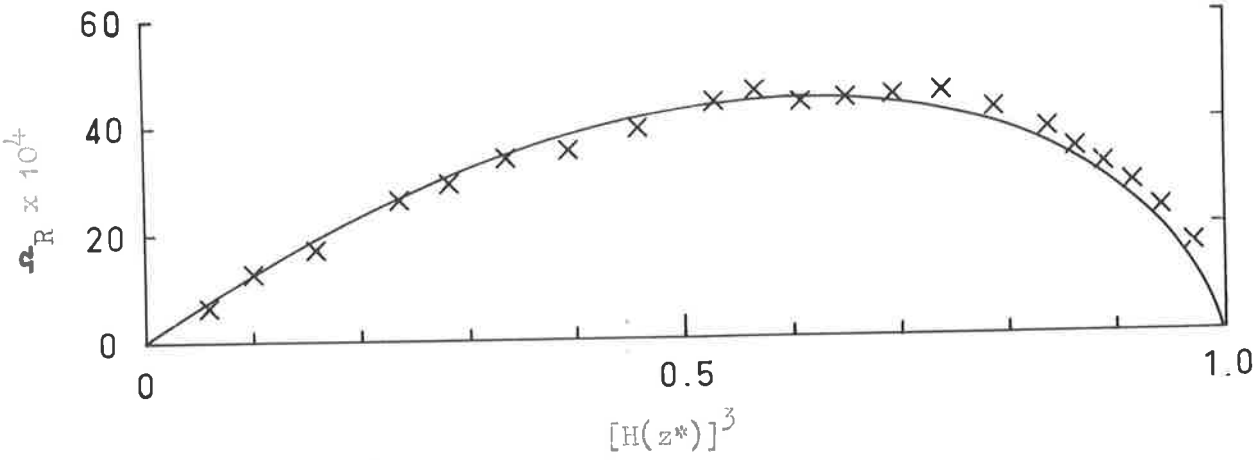


Fig. (VIII-5). Fringe deviation diagram for a urea-sucrose mixture ($a_{sucrose} = 0.0297$, $D_{urea}/D_{sucrose} = 2.844$). The line represents the predicted deviation, the crosses the mean of four experimental observations of the deviation.

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APPENDIX 1

AN EVALUATION OF THE APPLICABILITY OF THE DIFFUSION METHOD TO PROTEIN STUDIES

(1) The choice of ovalbumin as a 'model' system.

(2) The diffusion of ovalbumin at pH values near the isoelectric point. Results and discussion.

(3) The diffusion of ovalbumin at pH values different from its isoelectric point. The effect of charge on diffusion.

(4) General conclusions.

(1) The choice of ovalbumin as a 'model' system

The analysis procedure outlined in Section A of Chapter V has been applied to diffusion results obtained with the protein systems bovine plasma albumin, ribonuclease and β-lactoglobulin and indicated, in agreement with other observations,[1-6] that all of the preparations were heterogeneous.[7] The extent of the heterogeneity was defined by a limiting range of values for a_2: the corresponding r_2 values were also cited. It has, however, been strongly emphasized by Creeth[7] and Gosting[8] that the applicability of such procedures is subject entirely to the validity of the assumptions described earlier and, in particular, to the restriction that the solute flows do not interact. It would be of considerable interest, therefore, to diffuse an homogeneous protein (if this were available) and thereby demonstrate the validity of the basic assumptions.

Crystalline ovalbumin seems in many ways a suitable system to examine: reference to the requirements outlined in Chapter I exemplifies this. Thus, it may be prepared easily in satisfactorily large amounts by conventional salt fractionation techniques.[9] The value of the isoelectric point has been established[10-12] and the protein is soluble at this pH, as well as at higher and lower pH values. Moreover, information on the probable charge on the molecule over a wide pH range[13] and the molecular weight[14] is available. However, in this connection, the electrophoretic complexity (or micro-heterogeneity[15]) should be mentioned. At suitable pH values at least three components (designated A_1, A_2 and A_3 in order of descending negative mobility) may be distinguished.[16-19] This heterogeneity is

not reflected in the sedimentation behaviour of the protein, the ultracentrifuge patterns obtained with freshly prepared material showing essentially a single peak.[20,21] Moreover, a rational explanation of this behaviour was presented by Perlmann[22,23] on the basis of enzymatic dephosphorylation experiments: it was shown that A_1 could be converted to a protein with the mobility of A_2, with the simultaneous loss of one phosphate residue per molecule, and a similar relation existed between A_2 and A_3. The phosphorus analyses were such as to indicate that A_1, A_2 and A_3 contained, respectively, two, one and zero phosphate groups per molecule as suggested earlier.[24] Thus, on the basis of electrophoretic and analytical evidence, the only difference between the components of ovalbumin is in the number of phosphate groups per molecule; while minor chemical differences cannot be excluded[22,23] it seems reasonable to assume that in other respects these molecules are essentially 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.

A similar argument should apply to the diffusion coefficients. Diffusion experiments on ovalbumin, designed and performed (in conjunction with J.M. Creeth and D.J. Winzor[25]) primarily to demonstrate the identity of the diffusion coefficients of the ovalbumin complex and the A_1 component, strongly support this contention.[26] Summing up, it appears that in several respects the diffusion of ovalbumin may be potentially valuable in obtaining evidence, which directly pertains to the problem of 'flow interaction'. The

ovalbumin results are presented and discussed in this light.

(2) The diffusion of ovalbumin at pH values near the isoelectric point

(a) Experimental and Results.

Both twice recrystallized ovalbumin and the A_1 component (prepared by prolonged electrophoresis under conditions of reduced boundary anomalies; see experimental section) were diffused in a Rayleigh diffusiometer, and the experimental record analysed using the Creeth and Gosting approach, outlined in Section A of Chapter V. In this series of experiments any complicating factors, which might arise if the net charge on the protein were finite, were minimized by performing the diffusion at pH 4.5_9, a value close to the isoelectric point. For this purpose an acetate buffer was used which contained 0.01, 0.01 and 0.15 moles per litre of potassium acetate, acetic acid and potassium chloride, respectively.

All data obtained in these experiments are summarized in Table (A-1). The following points in relation to this table should be noted.

(i) The Δt values in column 4 represent the "zero time" corrections[27] and subject to the limitations of the doubled optical path operative in the diffusiometer give an indication of the sharpness of the initial boundary. It might be thought that these values are generally too large. However, with this apparatus, it has generally been found that the values are some two to five times greater than in the case with a straight-through optical system (a similar experience has been reported by Longsworth[28]). In addition,

Table (A-1)

Diffusion results on the ovalbumin complex and fractionated A_1

Expt. no.	Material	Total no. of fringes = J[a]	Δt sec.	$\mathcal{D}_A$[b] at 1.00° $cm.^2 sec.^{-1} \times 10^7$	$\Omega_{R\ max} \times 10^4$	r_2	a_2	D_1 $cm.^2 sec.^{-1} \times 10^7$
1	AC,[d] S1	83.23	60	3.96_0	37	1/2,1/6	0.078,0.015	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 (see Chapter II). [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.

for all solutes examined, including simple materials such as sucrose and urea, diffusion pictures taken at times such that $\sqrt{D\,t} < 0.1$ cm. have proved slightly inconsistent with later exposures and, therefore, have not been used in the calculations. This inability to use earlier exposures results in much greater difficulty in defining the zero time accurately (hence the wide spread of values), but as the duration of the experiments was at least four days, even the largest of these Δt values have a negligible effect on $\mathcal{D}_A$.

(ii) The essential identity of the $\mathcal{D}_A$ values for the complex and A_1 components lends considerable support to the other evidence outlined above, that the components constituting the ovalbumin complex differ only in very minor respects. If this is indeed true and there are no impurities in the samples of ovalbumin complex and A_1 component, the values of Ω_R over the whole boundaries in each case should be zero, provided that, in addition, the assumptions inherent in the analysis procedure[29] are completely fulfilled.

(iii) The deviation graphs shown in Fig. (A-1), where the crosses represent the mean observed deviations, and the maximum values of Ω_R in column 6, show immediately that one of these assumptions (at least) is incorrect. It proves informative at this stage to assume that the deviations are due solely to heterogeneity of the samples, i.e. $\mathcal{D}_A = D_A$, and to employ the curve fitting procedures described earlier to define the extent of the <u>apparent</u> heterogeneity. The graphs, Fig. (A-1), referring to the ovalbumin complex all show the maximum at the extreme right of the picture, which is characteristic of a rapidly diffusing impurity, i.e. $r_2 < 1$. (The diagram

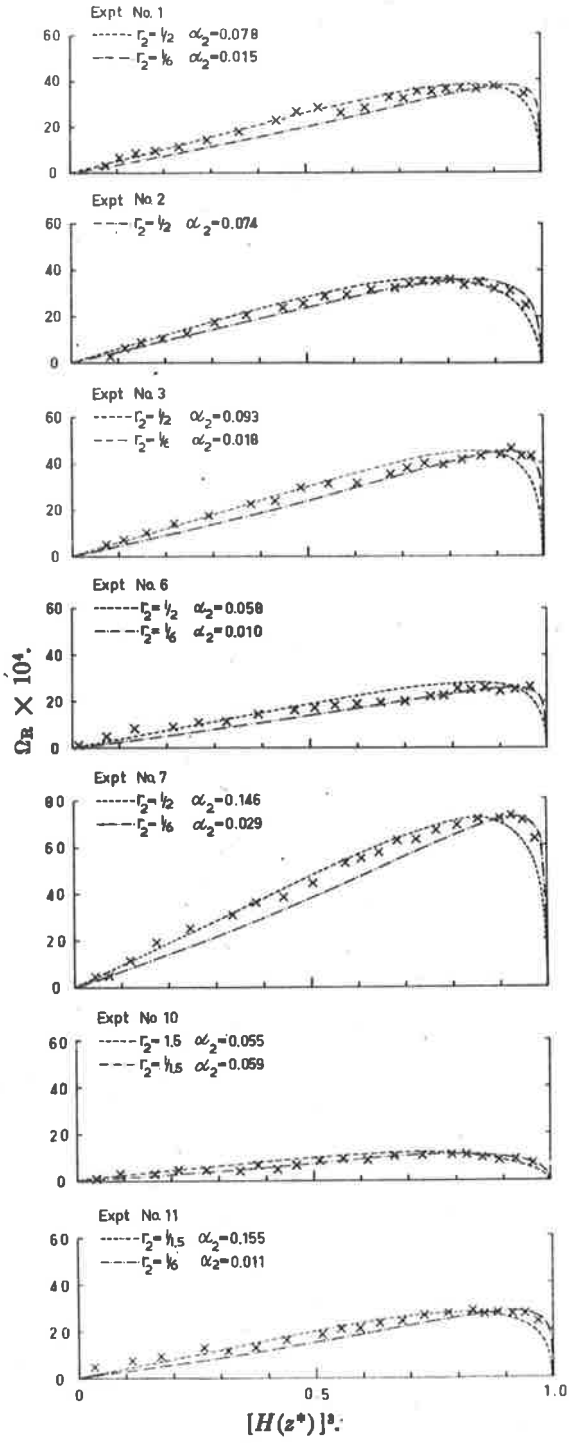

Fig. (A-1). Fringe deviation diagrams of the albumin complex and fractionated A_1, the experiment number referring to Table (A-1). The lines are attempts at curve fitting, the results of which are indicated in each case. In expt. no. 2, no unique pairs of values of α_2 and r_2 can be given for the dashed 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 diffusing impurities.

referring to Expt. no. 2 indicates the presence of some slow diffusing impurity in addition to the fast, which correlated with the lower value of $\mathcal{D}_A$). Columns 7-9 give the results of the actual curve fitting. Fitting the observed deviations to a unique pair of a_2 and r_2 has not been attempted for the reasons given in Chapter V: thus, the pairs of values are to be taken together (for example, for the first experiment reported, the observed deviations lie very largely within the two curves described by the characteristics $r_2 = \frac{1}{2}$, $a_2 = 0.078$ and $r_2 = 1/6$, $a_2 = 0.015$, while the corresponding values for D_1, obtained by using equation (11-V), are respectively 3.75 and 3.86 x 10^{-7} cm.^{2} sec.$^{-1}$). The deviation plots for the more concentrated samples of the A_1 component are also shown in Fig. (A-1) and comparison indicates that these are somewhat smaller in magnitude, particularly for Expt. no. 10, where, moreover, the shape of the curve is different from that of the parent material (AC 2). While the flatter curve may be interpreted as implying a more ideal diffusion, in the sense that the diffusion approximates quite closely to that of a single solute, curve fitting of such a deviation plot is very difficult as the abscissa corresponding to the $\Omega_{R\ max.}$ position is ill defined. The significance of the r_2 values quoted is therefore smaller than in the others and the values with corresponding a_2's are bracketed to indicate this unreliability. It should be noted that while the observed deviations fit most closely the curve for solutes of similar diffusion coefficient,[7,29] an error of only 3 x 10^{-4} in the Ω_R values for the three outermost points would result in a curve characteristic of $r_2 = 1/3$, while D_1 would

still lie in the range given. The second experiment on A_1, Expt. no. 11, for which reliable analysis is possible, performed at 25% higher concentration, shows the same kind of deviation plot as the parent material and curve fitting gives essentially the same value of D_1 as was obtained with this material.

(iv) Other conclusions from these results, such as the lack of concentration dependence of the diffusion coefficient of both the native material and fractionated component, while extremely significant in a study on ovalbumin,[26] are not directly pertinent to this study and will not be discussed. However, one other point must be made. It is important to establish that ovalbumin, which is considered to be very labile, is stable during the course of the diffusion run. It has been general experience throughout this work that the quantities $Y_t/\sqrt{t}$ and v_j were not time dependent. As mentioned previously, this represents a good indication that there was no variation of the diffusing solute with time.

(b) Discussion.

The major conclusion from this work is that interpretation of the diffusion deviation plots, on the basis of heterogeneity, leads to the observation that in the ovalbumin complex there is some 2-4% of an impurity with a diffusion coefficient four times that of the main component. However, this apparent impurity cannot be directly related to any component that is apparent in electrophoretic analysis. Fig. (VIII-1) is illustrative of the general finding that both materials are free of any impurity, possessing a markedly different electrophoretic mobility. The accuracy of

this estimation is improved by employing Rayleigh optics in conjunction with schlieren, and when this is done ½% of another electrophoretic component can be detected in a 100 fringe system. Certainly, the 'impurity' detected by the diffusion experiments cannot be correlated with the 15-20% of the A_2 component, evident in the complex (Fig. (VIII-1a)), because the same impurity was present in one of the isolated A_1 samples. It is possible (especially in view of the calculations presented in Chapter II) that the ovalbumin complex contains a relatively rapidly diffusing impurity, which has roughly the same electrophoretic mobility as the main component A_1: thus both the albumin complex and electrophoretically fractionated A_1 would both contain the impurity and therefore indicate such in their diffusion behaviour. However, the low deviation plot in Expt. no. 10 is not compatible with this hypothesis. Moreover, there is no independent evidence either chemical or physico-chemical that suggests the possibility of the existence of such an impurity.

Any other explanation must be based on the non-fulfillment of one or more of the assumptions on which the analysis theory is based. In this case, there seems no reason to doubt that the diffusion coefficient and the refraction increment of each solute are linear functions (of small magnitude) of the concentration of that component and are unaffected by the presence of others. It appears, therefore, that the assumption of independence of solute flows cannot be correct.

The question of interacting flows is one of great interest at the moment[8,30-32] and the work of Dunlop[33] seems particularly

relevant to this case. Dunlop found for an experiment in which raffinose diffused in the presence of potassium chloride, there being no increment of potassium chloride across the boundary, that appreciable deviation from single solute behaviour occurred: interpretation of the graph on the assumption that the deviations were due to an impurity would have led to the conclusion that some 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 generally shown the maximum at the extreme right of the picture thus becomes particularly significant: the implication is 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, as this is determined by a complex function of the diffusion coefficients;[32,33] 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 'flow interaction' between protein and buffer. This is a particularly significant finding.

It appears that detailed information on the heterogeneity of protein samples cannot be gained from diffusion measurements, until the effect of heterogeneity can be separated from that due to 'flow interaction'. Furthermore, the curve fitting procedures adopted to enable D_i values to be obtained from the observed $\mathcal{D}_A$'s are not justified. (It should be noted that these criticisms do not necessarily apply to all protein diffusion experiments. Thus,

the fact that other proteins investigated[7,34] have given deviation plots which show a maximum at an abscissa position characteristic of a slow diffusing impurity appears to rule out the possibility of 'flow interaction' in these cases.)

The exact nature of the phenomenon of interacting flows is unknown. This prevents the prediction of the possible occurrence of the phenomenon in individual systems and the conclusive interpretation of diffusion results impossible. Therefore, any information on the nature of the phenomenon is very valuable at this stage. It is generally accepted that coupling of flows results in part from the electrostatic coupling of ion flows,[30,35] although this need not be the complete answer, nor indeed in some cases part of the answer, as interaction has been observed in systems of non-electrolytes. Nevertheless, the contribution of electrostatic effects to interaction is particularly important in protein diffusion. For example in the ovalbumin system under consideration, the experiments were conducted at pH 4.5_9 at which it appears that A_1 was isoelectric under the conditions chosen;[10-12] however it follows that in experiments on the complex some 15-20% of the protein bore a net positive charge (probably about +1 per molecule[23]) and thus constituted an ionic species. Generally, the effect of charge on diffusion is also important as many proteins have been diffused at pH values away from their isoelectric points due to solubility and stability limitations. This is the case with urease.[36] Consequently, it is of fundamental importance, having found evidence for the existence of interactions in protein diffusion experiments, to extend the observations and attempt to assess the effect of charge on the diffusion results.

(3) The diffusion of ovalbumin at pH values different from its isoelectric point. The effect of charge on diffusion

The ovalbumin complex, sample 3, was diffused at several pH values to determine the effect of charge on $\mathcal{D}_A$ and on the fringe deviation diagrams. (It is true that a series of experiments on A_1 would be more desirable, as the charge on 15-20% of the molecules in the complex introduces an additional complication. However, sufficient quantities of A_1 are difficult to obtain and, at any rate, although the use of the complex does not represent the limiting case of a completely uncharged species, when at pH 4.5_9, it should serve as a valid standard for comparison with markedly charged species). The sample used had been stored for approximately 18 months, but the electrophoretic analysis was unchanged.

All the relevant information relating to this series of diffusion experiments is summarized in Table (A-2), which is largely self-explanatory. The values of $\mathcal{D}_A$ agree closely with those reported in Table (A-1) and it is at once clear that they do not show any significant dependence on the net charge of the protein (within the range 0 to -10). The insensitivity of $\mathcal{D}_A$ is in some respects an important finding, for if it were a general property of protein-buffer systems, it would imply that no additional error would be introduced into molecular weight determinations by employing the results of diffusion experiments performed at pH values away from the isoelectric point. However, little precise information on other systems is yet available (Charlwood's results[37] on human albumin cannot be analysed for deviations from Gaussian form, while

O'Donnell et al.[35] observed pH dependent values of $\mathcal{D}_A$ for bovine albumin).

In column 9 of Table (A-2) the maximum values of Ω_R are reported; the deviation graphs are given in Fig. (A-2), the crosses representing the mean observed deviations. The following points require comment. Expt. no. 1 of this series, performed under conditions where the protein was <u>essentially</u> uncharged, gives a deviation plot of the same general shape and magnitude as obtained previously (compare Fig. (A-1)). The experimental conditions differ only in that sodium salts have been used instead of potassium salts. Inspection of the deviations for experiments 2, 3 and 4 (Fig. (A-2)), where the protein bears a generally increasing net negative charge, shows a trend in which the maximum becomes shifted from the extreme right and, moreover, the value of $\Omega_{R\ max.}$ is decreased. This implies that the diffusion is more nearly "ideal" and appears at first sight contrary to the postulate that flow interaction results in part from electrostatic coupling of ion flows, since greater deviations would be expected in the case of charged systems.

However the diffusion experiment is conducted with dialysed solutions and accordingly the initial conditions require closer examination. Should the protein bear no net charge, the salt redistribution on dialysis is a measure of preferential 'binding' of the buffer constituents by the protein: depending on specific effects, this may lead to a greater or less buffer salt concentration within the dialysed protein solution. When the protein is charged, the Donnan effect[39] must always result in a lowered buffer salt concen-

Table (A-2)

Solution data diffusion results on ovalbumin

1 Expt. no.	2 Buffer composition[a] (molarities)	3 pH	4 Relative viscosity of buffer (η_{rel})	5 Apparent valence[b]	6 Total no. of fringes[c] = J	7 Δt[d]	8 $\mathcal{D}_A$[e] at 1.00° $cm.^2 sec.^{-1}$ $\times 10^7$	9 $\Omega_{R_{max.}}$[d] $\times 10^4$	10 $\Omega_{R_{max.}}$ cor. $\times 10^4$
1	0.15 NaCl 0.01 NaAc 0.01 HAc	4.5_9	1.011	0	68.53	140	3.89_5	37	37
2	0.09 NaCl 0.07 NaAc 0.01 HAc	5.4_0	1.034	$-5._1$	111.42	260	3.87_2	32	42
3	0.15 NaCl 0.01 NaV 0.02 HV	7.5_1	1.022	$-10._0$	113.36	150	3.95_4	19	48
4	0.12 NaCl 0.04 NaV 0.01 HV	8.4_2	1.040	$-10._5$	110.59	60	3.90_2	26	51

a Notation as in Table (II-2).

b Calculated on the basis of the method given by Abramson, Moyer and Gorin[13] using the mobility data of Longsworth.[12]

c As before, J = 100 corresponds to a concentration increment of 0.58_2 g./100 ml. for ovalbumin, based on Perlmann and Longsworth's value[38] for the specific refractive increment.

d The significance of these quantities is the same as in Table (A-1).

e Values corrected to water basis (by multiplying by η_{rel}).

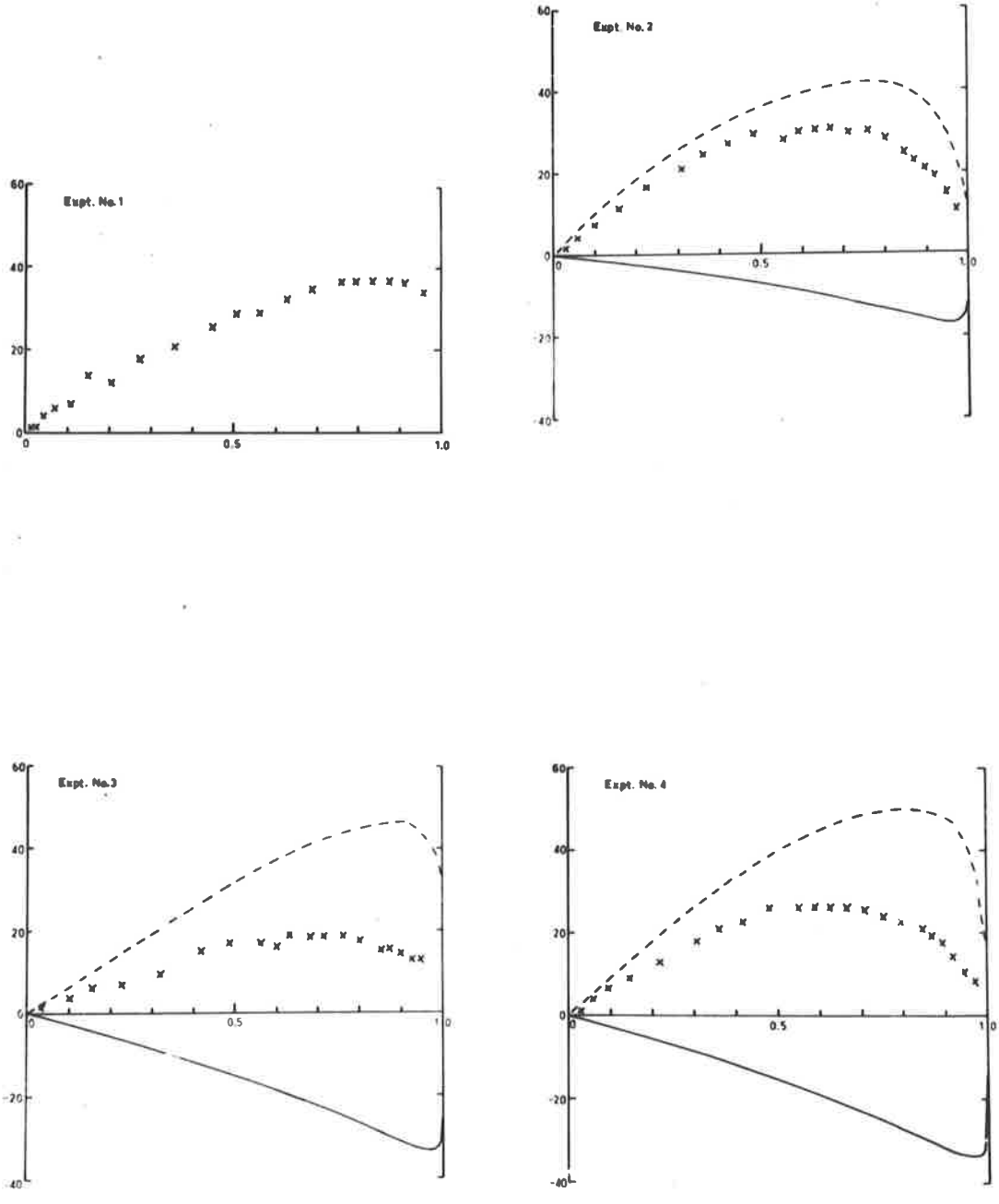

Fig. (A-2). Fringe deviation diagrams of ovalbumin, the expt. no. referring to Table (A-2). The crosses represent mean experimentally observed deviations, the solid line represents the deviation due to the buffer gradient, consequent on dialysis and the dashed line is a summation of the two. The vertical axes in each case are $\Omega_R \times 10^4$ and the horizontal axes $[H(z^*)]^3$.

tration within the dialysed solution. If the Donnan effect predominates, the conditions at the start of the experiment must involve a small negative concentration increment of salt superimposed on the positive concentration increment of protein. Qualitatively it is clear that, in diffusion, salt will only be transported in those regions where there is a protein concentration gradient, for elsewhere its chemical potential is constant. Strictly, the protein concentration gradient only reaches zero at infinite distance from the initial boundary, but the limits of optical resolution allow precise definition of regions of effectively zero gradient. The general solution for this type of problem is known,[32] but its application is not yet possible with protein systems; accordingly a precise interpretation of the results cannot be presented. Nevertheless it is of some interest to see how much of the observed deviations can be accounted for on the assumption that the flow of salt (due to the Donnan concentration gradient) proceeds independently of other diffusion processes.

The first step is to calculate a value for the initial concentration increment of salt, ΔC (assumed to be completely NaCl). Use is made of the Donnan membrane relation, as formulated by Svensson:[40]

$$(C_i)_{inner} = (C_i)_{outer} \left[1 - z_i C_p / 2 I\right] \qquad (1\text{-A})$$

where the terminology is as follows.

$(C_i)_{inner}$ is the concentration of either the Na^+ or Cl^- inside the dialysis bag (activity coefficients being assumed unity),

$(C_i)_{outer}$ represents the corresponding concentrations outside the bag,

z_i is the valence of the ion considered,

C_p is the concentration of the protein in electrochemical equivalents,

and I is the ionic strength.

The molecular weight of ovalbumin required to estimate C_p was taken as 45,000 [14] and the charge values used were those reported in Table (A-2). A value for the refractive index proportion a_2 was then found by using the relation

$$\Delta C = J\lambda/kd \qquad (2\text{-A})$$

where the value for the specific refraction increment, k, was taken as 1.011×10^{-2} l. mole^{-1}, [41] λ the wavelength of light used was 5461 Å, and d was the thickness of the cell. The diffusion coefficient ratio, r_2, was estimated to be 1/18 from the results of Vitagliano and Lyons [41] for NaCl. This is sufficient information to calculate a first approximation to the expected fringe deviation from equation (10-V). This function is shown in each case as the solid line in Fig. (A-2): it is of course zero for Expt. no. 1. From the nature of these calculations, it is obvious that these lines can only be approximately defined. If the observed deviations are regarded as the sum of the effects of 'flow interaction' and of an independently diffusing salt gradient, then the magnitude of the former effect may be obtained by subtracting the (negative) value of the latter from the observed deviation at a given value of z^*. By this means the dashed curves in the figure have been computed.

From the general appearance of these curves, it is clear that the assumption stated above leads to a rational qualitative interpretation of the diffusion results: the general trend shown by

the measured deviations in experiments 2-4 of Fig. (A-2) is what would be expected on the basis of increasing negative increments of NaCl combined with increasing positive deviations due to 'flow interaction'. The former would be expected on the basis of increasing negative charge on the protein, and the latter on the supposition that interaction (being due to coupled ion flows) will also increase with the charge on the protein ion. The maximum values of the dashed curves (reported as '$\Omega_{R\ cor.}$' in column 10 of Table (A-2)) show a fairly smooth pH dependence, while there is a marked similarity in the general shape of these curves and resemblance to the experimental curve obtained at the isoelectric point.

In this survey of the effects operating in the diffusion of a charged protein species, only molecules possessing a net negative charge have been considered, even though it would be of considerable interest to investigate the diffusion of positively charged macromolecules. This is particularly true in a study on ovalbumin, as the complex bears a small net positive charge at pH 4.5_9. However, as the primary concern of this work is urease (or more particularly, the modified derivative present at pH 7) this limitation is not important. A brief summary will not be given of the major conclusions pertinent to the diffusion studies on urease presented in Chapter V.

(4) General conclusions

In Section A of Chapter V, it has been shown that the analysis of data obtained from Rayleigh interferograms can give, in the case of three-component systems, both a diffusion coefficient for the main component and an estimation of the heterogeneity. Provided

that this procedure is valid, in the sense that the various assumptions are fulfilled, the information may be usefully employed in further characterization of the protein being studied. However, an experimental diffusion study on ovalbumin and a fractionated component provided evidence indicative of a flow of buffer salts resultant on the flow of protein. Consequently, in this case no meaningful interpretation of the finite deviation plot observed, in terms of a second solute impurity, could be made. Moreover, the curve fitting procedures used to estimate D_1 values were unjustified.

The general implication of these findings is that the interpretation of deviation plots in terms of heterogeneity must be done cautiously as the distinct possibility exists that the findings may not be meaningful. This is particularly pertinent if the fringe deviation diagram in the case of uncharged protein diffusion shows a maximum at the extreme right. Furthermore even if the deviation plot is characteristic of an apparent slow diffusing impurity (the $\Delta n_{R\ max.}$ position being more to the left), the possibility of 'flow interaction' cannot be excluded if the protein species diffused is charged. Thus in this case the effect of the initial buffer salt gradient, inherent on dialysis, may oppose that of interaction and the resultant experimentally observed deviation plot may be of this general shape.

It is apparent that a theory practically applicable to protein systems, which would enable 'flow interaction' effects to be separated from those due to heterogeneity, is essential before diffusion results can be analysed to provide the information which is potentially available. Various experimental approaches[26,33]

for the separation of the two effects have been postulated, but in each case the procedures are laborious and require considerable amounts of material.

While the conclusions presented stress the limitations of the results obtained from a diffusion experiment, and appear to suggest that at this stage of development such experiments would not contribute significantly to protein characterization, the following points should be noted. First, the deviation plots, even if complicated by interaction effects, may be used in a qualitative comparative manner: an example of this with urease was given in Chapter V. Secondly, although the reliability of the diffusion coefficient found by correcting the observed $\mathcal{D}_A$ (where $\mathcal{D}_A$ does not equal D_A) can be questioned, provided the corrections are small (as in the case of ovalbumin), it is almost certainly not markedly erroneous. Consequently, if a value at zero protein concentration can be determined, a reasonably accurate molecular weight can be found: even an approximate value is of considerable use in biochemical studies on proteins. In this connection, the observation that $\mathcal{D}_A$ is insensitive to charge effects, at least in the case of ovalbumin, is important as it lends some justification to the procedure frequently employed of estimating a molecular weight from diffusion data found with charged systems.

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A PHYSICO-CHEMICAL STUDY OF UREASE

Summary of Thesis presented by L.W. Nichol for the degree of Ph.D., University of Adelaide

Urease has been found to give single symmetrical schlieren peaks in electrophoresis over a wide range of pH and at an ionic strength of 0.1, but in sedimentation velocity experiments three main peaks, in proportions varying with sample, have been observed. The values of the sedimentation coefficients are compatible with those expected for association to dimers and trimers.

The effect of sulphite (and other S-nucleophilic bases) is to reverse the polymerization (so that only one peak is observed in the centrifuge patterns) and simultaneously to increase the specific activity. Evidence is brought forward to show that the mechanism of the polymerization is through the formation of intermolecular disulphide bridges by the oxidation (with molecular oxygen) of certain sulphydryl groups.

The sulphite modified form has been investigated by the methods of electrophoresis, diffusion and sedimentation velocity. It appears to differ from the comparable species in the polymeric mixture in that it possesses thiosulphate groups. The micro-heterogeneity detected in electrophoresis experiments confirmed the postulated mechanism for the unsymmetrical cleavage of disulphide bonds by sulphite. The material appeared homogeneous when subjected to a test designed essentially to detect the distribution of components with different sedimentation coefficients. Sulphite also has a very marked effect on the diffusion properties of the enzyme, altering

both the diffusion coefficient and the deviations from Gaussian form.

The sedimentation patterns observed on lowering the pH of urease solutions containing sulphite are interpreted on the basis of the cleavage of an inter-chain disulphide bond in urease. This and other observations are related to the published kinetic data on the mechanism of the hydrolysis of urea by urease.

Appendix 2

PUBLICATIONS

Sections of the following publications were used in this work.

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

2. "Physico-chemical Studies on Ovalbumin. II. Effect of Charge on Diffusion"
   L.W. Nichol, D.J. Winzor and J.M. Creeth, J.Phys.Chem., 64, 1080 (1960). (Reprints not available).

3. "The Chemical Interaction of Urease in Solution"
   J.M. Creeth and L.W. Nichol, Biochem. J., 77, 230 (1960). (Reprints not available).

Creeth, J. M., Nichol, L. W. & Winzor, D. J. (1958). Physico-chemical studies on Ovalbumin. I. Electrophoretic fractionation and characterization by diffusion. *The Journal of Physical Chemistry*, *62*(12), 1546-1553.