AN INVESTIGATION OF THE ASSOCIATION BEHAVIOUR AND HETEROGENEITY OF PAPAIN

by

John Christopher Swann, B.Sc.

Thesis presented for the degree of
Doctor of Philosophy
in the Department of Physical and Inorganic Chemistry,
University of Adelaide.

February, 1966.

CONTENTS

ABBREVIATIONS

The following abbreviations of chemical names have been used in this thesis:

SH	sulphydryl
PHMB	p-hydroxymercuribenzoic acid
PCMS	p-chloromercuribenzene sulphonic acid
IAA	iodoacetic acid
IAM	iodoacetamide
EDTA	ethylene diamine tetra-acetic acid
BAA	α-benzoyl arginine amide
CM	carboxymethyl
BSA	bovine serum albumin

SUMMARY

This thesis first considers the self-association of the proteolytic enzyme papain in aqueous solution. Association behaviour was studied qualitatively by the technique of sedimentation velocity. Quantitative studies were not proceeded with because of complications arising from the suspected heterogeneity of papain with respect to sulphydryl content. Consequently the heterogeneity of papain was further investigated and attempts were made to isolate two different forms of papain by electrophoresis and chromatography.

Sedimentation studies were conducted in several buffers. The schlieren optical system of the ultracentrifuge was used, and some optical distortion effects in schlieren patterns are discussed in a separate appendix. Reversible association was not detected at pH 4 and was only slight at pH 7, but became very marked at pH 8 which is near the isoelectric pH of papain. Contrary to the findings of previous investigators, no disaggregation was observed in the presence of cysteine or ethylene diamine tetra-acetic acid. There is thus no evidence that papain associates through disulphide or metal to protein bonding. However, the sulphydryl reagents iodoacetamide and iodoacetic acid were found to exert noticeable and opposite effects on the extent of association, indicating

the influence of charged groups near the active site. Hence it appears likely that the association behaviour may be complicated by the suspected heterogeneity of papain, i.e. the reported tendency for the active SH group to exist in two or more oxidation states.

The detection by physico-chemical methods of heterogeneity in papain has not previously been achieved; it was made possible in this investigation by combining the enzyme with the reversible inhibitor p-chloromercuri-benzene sulphonic acid (PCMS). It was expected that this anionic reagent would bind to the active, SH-containing form of papain, producing a species of different charge from the inactive, non-SH enzyme. Moving-boundary electrophoresis was then applied to separate the two forms.

In the pH range 4 to 7, PCMS modification of papain always produced another peak in the electrophoretic pattern on the trailing edge of the main peak. Recent theories about electrophoresis in interacting systems are used in discussing features of the moving boundaries.

The two peaks had very similar mobilities, but by application of back-compensated electrophoresis for 24 hours they were separated sufficiently to allow a sample of the material constituting the front one to be withdrawn. As this material had lower specific enzymic activity than

did the unfractionated solution, heterogeneity is strongly indicated.

Since a preparative fractionation could not be obtained by electrophoresis, the ion-exchange chromatography of PCMS-papain on carboxymethyl cellulose was attempted. Although some separation of activity occurred, the resolution of components was even poorer than in electrophoresis. An attempted fractionation of papain by chromatography on organomercurial-polysaccharide, a specific chromatographic material for SH-proteins, was also unsuccessful. Thus, although strong evidence has been obtained for the existence of active and inactive forms of papain, no preparative fractionation was achieved.

In a concluding chapter, the findings of this investigation are discussed in relation to structural features of the active site of papain.

This thesis contains no material previously submitted for a degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously written by another person, except when due reference is made in the text.

J. C. Swann

ACKNOWLEDGEMENTS

I wish to sincerely thank Dr. J. H. Coates for his encouragement and advice throughout the course of this work. I am grateful to Professor D. O. Jordan for his interest, particularly while Dr. Coates was absent on study leave in 1962. In addition, I have appreciated the opportunities for helpful discussion with many other staff members and fellow research students in the Department of Physical and Inorganic Chemistry.

I should also like to thank Mr. P. Rapp for his technical assistance, Miss Andrea Whittle for preparing the illustrations, and the staff of the glassblowing, engineering and electronics workshops for their help in the construction and maintenance of apparatus.

Financial assistance received from the Commonwealth Scientific and Industrial Research Organization, General Motors-Holden's Pty. Limited and the University of Adelaide is acknowledged with thanks.

J.C.S.

CHAPTER I

INTRODUCTION

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(a) The Field of Study

Research on chemically reacting protein systems has been stimulated in recent years by advances in both the theory and technique of physico-chemical methods[1]. Interest in this topic also stems from the increasing recognition of its possible role in metabolic processes[2]. A variety of reactions may be undergone by even a purified protein in solution, including self-association to dimers or higher aggregates, and interaction with small molecules or ions of the buffering medium.

It is now often rewarding to apply new and improved methods to an investigation of the solution properties of a protein of which some details of the primary, secondary, and tertiary structure are known. The chemical nature of any interactions can then often be understood, and this information may in turn help to give a clearer picture of the structure of the protein and its behaviour under physiological conditions. Examples of this approach are the work of Townend *et al*[3,4,5] who studied the association of β-lactoglobulin by light scattering, electrophoresis, and sedimentation velocity, and that of Jeffrey and Coates[6] who used sedimentation equilibrium to study the association of insulin. Both groups of workers were able to identify the species present in reversible

equilibrium in the system studied and to obtain thermodynamic parameters for the association reactions. Suggestions could then be made about the type of intermolecular bonding.

It was considered that a similar study of the association behaviour of papain, the proteolytic enzyme of papaya latex, would also be useful. Complex association behaviour of papain and its mercury derivative has been reported by Smith *et al*[7]. Later in this chapter, after an introduction to the molecular properties of papain, an account is given of the objectives of the present study and the approach adopted. However the main points of interest about the association behaviour of papain can be summarized as follows.

First it would be interesting to compare thermodynamic data for the association reactions with figures that have been obtained with other proteins to see if similarities exist. Secondly, some evidence has been presented by Smith *et al* for the participation of disulphide and metal to protein bonding in the association. This suggests that intermolecular bonding in papain may occur through the active site which is known to contain a reactive sulphydryl group[8]. In this event the influence of factors such as pH and added inhibitors on the association could help to elucidate the chemistry of the active site.

Thirdly, an understanding of the equilibria set up in the association of papain could be useful in testing current theories about the transport properties of systems in reversible association equilibrium[1]. Papain appears to be a very suitable protein for such tests because its association can be controlled and if necessary prevented by suitable adjustment of the pH of the solution and the presence of certain reagents[7].

A review of the properties of papain is given in the next section, at the end of which some of the unsolved problems in papain chemistry are pointed out and the structure of this thesis is outlined.

(b) A Review of the Structure and Chemistry of Papain

(i) The Peptide Chain

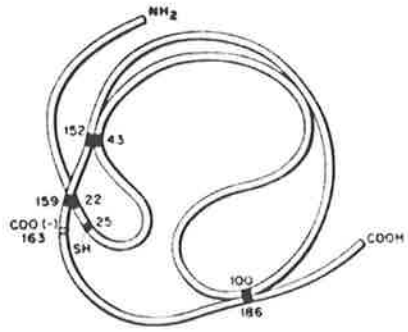
Papain is a globular protein having a molecular weight of about 21,000[9]. The molecule is believed to contain about 200 amino acid residues in a single peptide chain and, as a result of much investigation reviewed by Light *et al*[10], most of these residues can be placed in quite long sequences. The amino acid sequence of papain as far as is known at present is shown in Fig. I-1(a).

The folding of the peptide chain is largely influenced by the positions of three disulphide bonds

NH_2-Ileu-Pro-Glu-Tyr-Val-Asp-Trp-Arg-Gln-Lys-Gly-Ala-Val-Thr-
10
Pro-Val-Lys-Asn-Gln-Gly-Ser-CyS-Gly-Ser-CyS-Trp//Ala-Phe//
20
$(Ileu)_2$//Arg-Asn-Thr-Pro-Tyr-Tyr-Glu-Gly-Val-Gln-Arg-Tyr-CyS-
30 40
Arg-Ser-Arg-Glu-Lys-Gly-Pro-Tyr-Ala-Ala-Lys-Thr-Asp-Gly-Val-
50
Arg-Gln-Val-Gln-Pro-Tyr-Asn-Gln-Gly-Ala-Leu-Leu-Tyr-Ser-Ileu-
60 70
Ala-Asn-Gln-Pro-Ser-Val-Val-Leu-Gln-Ala-Ala-Gly-Lys-Asp-Phe-
80
Gln-Leu-Tyr-Arg-Gly-Gly-Ileu-Phe-Val-Gly-Pro-CyS-Gly-Asn-Lys-
90 100
Val-Asp-His-Ala-Val-Ala-Ala-Val-Gly-Tyr-Asn-Pro-Gly-Tyr-Ileu-
110
Leu-Ileu-Lys-Asn-Ser-Trp-Gly-Thr-Gly-Trp-Gly-Glu-Asn-Gly-Tyr
120 130
Ileu-Arg-Ileu-Lys-Thr-Gly-Asn-Leu-Asn-Gln-Tyr-Ser-Glu-Gln-Glu
140
Leu-Leu-Asp-CyS-Asp-Arg-Arg-Ser-Tyr-Gly-CyS-Tyr-Pro-Gly-Asp-
150 160
Gly-Trp//Ser-Ala-Leu//Val-Ala-Gln-Tyr-Gly-Ileu-His-Tyr-Arg-
170
Gly-Thr-Gly-Asn-Ser-Tyr-Gly-Val-CyS-Gly-Leu-Tyr-Thr-Ser-Ser-
180 190
Phe-Tyr-Pro-Val-Lys-Asn-COOH

(a)

--Tentative amino acid sequence of papain. Amino acid residues placed in sequence are separated by dashes; slant lines indicate unassigned peptides whose relative positions are not yet established.



(b)

--A diagramatic representation of the structure of papain, illustrating the relative positions of the disulfide bridges, the active sulfhydryl group, and the aspartyl group at position 163.

Fig. I-1 The Peptide Chain of Papain
(from Light et al.[10])

between half-cystine residues. The positions of these bonds have recently been located by hydrolysis of a papain derivative containing intact disulphide bridges[11]. Hence a diagrammatic representation of the folding of the peptide chain is now possible and is given in Fig. I-1(b). The gross picture of papain[10] is that of a compact molecule as can be observed from the folding back of the chain to form each disulphide bridge. One can visualize the occurrence of strong electrostatic and hydrophobic forces along the polypeptide chain producing an extremely compact structure. Such a structure is in accord with optical rotation studies of papain in denaturing systems which showed that only a small change occurred in the transition from the native to the denatured state[12].

(ii) <u>The Active Site</u>

The existence of a free sulphydryl group in the active site of papain has been established for many years and been demonstrated in several ways. Papain exhibits maximum proteolytic activity only in the presence of both a reducing agent such as cysteine and a metal complexing agent such as ethylene diamine tetra-acetic acid (EDTA)[13]. That these activators do not act as coenzymes was shown by Finkle and Smith[8] who used a reducing and deionizing column to prepare papain which was active in the absence of

activators. These observations suggested the presence in active papain of a sulphydryl group which must be freed from a reversibly blocked state such as disulphide or metal mercaptide.

The necessity for the free SH group is supported by inhibition studies which have shown that sulphydryl reagents such as p-hydroxymercuribenzoic acid (PHMB) inhibit the active enzyme. In fact Sanner and Pihl[14] showed the inhibition to be an almost linear function of the amount of PHMB added, and also demonstrated that papain combined with this reversible inhibitor was protected from irreversible inhibition e.g. by ionizing radiation[15,16]. Furthermore, kinetic studies[17,18] of the papain catalysed hydrolysis of synthetic substrates over a wide range of pH have indicated the participation of a sulphydryl group and an ionized carboxyl group. The identification of groups by heats of ionization calculated from titration pK's is however sometimes unreliable[19], and Sluyterman[20] has commented that although the existence of an ionized carboxyl group in the active site seems certain, the kinetic evidence implicating an SH group is less sound.

The position of the active SH group in the polypeptide chain has been found by hydrolysis of a papain derivative in which the appropriate cysteine residue was alkylated, using C^{14}-labelled iodoacetate, to distinguish

it from the other half-cystine residues[11]. Fig. I-1(b) shows that the active SH group is at position 25, and that the disulphide bridge between positions 22 and 159 probably brings it close to the carboxyl group of the aspartic acid residue in position 163.

The participation of an imidazole group of histidine in the catalytic mechanism has recently been suggested from kinetic results[20,21]. This idea is supported by photochemical inactivation studies[22] which also indicated that one tryptophan residue may be essential. The latter postulate is interesting since there is a tryptophan residue at position 26, adjacent to the active SH group, and another at position 165 which may also be near the active site. It is more difficult to visualize either of the histidine residues at positions 106 and 175 being near the active SH group in the folded structure shown in Fig. I-1(b).

It is considered likely that the active SH group acts on the substrate by forming an acyl intermediate[23]. Lowe and Williams[24] have recently proposed a mechanism in which the acylation, or formation of a thiol ester, is followed by a deacylation, both steps being catalysed by an imidazole group. An ionized carboxyl group is believed to assist deacylation probably by interacting with another part of the substrate molecule and thereby specifically

orienting the thiol ester bond. This mechanism is however only tentative.

The active SH group of papain reacts much faster with inhibitors than does the SH group of cysteine[8,25], an observation which led Smith[26] to suggest that it might exist in the enzyme in a "high energy" bond of the thiol ester type. However this hypothesis has been discounted on the basis of chemical evidence that thiol esters are not cleaved by PHMB[27], and studies of the ultraviolet difference spectra of papain in the presence of an activator (cyanide) and a thiol ester reagent (hydroxylamine)[28]. It seems more likely that the high reactivity of the active SH group of papain is due to the electronic effects of neighbouring groups on the enzyme surface.

(iii) Sulphur Content

The sulphur content of papain, which is important in view of the sulphydryl nature of the enzyme and the structural influence of the disulphide bridges, has proved difficult to determine. Elementary analysis[8,13] showed 1.2 - 1.3% of sulphur, corresponding to 8 atoms of this element per molecule of papain, but most reported determinations of the number of half-cystine residues in papain have given a figure of less than 8. The most recent and reliable analysis appears to be that reported by Glazer and

Smith[29] who, using an amino acid analyzer to determine cysteic acid in hydrolysates of performic acid oxidized papain, found 7.0 ± 0.2 moles per mole of papain. The same investigators have shown, using a coloured disulphide compound, that papain contains 7.8 ± 0.2 groups capable of undergoing a disulphide interchange reaction. It is not clear why this figure should differ from the number of half-cystine residues, but Glazer and Smith have emphasized that the disulphide interchange method gives high results with some other proteins. Since no other sulphur containing amino acid or inorganic sulphate could be detected in papain[9] the sulphur analysis is also not fully explained.

The distribution of half-cystine residues between the reduced (sulphydryl) and oxidized (disulphide) forms has also been the subject of much investigation. Balls and Lineweaver[30] first reported a result of one SH group per mole of papain by iodine titration of papain and iodoacetate-inhibited papain. Their calculations were based however on an incorrect molecular weight for papain, and subsequent recalculation by Kimmel and Smith[31] showed that only 0.6 to 0.8 equivalents of iodoacetate reacted. Numerous other methods have since yielded a figure between zero and 1.0 for the number of sulphydryl groups in papain.

Finkle and Smith[8] estimated free SH groups in papain which had been activated by use of a mixed bed reducing and deionizing column. When the enzyme in eluates from this column was treated with sulphydryl reagents, e.g. PHMB and iodoacetamide, it was observed that 0.4 to 0.8 molar equivalents of a reagent reacted rapidly with papain completely inhibiting it. The combining ratio varied with the papain preparation. In the same paper it was noted that prolonged treatment with PHMB in 70% ethanol produced a papain derivative containing 6 molar equivalents of the mercurial. It was concluded that papain had 6 SH groups, most of which were "masked" or buried inside the enzyme. In a later paper[29] however this high combining ratio under such severe conditions was attributed to reactions of PHMB with other groups, such as the cleavage of disulphide bonds commented on by Cecil and McPhee[32].

Glazer and Smith[29] estimated the SH content of papain by spectrophotometric titrations with PHMB and N-ethyl maleimide, and by amperometric silver ion titration. They found that unreduced papain (i.e. papain not subjected to any activating procedure) contained 0.1 to 0.4 SH groups per molecule. The SH titre was not increased by denaturation, thereby precluding the presence of masked SH groups. With column-reduced papain the SH titre increased to 0.5 to 1.0 groups, this figure also being unaffected by denat-

uration. Sanner and Pihl[14] have also performed spectrophotometric PHMB titrations of unreduced papain, and found 0.1 to 0.5 SH groups per mole in different preparations.

One remarkable feature of all these results is the variability of sulphydryl content in different papain samples, a finding which assumes more significance in view of the proportionality which has been noted between sulphydryl content and proteolytic activity of papain[8,14]. The sulphydryl analyses also support the hypothesis that in native papain some of the active SH groups are in a reversibly blocked state which can be restored to the sulphydryl form by some reducing agents. These points will be discussed more fully in Chapter III in connection with the heterogeneity of papain.

The number of disulphide bonds in the intact papain molecule has been determined by Glazer and Smith[29] by amperometric titration with silver nitrate in the presence of sulphite[33]. This method showed 2.4 to 3.0 disulphide bonds in native or urea-denatured papain. However 3.8 disulphide bonds were titrated in acid-denatured papain.

It is therefore apparent that the structure of papain represented by Fig. I-1, which has 7 half-cystine residues forming 3 disulphide bridges and the active SH

group, is in accordance with most analyses of the sulphur distribution, but it is possible that there is one more sulphur atom in papain.

(iv) Compounds with Mercury

A crystalline mercuric complex of papain was first prepared by Kimmel and Smith[13]. This compound, mercuripapain, was found to have the empirical formula $Hg(Papain)_2$. It was enzymically inactive, but the usual activating agents, cysteine and EDTA, restored the activity presumably by removing the mercury. A structure of the form Papain-S-Hg-S-Papain was suggested in accordance with the well known affinity of the Hg^{2+} ion for SH groups. However native papain contains only a small proportion of SH groups reactive towards reagents like PHMB; the formation of a dimeric mercaptide in high yields therefore seems unlikely.

A 1:1 complex of papain with mercury has been prepared by Finkle and Smith[8] using a mixed bed column. Attempts to crystallize this compound yielded only precipitates of the 1:2 derivative, mercuripapain. Finkle and Smith considered that the latter was probably the least soluble of several possible mercury compounds of papain. Kimmel and Smith[9] have since reported that the mercury content of a mercuripapain preparation appears to

be variable and related to the specific enzymic activity. It was suggested that the original mercuripapain may not have been a dimer but instead mixed crystals of papain and the 1:1 complex.

It now seems unlikely that in mercuripapain the mercury is bound specifically at the active site. Recent X-ray crystallographic work[34] on mercuripapain has shown that the mercury is not incorporated into the papain crystal at specific sites. This is not unexpected since the complex is prepared in 70% ethanol. It has been mentioned in section (iii) that under these conditions the reaction of PHMB with papain is apparently unspecific.

(v) Solution Properties

Physical studies by Smith *et al*[7] have shown that twice recrystallized papain gives single peaks in the ultracentrifuge and in the electrophoresis apparatus. Electrophoresis of papain in univalent buffers at an ionic strength of 0.1 showed its isoelectric point to be at pH 8.75. This high pH is consistent with the predominance of basic amino acids in papain[9].

Although under certain conditions the sedimentation behaviour of papain indicated the presence only of monomers, the enzyme generally displayed a tendency to associate reversibly. This topic will be taken up in

Chapter II, but it must be mentioned at this stage that Smith *et al* found the reagents cysteine and EDTA to be capable of suppressing the association under some conditions. It was therefore suggested that intermolecular disulphide and metal to protein bonds were formed in the association of papain.

The molecular weight of papain was found to be 21,000 from sedimentation and diffusion data obtained under non-associating conditions. This is in excellent agreement with the molecular weight of 20,900 found from amino acid analysis[9]. A determination of the molecular weight by the approach to equilibrium method in the ultracentrifuge has yielded a figure of 20,700[35].

(vi) Some Unsolved Problems

Whereas it can be realized from the foregoing account that the structure of papain is by no means obscure and is better understood than that of many proteins, several investigators have pointed out still unanswered questions about the chemistry of this enzyme. Outstanding among these are (1), the nature of the "blocked" SH groups apparently present in a fraction of the molecules in any papain sample, and (2), the chemical sequence of events associated with the activation of papain.

(1) Blocking of the SH Group

Since the SH titre of papain preparations can be increased by reaction with a thioglycollate resin[8], excess of a small molecular thiol[36], or sodium borohydride[29], the blocked SH groups are probably in an oxidized state. This is further supported by the observation that the activity of papain can be increased by electrolytic reduction[37]. Again if activity is used as an index of sulphydryl content, it can be demonstrated that papain is reversibly oxidized by hydrogen peroxide and reduced again by cysteine[14,29]. All these observations seem consistent with the oxidation of a sulphydryl compound to a disulphide, a well recognized reaction. However the difficulty[28] with this mechanism is in finding a partnering sulphur atom which could form a disulphide bond with the active SH group.

Sedimentation studies[7] have shown that although papain undergoes reversible association the predominant species in solution must be the papain monomer except perhaps at pH values near its isoelectric point at concentrations above 0.5 g/100 ml. Hence intermolecular disulphide bonding cannot be an important factor in the blocking of SH groups in papain. Intramolecular disulphide bonding seems equally unlikely since no eighth sulphur atom has been found elsewhere in the molecule. More significantly, activator-free, reduced papain, prepared by

reduction with sodium borohydride or on a thioglycollate column, has been found to contain less than one molar equivalent of SH groups. Reduction of an intramolecular disulphide bond should yield two SH groups. On the other hand the presence of a fourth disulphide bond in papain would be consistent with the results cited in section (iii) for the sulphur analysis and some of the disulphide estimations.

A mixed disulphide of the type PROTEIN-S-S-R, where R is a small molecular group has recently been described in a streptococcal proteinase[38]. Glazer and Smith[29] have pointed out that the existence of a bond of this type in papain could explain some of the peculiarities in the activation process. However there are experimental reasons for doubting the importance of this type of group in papain.

First, an analysis for mixed disulphide in papain has been performed by Glazer and Smith[29] who oxidized papain with performic acid and examined the products by paper electrophoresis. The only products were oxidized papain and a small amount of cysteic acid. Thus papain does contain a mixed disulphide with cysteine but it was considered that the amount was too small to account for the variability in SH titre. Secondly, the detection of less than one molar equivalent of SH groups after reductive

activation is inconsistent with the formation of PAPAIN-SH and R-SH. Although this could be rationalized by assuming the latter compound to be volatile like the corresponding product of streptococcal proteinase, the explanation of some other points would become difficult. Specifically, it would be difficult to account for the sulphur analysis of column-reduced papain[8] which still indicated a content of 8 sulphur atoms per papain molecule, or for the reversible inactivation of borohydride-reduced papain by hydrogen peroxide, if the RSH compound had been lost from the solution. Hydrogen peroxide inactivation was shown[29] not to cause dimerization of papain. Thus an explanation other than intermolecular or mixed disulphide formation is required.

It has been postulated several times[26,28,29] that the inactivation of papain may be due to conversion of the SH group to a higher oxidation state corresponding to a sulphenic acid (RSOH) or a sulphinic acid (RSO.OH). However no evidence has been reported to either support or dispute this hypothesis.

The position is further complicated by the likely existence of another oxidized form of papain which cannot be reduced to the sulphydryl form by the usual activators. This suggestion, which was first based on the variability in the SH titre of reduced papain will be considered more

fully in Chapter III. Recently Sanner and Pihl showed that inactivation of papain by hydrogen peroxide[14] and X-ray irradiation[16], though largely reversible in the early stages, becomes irreversible on prolonged or excessive exposure to the cause of inactivation. Reaction with the SH group was clearly shown to be the cause of inactivation by the protecting effect of a number of sulphydryl reagents including PHMB.

To summarize this first problem it can be said that the active site of papain contains a sulphydryl group which is partly free and partly bound in other linkages which probably correspond to higher oxidation states of sulphur. The nature of these linkages is not yet defined.

(2) Mechanism of Activation

The closely related problem of the mechanism of the activation process is also basic to an understanding of the mode of action of papain. Although the usual activators, cysteine and EDTA, undoubtedly act by liberating in papain active SH groups which have become oxidized or bound to metal ions, there are some grounds for believing that this is not the only effect of activators.

Finkle and Smith[8] found that the activity of column-reduced papain was increased by cysteine addition. This was noted by Sanner and Pihl[14] who further showed that

different sulphydryl compounds activated papain to different extents. The latter authors also showed that the specific activity of papain in the absence of cysteine was only about 60% of that found by Finkle and Smith for papain of corresponding sulphydryl content when assayed in the presence of cysteine. Sanner and Pihl extended these observations to a systematic study of the effect of cysteine on the kinetic parameters for the papain-catalysed hydrolysis of a synthetic peptide. Their results showed that activation by cysteine was not only due to the liberation or more SH groups in papain but also to an increase in the rate of cleavage of the enzyme-substrate complex. It was suggested that an enzyme-substrate-activator complex was formed which was then cleaved with a higher rate constant than the enzyme-substrate complex.

However conflicting evidence has been obtained by Whitaker and Bender[39] who, using activator-free reduced papain[36], found little or no effect of cysteine on kinetic parameters for papain-catalysed hydrolyses. Thus the activating effect of thiols on papain is still not entirely understood. Even the reductive part of this process may not be a simple equilibrium reaction of the form

$$2\ PS_{ox} + 2\ RSH \rightleftharpoons 2\ PSH + RSSR$$

where PS_{ox} denotes papain with the SH group reversibly

oxidized. Carty and Kirschenbaum[40] consider that an equilibrium of this type is not in accord with the feeble inhibiting effect of a disulphide on papain. They have suggested that the activation and inactivation of papain may proceed by different mechanisms.

(c) Outline of the Present Investigation

As mentioned earlier, the initial aim of this investigation was to obtain quantitative molecular weight data which might help to elucidate the mechanism of association of papain. This, it was thought, might yield information about groups in the active site and the mechanism of activation. Since Smith *et al*[7] found that the usual activators of papain also suppressed its reversible association under certain conditions, it appeared that the phenomena of activation and association of papain were closely related.

Accordingly the first stage in this investigation was a qualitative reinvestigation of the sedimentation behaviour of papain in the ultracentrifuge. This work is described in Chapter II. A discussion of some optical corrections which appear to be pertinent to the accurate determination of sedimentation coefficients of small protein molecules such as papain has been placed in a separate appendix.

Some notable differences were observed in the sedimentation behaviour of papain from that reported by Smith *et al*[7]. In particular, it is shown that the active SH group is not required for association and that cysteine and EDTA did not affect the association of papain under the conditions studied. Thus it is doubtful whether association studies can help to elucidate the activation process. However it is shown that slight differences in the nature of groups near the active site can noticeably influence the association of papain. Since the active SH group of papain is believed to exist in other chemical states, it is the author's opinion that any study of the association behaviour of papain requires first an understanding of what different chemical states of papain exist and, if possible, a separation of the different forms. The rest of this thesis is concerned with an investigation of these topics.

Thus Chapter III deals with experiments designed to demonstrate any possible heterogeneity of papain with respect to sulphydryl content, by electrophoresis of a papain derivative prepared by modification with p-chloromercuribenzene sulphonic acid (PCMS). The results provide strong evidence for the existence of sulphydryl and non-sulphydryl forms in papain isolated by the usual method[41]. However a clear-cut preparative fractionation could not be

achieved by electrophoresis. It was considered that this failure, together with some other peculiarities in the electrophoresis of papain and PCMS-papain, was a possible sign of a chemically interacting system. Accordingly part B of Chapter III comprises a discussion of the electrophoretic behaviour of interacting systems, and the possible relevance of some theories to the results presented earlier in the same chapter.

Chapter IV is an account of attempts to separate papain into two forms by chromatography. However, although the results of this work were consistent with the electrophoretic results, the separation of components was even poorer than in electrophoresis. Thus this thesis supports the hypothesis of sulphydryl heterogeneity in papain but does not provide a practicable method for fractionating papain into active and inactive species.

Finally in Chapter V the contribution of the present study to an understanding of the chemistry of the active site of papain is discussed.

CHAPTER II

THE ASSOCIATION BEHAVIOUR OF PAPAIN IN SOLUTION

(a) Summary of Previous Work

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    (3) Effect of Cysteine

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  (iii) Sedimentation of Papain at pH 8

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    (2) Effects of Activators and Inhibitors

    (3) Detailed Consideration of Boundary Shapes

    (4) Size and Nature of the Aggregates

(e) Conclusions

(a) Summary of Previous Work

From the preceding chapter it is evident that the physico-chemical studies of papain reported by Smith et al[7] form the basis for interpreting much of the chemical data on this enzyme. These investigators recognized association behaviour of papain from sedimentation velocity experiments.

It was found that native papain in buffer solutions of pH 4.0 and 5.4 gave a single sedimenting boundary; the behaviour was characteristic of a rapidly reversibly associating system (see next section) in that the sedimentation coefficient increased with increasing protein concentration in dilute solution. At pH 4.0 this concentration dependence of the sedimentation coefficient was abolished by the presence of 0.02 M cysteine. It was thus inferred that papain underwent association at pH 4.0 by the formation of intermolecular disulphide bonds. The reducing agent cysteine would be expected to suppress formation of this type of bonding. At pH 8 the association of papain was more noticeable, a second, faster-moving, peak being observed in the sedimentation pattern. Considerable disaggregation was apparent in a solution to which 0.001 M EDTA had been added, thereby indicating the participation of a metal ion in the association at pH 8. The metal ion was not identified.

The sedimentation behaviour of the 1:2 mercuri-papain complex was also examined. At pH 4 this derivative gave unimodal sedimentation patterns with no sign of association. However at pH 8 mercuripapain exhibited a bimodal sedimentation pattern, the fast component being more prominent and having a higher sedimentation coefficient than in the case of papain.

For the several reasons mentioned in the last chapter it was intended to conduct a detailed study by the sedimentation equilibrium technique of the association behaviour of papain, and later of the more involved mercuripapain system. However, since some of the conclusions drawn by Smith *et al* were based on very few observations, it seemed advisable to first establish with more certainty the conditions under which association of papain occurred. Sedimentation velocity studies were thus undertaken to obtain this qualitative information.

(b) Use of the Sedimentation Velocity Method to Detect Associating Protein Systems

Reversible association of a protein in solution can usually be detected in the ultracentrifuge by determining the weight-average sedimentation coefficient (S) at varying solute concentration (c). Non-associating proteins show a negative concentration dependence of S

resulting from hydrodynamic effects[42]. The variation of S with c can be described by equations of the form

$$S = S^{o}(1 - kc), \quad \text{or} \quad S = \frac{S^{o}}{1 + kc}$$

where S^{o} is the sedimentation coefficient at zero concentration of solute, and k is a positive constant.

It is however found that for some protein systems the sedimentation coefficient increases with concentration. Such behaviour signifies the occurrence of reversible association-dissociation reactions of the form

$$nP \rightleftharpoons P_n$$

It is presumed that increasing solute concentration causes a readjustment of the equilibrium in favour of the larger aggregates which sediment faster, resulting in an increase in the observed sedimentation coefficient.

Clearly if adjustment of the equilibrium is slow in comparison with the rate of separation by sedimentation, then separation of the aggregated from the monomeric form can be expected, giving two boundaries. On the other hand, with rapid equilibria this is not the case. Gilbert[43] has shown that if a rapid reversible equilibrium favours the dimer to the near exclusion of higher oligomers, only a single sedimenting boundary with a positive concentration

dependence of sedimentation coefficient will be observed. However if the equilibrium favours one particular aggregate higher than the dimer, a bimodal sedimenting boundary may be observed regardless of the rate at which equilibrium is adjusted. The proportion of the faster component will increase as the solute concentration is increased and a minimum may be visible in the concentration gradient curve. The gradient however will not drop to zero at any point in the boundary.

Thus to test for association behaviour of a protein by sedimentation velocity, measurements should be made of two characteristics, viz. boundary shape and sedimentation coefficient, both at several protein concentrations. Reversible association is signified by either a bimodal boundary in which the relative size of the faster peak increases with increasing solute concentration, or by a single boundary which is shown to give a positive concentration dependence of the sedimentation coefficient. A number of theories can be applied to deduce further details about the association. Nichol *et al*[1] have mentioned most of these in a recent review.

It is emphasized that the sedimentation coefficient referred to in the foregoing discussion is the weight-average sedimentation coefficient of the species present

in the plateau region[44] of the ultracentrifuge cell. This quantity is strictly found from the rate of movement of a point whose radial position is given by the square root of the second moment of the concentration gradient curve[45]. Calculation of the position of this point is however so laborious that the maximum ordinate of the gradient curve is usually taken as the boundary position in qualitative applications of the sedimentation velocity method. For symmetrical gradient curves little error is incurred by using the maximum ordinate[45].

In the present study the sedimenting boundaries observed with papain at pH 4 and pH 7 were apparently symmetrical (Fig. II-1). Hence the position of the maximum ordinate has been used in the determination of sedimentation coefficients. At pH 8 where the boundary was markedly asymmetrical (Fig. II-5) the shape has been imagined to arise from two symmetrical peaks. The areas of each were measured as described later in this chapter. The sedimentation coefficient of the slow peak was calculated from the movement of its maximum ordinate, and the fast peak was treated similarly whenever sufficiently prominent to be measured.

(c) <u>The Solubility of Papain and the Choice of Experimental conditions</u>

There are few published solubility data on papain,

although it has been noted[7] that electrophoresis of papain at pH values above 7 proved difficult because of the low solubility of this enzyme in the cold in the region of its isoelectric point. In the present study it was found that the experimental conditions used in ultracentrifugal and in later electrophoretic studies (Chapter III) were frequently dictated by the low solubility of papain. The author's observations on the solubility of papain are therefore summarized in Table II-1.

It is generally advisable in transport experiments with charged proteins to use buffer solutions of ionic strength at least 0.1, in order to minimise charge-separation effects[47]. The solubility of papain does not always permit this. In the present study, the highest ionic strengths allowing reasonable solubility of papain have been used even though this necessitated the use of different ionic strengths at different pH's. The alternative would have been to use a single, low ionic strength for all experiments, but this might have produced anomolous charge-separation effects at pH values far below the isoelectric point.

TABLE II-1

Observations on the Solubility of Papain

pH	I	Buffer Composition (molarities)	Solubility (g/100 ml)		Buffer useful for	
			20°C	1°C	Sed.	Elec.
4	0.1	0.10 NaAc 0.43 HAc	~2.0	> 0.5	+,‡	+,‡
4	0.1	0.02 NaAc 0.08 NaCl 0.08 HAc	~1.5	< 0.5	+	-
5	0.1	0.10 NaAc 0.043 HAc	No data	~ 0.5		+,‡
6	0.1	0.011 Na_2HPO_4 0.066 KH_2PO_4	No data	< 0.5		-
6	0.05	0.005 Na_2HPO_4 0.035 KH_2PO_4	No data	> 0.5		+,‡
7	0.1	0.027 Na_2HPO_4 0.018 KH_2PO_4	< 1.0	< 0.25	-	-
7	0.05	0.013 Na_2HPO_4 0.010 KH_2PO_4	~1.8	~ 0.3	+,‡	+,‡

(continued) ...

Table II-1 (continued)

pH	I	Buffer Composition (molarities)		Solubility (g/100 ml)		Buffer useful for	
				20°C	1°C	Sed.	Elec.
8	0.05	0.086 0.050	Tris HCl	~1.0[a]	< 0.1	-	-
8	0.02	0.035 0.020	Tris HCl	~1.2	No data	+,*	

[a] Slow precipitation occurred over a period of 24 hours.

I: Ionic strength

Ac: Acetate, Tris: Tris-hydroxymethyl-aminomethane

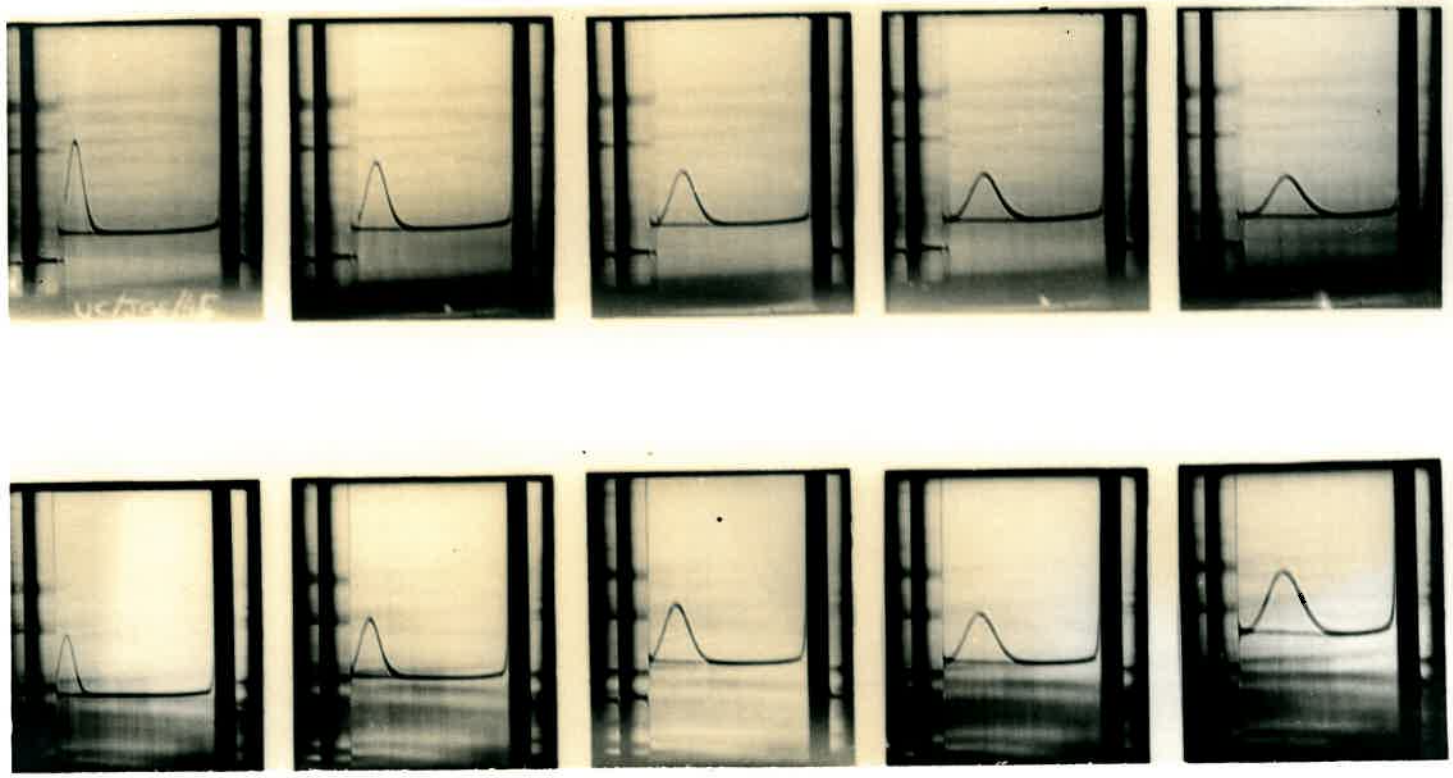
A + sign inserted in the column headed "Sed." or "Elec." denotes suitability of the buffer for sedimentation or electrophoresis respectively. A - sign denotes unsuitability.

* denotes that the buffer was chosen as the most suitable of that pH for sedimentation or electrophoresis of papain. In the text when a buffer is referred to as having a particular pH and ionic strength, its composition will be that of the buffer asterisked in Table II-1, unless otherwise stated.

Explanatory Notes -

Sedimentation velocity studies over a range of solute concentrations require a protein to be soluble to about 1% (w/v) at 20°C. The lowest concentration which can be used with schlieren optics is about 0.1%.

Electrophoresis in the apparatus available (see Chapter VI) is best carried out with approximately 0.5% solutions, but satisfactory results can be obtained at concentrations as low as 0.1%.



(a)

(b)

Fig. II-1 Sedimentation patterns of pepsin, (a) at pH 4, ionic strength 0.1, (b) at pH 7, ionic strength 0.05. The first photograph in each series was obtained [illegible] ca. 20 minutes at 59,780 r.p.m., the others at 12 minute intervals later the first. The initial pepsin concentration was ca. 1.1 g/100 ml in both experiments. In (a) the diaphragm angle was the same (70°) for all photographs; in (b) it [illegible] 72° to 55° during the run. The direction of sedimentation is from left to right.

(d) Experimental Results and Discussion

(1) Sedimentation of Papain at pH 4

A typical series of photographs from a sedimentation velocity run at pH 4 is shown in Fig. II-1(a). Details of the methods and controls in sedimentation velocity experiments and of the calculation of sedimentation coefficients are given in Chapter VI. In accordance with the usual procedure, sedimentation coefficients were corrected to a standard basis corresponding to a solvent with the viscosity and density of water at 20°C. Corrections were made using the equation[48]

$$S_{20,w} = S \frac{\eta}{\eta_{20,w}} \frac{(1 - \bar{v}\rho_{20,w})}{(1 - \bar{v}\rho)} \quad \text{.... II-(1)}$$

where $S_{20,w}$ is the corrected or standard sedimentation coefficient,

S is the sedimentation coefficient under the conditions of the experiment,

η and ρ are respectively the viscosity and density of the solvent at the temperature of the experiment,

$\eta_{20,w}$ and $\rho_{20,w}$ are the corresponding quantities for water at 20°C,

and $\bar{v}$ is the partial specific volume of the protein in the solvent used.

Determination of the required viscosities and densities is described in Chapter VI. The figure used for $\bar{v}$ was 0.724, calculated[49] from the amino acid composition of papain. This figure may be slightly inaccurate since subsequent work[10] has given a revised estimate of the amino acid composition. Error could also arise from the assumption that $\bar{v}$ was the same in all buffers and at all protein concentrations. However provided that ρ is not too different from $\rho_{20,w}$, $s_{20,w}$ calculated from equation II-(1) is not sensitive to a small error in $\bar{v}$. Evaluation of a few results using $\bar{v}$'s of 0.724 and 0.75 for papain gave the same $s_{20,w}$ figures within 0.01 S. Nevertheless in experiments at 5°C a temperature correction was applied to $\bar{v}$ by assuming $\frac{d\bar{v}}{dT} = .0005$ ml/gm/°C[50].

(1) Concentration Dependence of $s_{20,w}$

The variation with initial solute concentration of the standard sedimentation coefficient of papain in acetate buffer of pH 4.01 and ionic strength 0.1 is shown in Fig. II-2. The concentration of the most concentrated solution was determined by refractometry and the other solutions were prepared by dilution of this solution with diffusate. Dilutions were made by weight, the densities of all aqueous solutions being taken as 1 g/ml. The error lines shown on the points in Fig. II-2 are 95%

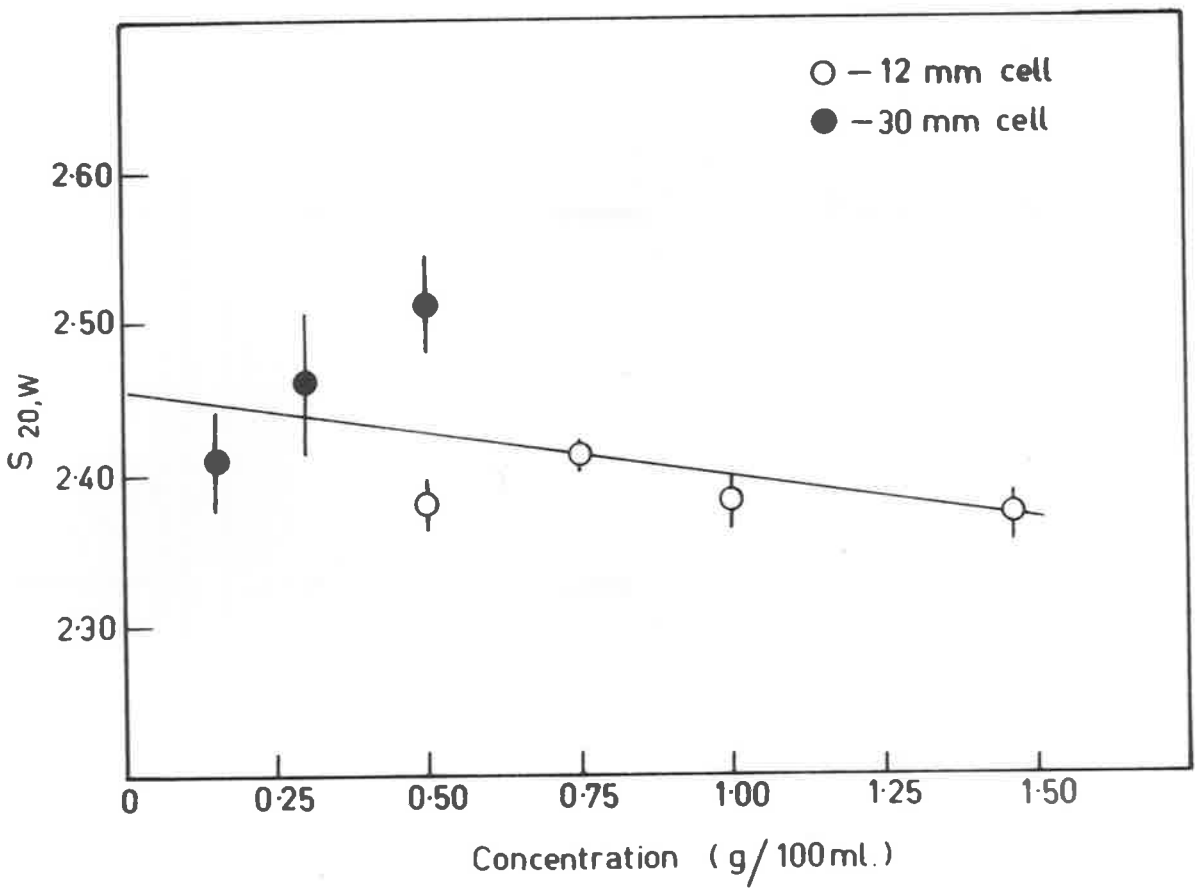


Fig. II-2 Variation of the standard sedimentation coefficient with concentration for papain in acetate buffer pH 4, ionic strength 0.1.

fiducial limits calculated from the regression of the logarithm of the boundary position on time (see Chapter VI).

It can be seen from Fig. II-2 that, over most of the concentration range 0 - 1.5%, $S_{20,w}$ decreases with increasing c. Thus, reversible association of papain either does not occur or is so slight that it is masked by hydrodynamic effects. The three points represented by solid circles appear to imply a slight positive slope at low concentrations. However, these points correspond to determinations in the 30 millimetre light path ultracentrifuge cell. It is considered that, in view of optical corrections (see Appendix) which assume importance when 30 mm cells are used in the determination of sedimentation coefficients of small proteins like papain, these points should not be given undue emphasis.

The straight line in Fig. II-2 corresponds to the equation

$$S_{20,w} = 2.46 - 0.059\ c \qquad \ldots\ \text{II-(2)}$$

which is obtained by a linear regression of $S_{20,w}$ on c. By the conventional statistical methods[51] the standard error in the slope is estimated to be $\pm .04_3$, and that in the intercept at c = 0 to be $\pm .03_9$. An attempt was originally made to use the previously estimated standard

errors in the individual points to obtain a weighted regression line fitting the points in Fig. II-2. However the usual method, that of weighting the points inversely as the square of their standard errors[51], was found to be inapplicable because the estimated standard errors could not satisfactorily account for the observed large deviations of points from the weighted regression line. It is believed that the optical distortion effects discussed in the Appendix may have contributed to the experimental scatter of points.

The extrapolated $S^{o}_{20,w}$ of $(2.46 \pm .03_9)$ S agrees well with the figure $(2.42 \pm .04)$ S found by Smith *et al*[7] for papain at pH 4 in the presence of 0.02 M cysteine. However the results differ from those reported by Smith *et al* in that they indicate no association of papain at pH 4 in the absence of cysteine. Although the evidence presented by the above authors for association under these conditions was slight, since $S_{20,w}$ was determined at only two papain concentrations, some further investigation seemed warranted to see if the two sets of results could be reconciled.

(2) Effect of Low Temperature and Different Buffer Composition

More sedimentation studies were therefore carried out with papain at pH 4, but employing lower temperature and different buffer composition. By analogy with the

known behaviour of β-lactoglobulin[3] and insulin[6], it was thought that the use of low temperature might promote association of papain. In addition, since papain tends to precipitate in the presence of chloride ions (see Table II-1 and reference 41), there was some basis for believing that its association might be more pronounced in a buffer containing chloride ions.

Table II-2 shows sedimentation coefficients obtained under the following conditions:

(1) at 5°C in the same buffer as that used in obtaining the results of the previous section,

and (2) at 20°C in a buffer of the same pH and ionic strength, but containing 0.08 M sodium chloride.

TABLE II-2

Effect of Temperature and Buffer Composition on the Sedimentation of Papain at pH 4.00 ± .01 and ionic strength 0.1

C (g/100 ml)	T (°C)	Buffer Composition (molarities)	$s_{20,w}$ obs. (S)	$s_{20,w}$ calc.[a] (S)
0.96	5.3	0.10 NaAc	$2.37 \pm .00_9$	2.40
0.67	5.3	0.43 HAc	$2.36 \pm .02_4$	2.42
1.19	19.9	0.08 NaCl	$2.61 \pm .01_6$	2.39
0.70	19.9	0.02 NaAc 0.08 HAc	$2.57 \pm .02_3$	2.42

[a] Calculated for corresponding papain concentration, at 20°C, in pure acetate buffer of pH 4.0 and ionic strength 0.1, by means of equation II-(2).

It is seen that there is good agreement between sedimentation coefficients obtained at 20°C and 5°C in the same buffer. Thus no association of papain in 0.1 M sodium acetate buffer of pH 4 is induced by lowering the temperature to 5°C. On the other hand the $S_{20,w}$ figures obtained with the buffer containing 0.08 M NaCl are about 0.2 S higher than those obtained with the chloride-free buffer at corresponding papain concentrations in the same ultracentrifuge cell and rotor. This increase, which appears to be greater than experimental error, could arise in at least two ways.

(1) Binding of chloride ions to the positively charged papain molecule. This might cause an increase in the sedimentation coefficient if the bound ion were denser than the solvent[52] or if the protein molecule were enabled to shrink to a more compact shape.

(2) A slight amount of reversible association. Gilbert[53] has recently reported a pronounced acetate buffer effect on the association of human oxyhaemoglobin in neutral and slightly acid solution. Aggregated complexes appeared to dissociate in the presence of high concentrations of acetic acid at constant pH and ionic strength. This is mentioned because the acetate-chloride buffer used in obtaining the data in Table II-2 inevitably contained less acetic acid than did the pure acetate buffer. To test

for the occurrence of a similar phenomenon with papain would require very precise measurements of sedimentation coefficients since the increase is so small.

(3) Effect of Cysteine

From the results of the previous section it appears possible, though unlikely, that the use of an acetate-chloride buffer may have promoted the association of papain at pH 4 reported by Smith et al[7]. (The composition of the buffer is not given in this paper; it is referred to as an acetate buffer of pH 4.0 and ionic strength 0.1). However the suggested mechanism of intermolecular disulphide bonding seems to be inconsistent with the results of the present investigation which indicate little or no association of papain in a sodium acetate-acetic acid buffer at pH 4. It was therefore decided to re-investigate the reported decrease in sedimentation coefficient of papain at pH 4 on cysteine addition.

Fig. II-3 shows standard sedimentation coefficients of papain determined at a constant solute concentration (1.07 g/100 ml) in an acetate-chloride-acetic acid buffer of pH 4.00 with concentrations of cysteine varying from zero to 0.08M. The solutions were prepared by mixing more concentrated stock solutions of papain and cysteine, the volumes being measured with a micrometer syringe. To

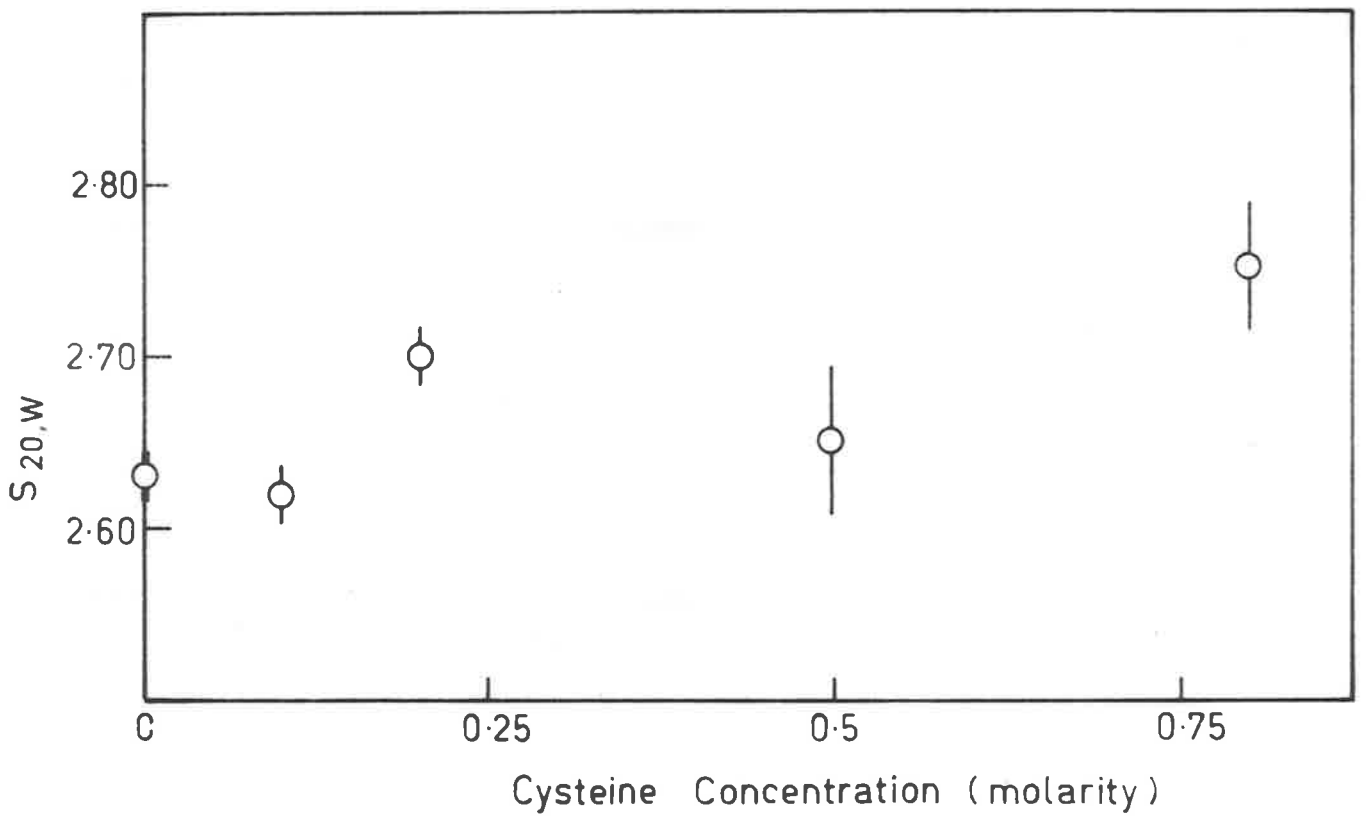


Fig. II-3 Effect of added cysteine on the standard sedimentation coefficient of papain (1.07 g/100 ml) in acetate buffer pH 4.01, ionic strength 0.1.

prevent autolysis of papain in the presence of the cysteine activator[8], the mixtures were made immediately before commencing each sedimentation experiment. As a precaution against oxidation of cysteine, the preparation of and all manipulations with this solution were carried out in a nitrogen-filled box. The stock cysteine solution was stored in a refrigerator under a nitrogen atmosphere. At the beginning and completion of the above experiments its free sulphydryl content was checked by iodine titration[54]. It was found that less than 6% of the initial SH titre was lost during the several days of storage.

Fig. II-3 shows that $S_{20,w}$ is substantially unaffected by cysteine under the foregoing conditions, there being only a small increase of about 0.1 S as the concentration of cysteine is varied from zero to 0.08 M. This almost insignificant increase is not necessarily due to added cysteine as the ionic strength and sodium chloride concentration were not constant in this set of experiments. Cysteine was added in the form of the more stable hydrochloride neutralized to pH 4, which meant that as the cysteine concentration was varied from zero to 0.08 M, the sodium chloride concentration changed from 0.04 to 0.12 M and the ionic strength changed from 0.08 to 0.16.

Since cysteine addition caused no decrease in the standard sedimentation coefficient of papain in the

acetate-chloride buffer at pH 4, there is no basis for believing that intermolecular disulphide bonding occurs in this buffer. The papain system at pH 4 does not seem to warrant a more detailed study by the sedimentation equilibrium method since any association which does occur is hardly detectable. Accordingly it was decided to make further qualitative studies at pH 7. It has been reported[7,9] that association at this pH is more pronounced even though a single peak is still observed in the sedimentation pattern.

(ii) Sedimentation of Papain at pH 7

Fig. II-4 shows the variation of $S_{20,w}$ with initial solute concentration for papain in phosphate buffers of ionic strength 0.05 and pH 7.00 to 7.05. Solutions were prepared by weight-dilution as described for the corresponding set of runs at pH 4. The points in Fig. II-4 were obtained in two separate series of runs. The first, represented by circles, was with a commercial papain preparation[55] which was later found by electrophoresis to contain about 4% of impurities. Subsequently the series was repeated using a papain sample prepared by the author using a method (see Chapter VI) which had been shown to give electrophoretically homogeneous samples. The second set of results, represented by squares in Fig. II-4, is

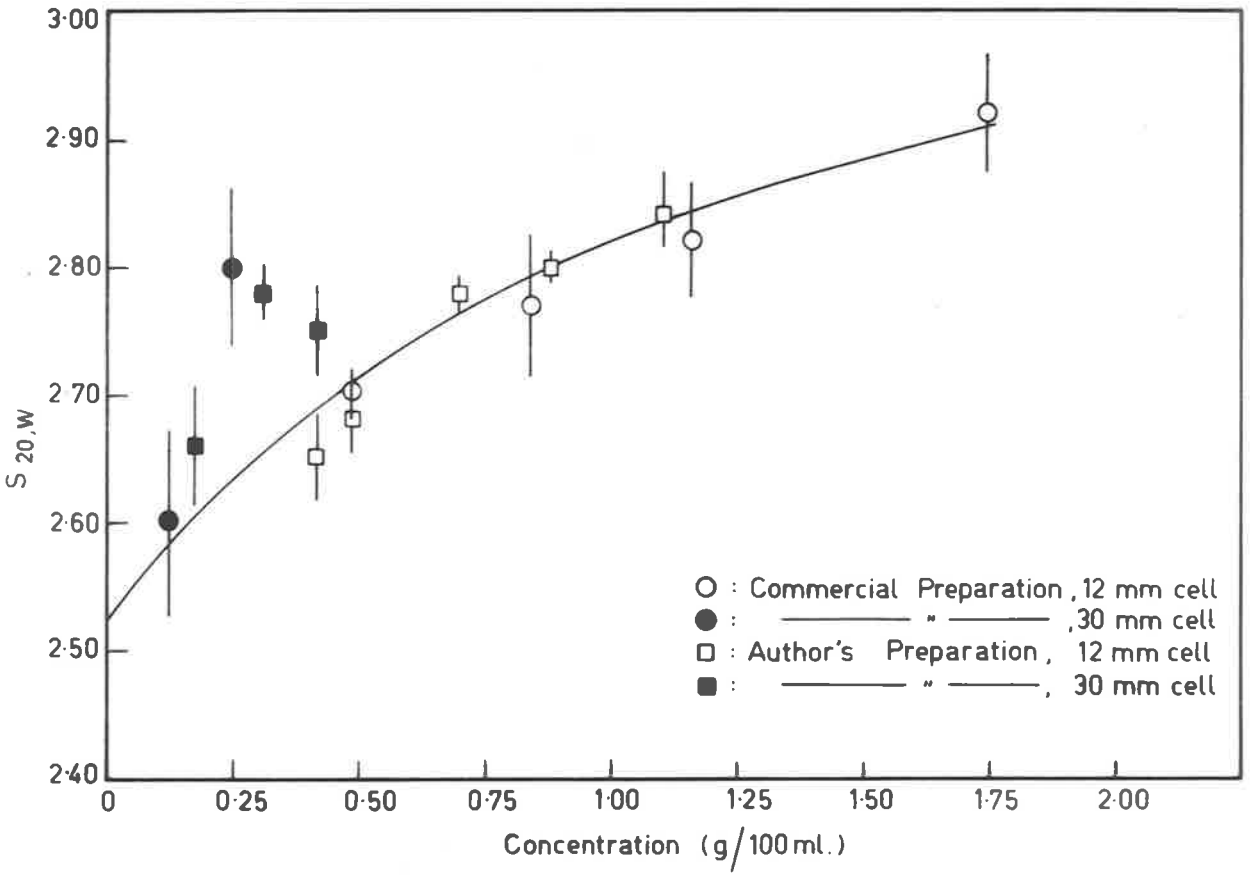


Fig. II-4 Variation of the standard sedimentation coefficient with concentration for papain in phosphate buffer pH 7.00-7.05, ionic strength 0.05.

seen to agree closely with the first. An increase of $S_{20,w}$ with papain concentration is evident in both cases. Thus it appears that papain undergoes a rapid reversible association under these conditions.

Extrapolation of the curve in Fig. II-4 to zero papain concentration is difficult because of the experimental scatter of points at low concentration. However it appears that $S^{o}_{20,w}$ would be approximately 2.5 S which is close to the figure $2.46 \pm .03_9$ found at pH 4. As was the case at pH 4 there seems to be a discontinuity in the graph at the point where a change was made to a longer light path cell and rotor. Again two runs were done at the same concentration to confirm that the effect was an artifact and not due to a change in the properties of the sedimenting material. Possible causes of this discontinuity are discussed in the Appendix.

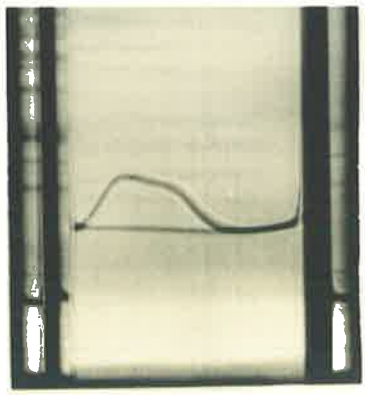
Although the association of papain at pH 7 still appears slight, and therefore difficult to study quantitatively, this system does seem a possible subject for further investigation by the sedimentation equilibrium technique. However it was decided to first proceed with sedimentation velocity experiments at pH 8 which, as mentioned in section (a), are reported to give bimodal reaction boundaries with papain. Such a system would be more suitable for studies of the effects of specific reagents on the association

since any change in the amount of association should cause a change in the shape of the sedimenting boundary. This should be a more readily observable and measurable change than a small difference in sedimentation coefficient which is all that could be expected at pH 7.

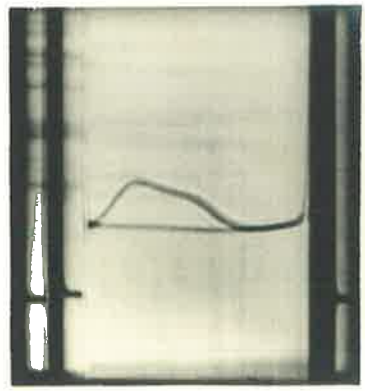
(iii) Sedimentation of Papain at pH 8

(1) Concentration Dependence of Sedimentation Characteristics

The sedimenting boundaries observed with papain solutions of four different concentrations at pH 8.00 and ionic strength 0.02 are shown in Fig. II-5. A tris-hydroxymethyl-aminomethane buffer was used, and the more dilute papain solutions were prepared from the most concentrated by weight dilution in the manner previously described. It is seen that at the highest concentrations (which were dictated by the solubility of papain), aggregation is clearly indicated by a prominent shoulder on the leading edge of the slow peak. As the solute concentration is decreased the shoulder diminishes in the manner characteristic of a reversibly associating system (section (b)). This behaviour is apparently in agreement with that reported by Smith *et al*[7] for papain at pH 8 in a tris buffer of unspecified ionic strength, although no photographs of the sedimentation patterns under the above conditions were published. Their paper gives only

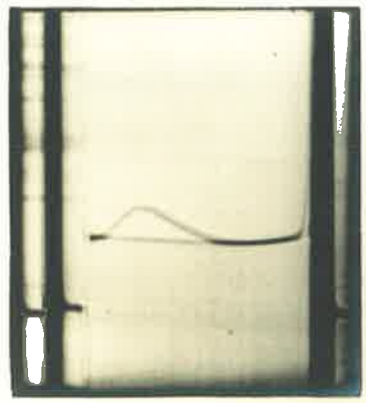


(a)



(b)

(c)



(d)

Fig. II-5 Sedimentation patterns of papain in tris buffer pH 8.00, ionic strength 0.02. Initial papain concentrations in g/100 ml. (a) 1.17, (b) 0.98, (c) 0.70, (d) 0.50.
All the above photographs were taken with a diaphragm angle of 55°, after ca. 70 minutes at 59780 r.p.m. The direction of sedimentation is from left to right.

numerical percentage areas for the two peaks with no details of how the area was divided which prevents any strict comparison with the results of the present study.

(2) Effects of Activators and Inhibitors

With the idea of gaining some information about the mechanism of association of papain it was decided to ascertain the effects of certain activators and inhibitors of papain on its sedimentation behaviour at this pH and ionic strength. The effect of cysteine and of EDTA was considered to be of interest in view of the reported ability of these compounds to cause dissociation of papain aggregates as mentioned in section (a). Furthermore from the effect of SH-specific inhibitors on the association it was hoped to determine whether the active SH group was required for association of papain. The alkylating reagents iodoacetamide (IAM) and iodoacetic acid (IAA) were chosen for this purpose for the following reasons:

(1) Their reaction with papain is irreversible and specifically with the SH group[25].

(2) Although very similar reagents, at pH 8 they should differ in charge. IAA should produce a negatively charged site ($-S-CH_2-COO^-$) in the papain molecule, while IAM should produce an uncharged site ($-S-CH_2-CONH_2$). Thus a comparison of their effects could be expected to show whether the association were sensitive to the nature of charged groups at the active site of papain.

The alkylated forms of papain were in each case prepared by incubating a mixture of papain (1 mole), cysteine (15 moles) and EDTA (3 moles) in tris buffer (pH 8, I 0.02) with the inhibitor (25 moles) at 39°C for 60 minutes. More cysteine (11 moles) was then added in accordance with the procedure of Thompson and O'Donnell[56] as a precaution against unspecific alkylation. The resulting mixture, in which the papain concentration was approximately 1.2%, was then transferred to dialysis tubing and dialysed at 4°C against 100 volumes of tris buffer for 48 hours with a change of diffusate after 24 hours. The inhibited preparations were assayed in the usual way (see Chapter VI) and found to retain less than 2% of the activity of the untreated enzyme.

The sedimentation patterns obtained with IAA- and IAM- inhibited papain, and with papain in the presence of cysteine and EDTA are shown in Fig. II-6, together with a pattern obtained with untreated papain. It is evident that the sedimentation pattern is essentially unaffected by the presence of 0.005 M cysteine. Similarly, addition of 0.001 M EDTA has little or no effect, whether the solution is run in the ultracentrifuge immediately after mixing or after being kept for 24 hours at 20°C. The latter result is in disagreement with the effect of 0.001 M EDTA reported by Smith *et al*.[7] Fig. II-6 however shows that the

Fig. II-6 Effects of Activators and Inhibitors on the Sedimentation of Papain at pH 8.

(a) Papain (0.98 g/100 ml)

(b) Papain (0.98 g/100 ml) + cysteine (0.005 M)

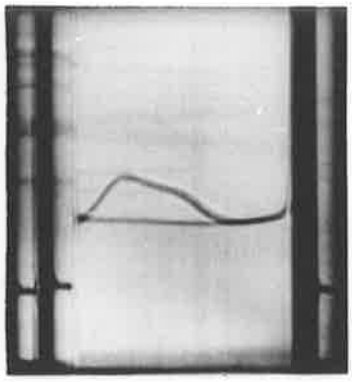
(c) Papain (0.98 g/100 ml) + EDTA (0.001 M)

(d) Papain (0.98 g/100 ml) + EDTA (0.001 M) Solution kept at 20°C for 24 hours after addition of EDTA before ultracentrifuge run.

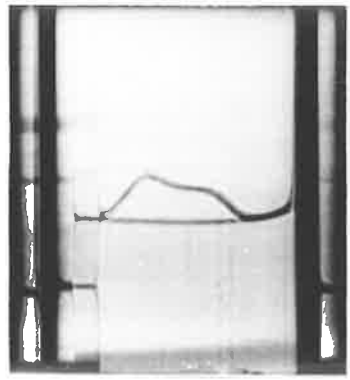
(e) Iodoacetamide-inhibited papain (0.94 g/100 ml)

(f) Iodoacetic acid-inhibited papain (0.99 g/100 ml)

A tris-hydroxymethyl-aminomethane buffer of pH 8.00, ionic strength 0.02 was used in all cases. The photographs shown were all obtained with a diaphragm angle of 55°, after approximately 70 minutes at 59780 r.p.m. The direction of sedimentation is from left to right.



(a)



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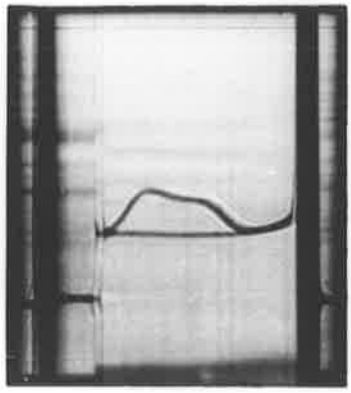
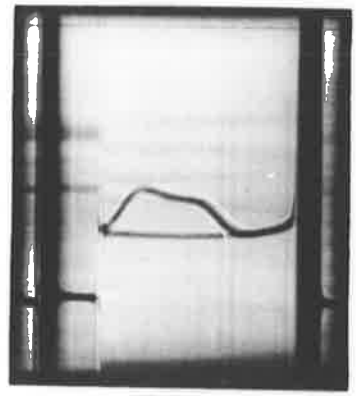
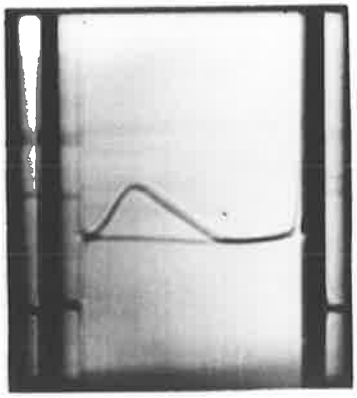


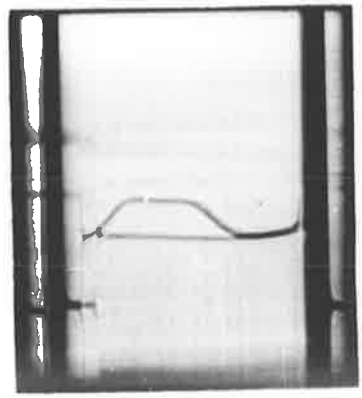
(c)



(d)



(e)



(f)

Fig. II-6

sedimentation behaviour of papain in this buffer is markedly influenced by inhibition and by the nature of the inhibitor. The two inhibitors are seen to exert opposite effects, the uncharged inhibitor (IAM) reducing the prominence of the leading shoulder while the anionic inhibitor (IAA) increases its prominence.

(3) Detailed Consideration of Boundary Shapes

In order to express the sedimentation data on papain at pH 8 in a semi-quantitative manner, the observed boundaries were divided into symmetrical peaks. The method of subdivision used was that outlined by Svedberg and Pedersen[57]. From each run the particular photograph was selected in which the slow peak had moved (0.264 ± .012) cm (distance in the cell) from the meniscus. Thus the resolutions of peaks were comparable and errors due to radial dilution were as similar as possible. An enlarged drawing of the photographed pattern (1 cm : 1 mm along both axes) was made on graph paper from comparator measurements at 0.5 mm intervals along the radial axis. The data was plotted as (peak height - baseline height) against radial distance. A vertical bisector of the back peak was then drawn and a leading edge symmetrical with the trailing edge was constructed. The ordinates of the so constructed leading edge were subtracted from those of the whole

boundary to obtain the trailing edge of the front peak. Since this trailing edge appeared in all cases to be symmetrical with the observed leading edge of the pattern, no further subdivision of the front peak was attempted. The area of each peak was then measured with a planimeter. To minimise personal bias in selecting the position of the bisector of the back peak, the point selected was the boundary position read from the graph used in determining the sedimentation coefficient of the back peak (see Chapter VI). Thus the data from all other photographs from the run was used in selecting this position.

The sedimentation coefficient of the back peak was determined in the usual way although the experimental error was inevitably larger than in those experiments which yielded a symmetrical peak. In patterns such as those obtained with papain at pH 8, in which there is not a distinct minimum between the two peaks, it is of more quantitative significance to determine the weight-average sedimentation coefficient from the entire gradient curve. However when only qualitative information on the association is required, the method used in the present study has the advantage of necessitating less calculation and being in some ways more illustrative. It must be appreciated however that the peak areas obtained in this way are only a means of describing the shape of the boundary and <u>do not</u>

represent the concentrations of aggregated and monomeric species.

The area measurements so obtained from the experiments described in sections (1) and (2) are represented in Fig. II-7 which also shows the sedimentation coefficients. In this diagram the areas of the individual peaks have been plotted against the total area of the corresponding pattern. Since all the photographs were taken with the schlieren diaphragm set at the same angle (55°), the total area is proportional to the papain concentration in the plateau region of the cell. This in turn is proportional to the initial concentration except for small differences in radial dilution, or slight precipitation of papain which occurred in some solutions. For the two lowest papain concentrations, only sedimentation coefficients have been evaluated. The peaks appeared reasonably symmetrical, and no attempt was made to subdivide them since the data, being obtained at a different centrifugal field strength in a different cell, could not be compared with that from other runs. Lines have been drawn in Fig. II-7 connecting points derived from experiments with each particular sedimenting species i.e. papain, IAM-modified papain, and IAA-modified papain. Fig. II-7 illustrates in a semi-quantitative manner the same features which were observed qualitatively from the

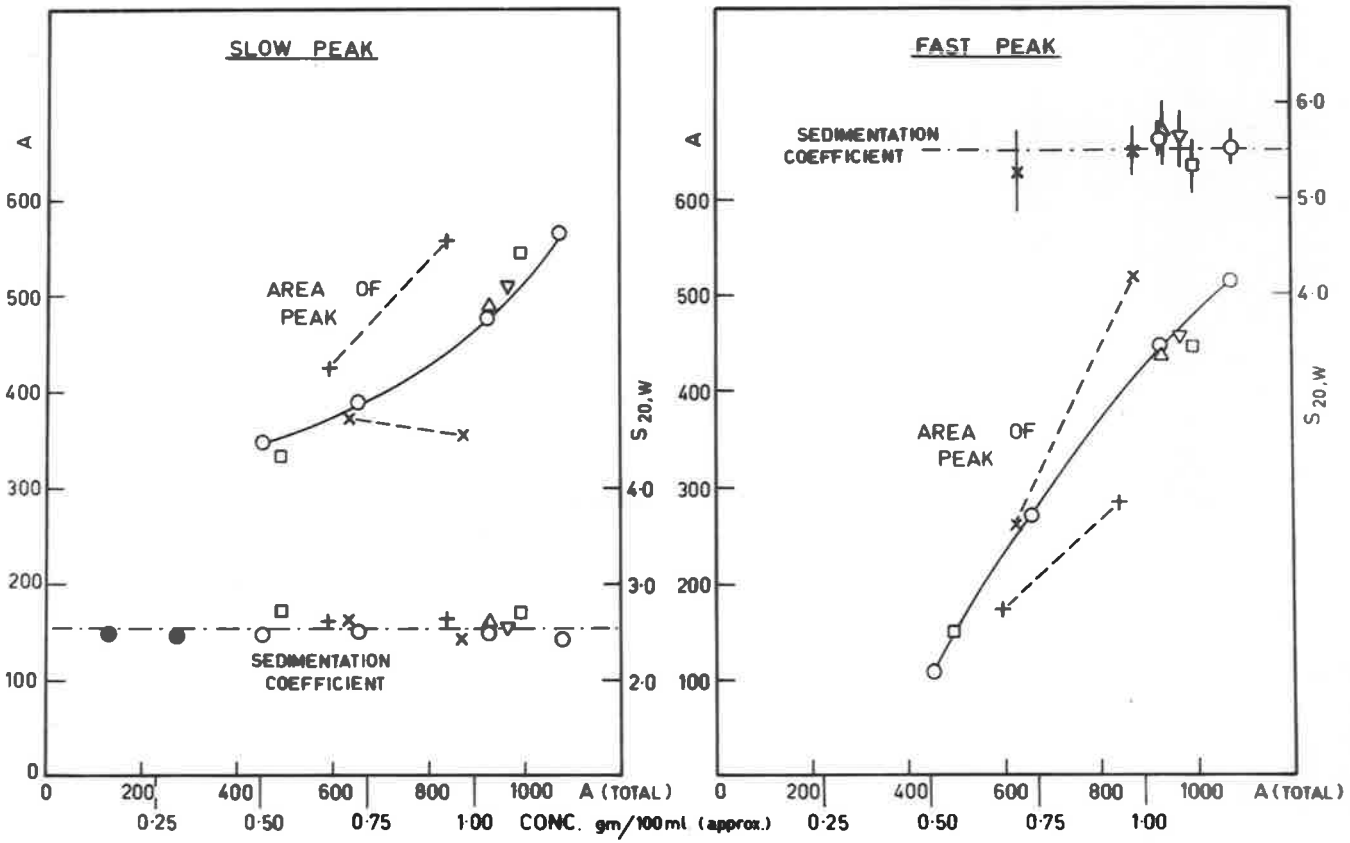


Fig. II-7 Peak areas (arbitrary units) and sedimentation coefficients for papain in tris buffer pH 8, ionic strength 0.02. ○: Papain (12 mm cell), ●: Papain (30 mm cell), □: Papain + .005 M cysteine, Δ: Papain + .001 M EDTA, ▽: Papain + .001 M EDTA (soln. kept for 24 hr. at 20°C before centrifuge run), +: Papain inhibited with iodoacetamide, ×: Papain inhibited with iodoacetic acid (sodium salt).

shapes of the peaks, namely that the extent of association, as judged from the relative areas of the peaks (section (b)), increases with increasing papain concentration, is apparently unaffected by added cysteine or EDTA, is decreased by IAM-modification, and increased by IAA-modification.

The apparent sedimentation coefficient derived from the fast peak is in all cases about 5.4 S. The intrinsic sedimentation coefficient of the largest aggregate in the solution cannot be less than this[58]. Since this sedimentation coefficient of the largest aggregate is more than twice that of the papain monomer (2.5 S), its molecular weight must also be more than twice that of papain, assuming there is not a gross shrinkage or a configurational change sufficient to make the frictional coefficient[59] of the aggregate less than that of the monomer. In other words, aggregates larger than a papain dimer are almost certainly formed in this system.

The absence of a distinct minimum in the concentration gradient curve is of interest since Fujita[60] has shown that a simple associating system of the form

$$jP \rightleftharpoons P_j \quad , \quad j > 2$$

will give a minimum in the sedimentation velocity pattern provided that the concentration of oligomer is more than

one fourteenth that of the monomer. (The lowest necessary oligomer concentration is less than this for most values of j, as can be ascertained by substituting numerical values of j in equation 4.82 of the above reference). Fujita's theory neglects diffusion and considers only very rapid reactions. The first of these assumptions limits application of his theory to the present problem, but in connection with the second one it is likely that the inclusion of kinetic terms could only enhance the predicted resolution[61]. Nevertheless at least three reasons seem possible for the absence of a minimum in the sedimentation patterns of papain.

(1) The total protein concentration is still below that required to produce a minimum (i.e. by Fujita's theory the equilibrium concentration of oligomer is still less than one fourteenth that of the monomer).

(2) The minimum is obliterated by diffusion.

(3) Intermediate oligomers exist in the system. Rao and Kegeles[62] showed that although a monomer-trimer equilibrium yields a bimodal sedimentation pattern, a monomer-dimer-trimer system with suitably adjusted parameters might show only a single peak.

A possible means of deciding whether the first of the above reasons applies in the present case is by measuring the area of the slow peak at different papain concentra-

tions. Nichol and Bethune[58] have shown that in a rapidly reversibly associating system, the area of the back peak in the sedimentation pattern is independent of protein concentration provided the latter is greater than the concentration at which the minimum occurs. Although this result was formulated for a simple monomer-oligomer equilibrium, Nichol and Roy[63] observed a constant back peak area in the sedimentation of sulphatase A when it was considered that several species probably coexisted in equilibrium.

Reference to Fig. II-7 shows that constant back peak area was not observed with papain. This could indicate that the papain concentration is still below the value required for a minimum, or it might be a consequence of the arbitrary method of subdivision which was used. However two other explanations which should be considered are (1), that the association reaction is not sufficiently fast to maintain equilibrium at all points in the cell, and (2) that a non-associating or differently associating impurity is present. The latter explanation, which bears a possible analogy to the case of β-lactoglobulin[3], also provides a fourth possible reason for the failure to observe a minimum in the sedimentation pattern of papain.

(4) Size and Nature of the Aggregates

It can be said from the above discussion that the

polymerization of papain at pH 8 and ionic strength 0.02 apparently yields aggregates larger than the dimer. However, the failure of peaks in the sedimentation pattern to resolve, together with uncertainty about the rate of the association or the presence of differently associating impurities make further formulation difficult.

The active SH group of papain is clearly not required for association since the IAA inhibited derivative associates more than does untreated papain. The IAM derivative however shows a decreased tendency to associate which clearly indicates that the nature of chemical groups near the active site of papain exerts an effect on its association at pH 8. It has, of course, not been shown that the position of the substituted groups is significant. Papain at pH 8 is still below its isoelectric point; accordingly the introduction of a negatively charged group into any part of the molecule probably reduces its net charge. The repulsive force between neighbouring molecules may thus be reduced, resulting in increased association.

Since the reagents cysteine and EDTA, when present in 2 to 10 fold molar excess, exert no significant effect on the association of papain, there is now no basis for believing that intermolecular disulphide or metal to protein bonds are formed. Further evidence against the participation of a metal ion in the association is provided

by the results of a spectrographic trace metal analysis carried out[64] on a commercial papain sample which had been shown by the author to undergo association at pH 8. The only metals found were aluminium (0.01%) and calcium (0.004%). Since an aluminium content of 0.01% corresponds to only one metal atom in 13 molecules of papain, it is unlikely to significantly affect the association behaviour. It may yet transpire that hydrophobic forces are responsible for the association of papain as they appear to be for that of insulin[6].

(e) Conclusions

The main findings of this chapter can be summarized as follows.

(1) Sedimentation of papain in acetate buffer of pH 4 and ionic strength 0.1 revealed no significant indication of association.

(2) Similar experiments at pH 7 showed slight association of papain. The behaviour was characteristic of a rapidly reversibly associating system in which no particular aggregated species is favoured other than possibly the dimer.

(3) Papain undergoes considerable reversible association at pH 8 probably yielding some aggregates larger than the dimer. This behaviour is manifested as a pronounced leading shoulder in the sedimentation pattern. It is most

unlikely that intermolecular disulphide or metal to protein bonds are formed in the association. However the nature of chemical groupings near the active site of papain exerts an influence on its association at pH 8.

Thus, over the pH range studied, association of papain increases with increasing pH, becoming most pronounced as the isoelectric point is neared. This suggests that electrostatic repulsion may be opposing the forces which promote association. Further information on the association of papain at pH 7 and pH 8 could probably be obtained from sedimentation equilibrium studies. However complications are introduced by the fact that the association is so greatly influenced by a slight change in the nature of one group in the active site. As noted in Chapter I there are grounds for believing that the active SH group of papain can exist in different oxidation states in proportions which vary among given preparations. If these different oxidized forms of papain should differ in their propensity for undergoing association then it would be extremely difficult to obtain meaningful quantitative data for the association reactions.

Quantitative studies of the association behaviour of papain were therefore not proceeded with. Instead, as mentioned in Chapter I, it was decided to investigate the more fundamental problem of the heterogeneity of papain.

Accordingly the next chapter deals with attempts to demonstrate heterogeneity in papain by electrophoresis of a chemically modified derivative, and to fractionate papain into sulphydryl and non-sulphydryl forms.

CHAPTER III

CHEMICAL MODIFICATION OF PAPAIN WITH PCMS: ELECTROPHORETIC STUDIES

PART A:

Electrophoretic Heterogeneity of PCMS-modified Papain.

PART B:

Secondary Effects in the Electrophoretic Analysis of Papain and PCMS-Papain.

CHAPTER III - PART A

ELECTROPHORETIC HETEROGENEITY OF PCMS-MODIFIED PAPAIN

(a) Previous Evidence for Sulphydryl Heterogeneity in Papain.

(b) Choice of a Sulphydryl Reagent.

(c) Comparative Electrophoretic Studies of Papain and PCMS-Papain.

(i) Results

(ii) Discussion

(1) Boundary Shapes

(2) Electrophoretic Mobilities

(iii) Charge Calculations

(d) Back-Compensated Electrophoresis of Papain and PCMS-Papain.

(i) Elimination of the Stationary Boundaries

(ii) Compensation

(iii) Sampling

(e) Conclusions.

(a) Previous Evidence for Sulphydryl Heterogeneity in Papain

It was mentioned in Chapter I that the sulphydryl content of papain has been found to vary between zero and 1.0 molar equivalents in different preparations. Finkle and Smith[8] observed that the specific activity of papain isolated from different batches of latex was also variable and that a rough proportionality existed between specific activity and sulphydryl content. It was postulated that any papain preparation contains molecules of two types: an active SH-containing species, and an inactive species differing from the former in that its SH group has become irreversibly inactivated. The proportion of molecules containing SH groups would thus determine the activity.

The above hypothesis was formulated only for papain samples which had been subjected to a prior activating procedure, since the sulphydryl analyses were carried out on column-reduced papain, and activity measurements were performed in the presence of the usual activators (0.005 M cysteine and 0.001 M EDTA). However recent work by Sanner and Pihl[14] has also shown a linear dependence of activity on sulphydryl content, in this case for papain which had not been subjected to any reducing or activating procedure. This result also was

interpreted in terms of the existence of active SH-containing and inactive, non-SH forms of papain.

Glazer and Smith[29] showed by reducing papain with sodium borohydride that the proportionality between activity and sulphydryl content no longer holds if reduction of structure-stabilizing disulphide bridges occurs. In prolonged reduction of papain the maximum activity occurred when the SH titre was about 0.85 to 1.0 molar equivalents. Further reduction caused a drop in activity. Thus the specific activity of papain is proportional only to the molar content of active SH groups (i.e. to the proportion of molecules having free SH groups in their active sites).

Despite this analytical and enzymic evidence for the existence of active and inactive forms of papain, there have been no reports of heterogeneity in recrystallized papain being demonstrated by physico-chemical methods. Techniques which have been used for the examination of papain include sedimentation velocity[7], electrophoresis both in free solution[7] and on starch gel[20], and cation exchange chromatography both on a polymethacrylate resin[8] and on carboxymethyl cellulose[20]. Although some of these methods have revealed slight traces of impurities, no separation into two substantial components has been achieved. In addition, immuno-

chemical studies[65] of recrystallized papain and mercuripapain have revealed a homogenous antigen-antibody system by a number of criteria including complete precipitation as well as complete inhibition of the enzyme. It is thus apparent that any differences which exist between the active and inactive forms of papain are very slight, probably not extending beyond the chemical state of the active SH group.

This chapter describes the detection of heterogeneity in papain after chemical modification with p-chloromercuribenzene sulphonic acid (PCMS). It was expected that this anionic reagent would bind to the active, SH-containing form of papain, producing a species of different charge from the inactive, non-SH enzyme. (It is reasonable to suppose that the different forms of papain originally have the same charge since electrophoresis shows single peaks[7].) Moving-boundary electrophoresis was then applied to separate the two forms. It has been reported[7] that electrophoresis of mercuripapain of empirical formula $Hg(Papain)_2$ showed a double peak pattern. However as mentioned in Chapter I, the reaction of papain with mercury is obscure and apparently unspecific. Herblin and Ritt[66] have recently demonstrated the capacity of papain to bind three mercuric ions per molecule. Thus a more specific organomercurial reagent has been used in the present study.

(b) Choice of a Sulphydryl Reagent

The aim of chemical modification of papain was to alter the charge on each molecule of active papain thereby making it different from that on the inactive form. A charge difference is readily detectable, and in favourable cases measurable, by electrophoresis in free solution. It was therefore desirable that the modifying reagent should have the following properties.

(1) It should react only with SH groups.

(2) It should carry a different charge from that of the SH group in the pH range 4 to 7 (i.e. where papain is most soluble).

(3) It should be readily soluble in buffers of pH 4 to 7, thus enabling modification to be carried out in the presence of activators of papain by addition of an excess of reagent. It is clearly essential to activate the papain sample before modification since a large proportion of the SH-groups in a given papain preparation are reversibly blocked (see Chapter I).

(4) Modification should be reversible in order to allow any fractionation to be followed by activity measurements.

(5) The extent of modification should be measurable by an analytical method.

Organomercurial reagents fulfil the criteria of being reversible inhibitors and are specific for the active SH group of papain provided the conditions of reaction are properly controlled[8]. PCMS was chosen in preference to PHMB as it is more soluble[67] and contains a sulphonic acid group which should be negatively charged at pH's as low as 4[68], where SH groups are predominantly uncharged. One disadvantage of PCMS relative to PHMB is the greater difficulty in estimating the binding of the former reagent by Boyer's spectrophotometric method[67]. Both reagents undergo an absorption change on combination with SH groups but with PCMS the maximum change occurs in a slightly different spectral region where absorption by proteins is much greater (see Chapter VI).

(c) Comparative Electrophoretic Studies of Papain and PCMS-Papain

(1) Results

Electrophoretic patterns observed with papain and PCMS-papain in the pH range 4 to 7 are shown in Figs. III 1-4. The photographs shown were each obtained after the boundary had moved the entire optical length of the cell. Apparent mobilities of the various peaks are given in Table III-1, which also records spectrophotometric analyses of bound PCMS. In the examination of a protein

for heterogeneity it is advisable to conduct electrophoresis over a range of pH's, as has been done in the present study, since the extent of resolution often depends on the pH, ionic strength and chemical composition of the buffer[69].

Before PCMS-modification, papain was activated by treating an approximately 1% solution of the enzyme with a 10-fold molar excess of cysteine (0.005 M in the activation mixture) and a 2-fold molar excess of EDTA, all solutions being made up in acetate buffer at pH 5 and ionic strength 0.1. To this solution at room temperature was then added one of the same pH containing 1½ to 3 times the quantity of PCMS necessary to react with all the SH groups in the solution. The solution was immediately transferred to dialysis tubing and dialysed at ca. 4°C for 2 days against 40 to 100 volumes of diffusate, with a change of diffusate after 24 hours, to remove excess reactants and non-protein products. The diffusate, which was of course the buffer to be used in electrophoresis, contained 3×10^{-5} M PCMS to suppress dissociation of the papain-PCMS complex. When permitted by solubility the concentration of either papain or PCMS-papain was about 0.5 g/100 ml in the final solution. In some early experiments there were minor differences to the procedure. These are recorded in footnotes to Table III-1. Details of the methods and controls in electrophoresis are given in Chapter VI.

Fig. III-1 Electrophoresis of Papain and PCMS-Papain at pH 4.

Top Row

Papain in acetate buffer pH 4.01, ionic strength 0.1. Electrophoretic patterns after migration for 310 minutes at a field strength of 6.2 volt/cm.
(a) Ascending limb; (b) Descending limb.

Middle Row

PCMS-papain in acetate buffer pH 4.00, ionic strength 0.1. Electrophoretic patterns after migration for 358 minutes at a field strength of 6.2 volt/cm.
(c) Ascending limb; (d) Descending limb.

Bottom Row

Papain, treated with cysteine (10 moles) + EDTA (2 moles) and dialysed to remove these reagents, in acetate buffer pH 4.00, ionic strength 0.1. Electrophoretic patterns after migration for 327 minutes at a field strength of 6.3 volt/cm.
(e) Ascending limb; (f) Descending limb.

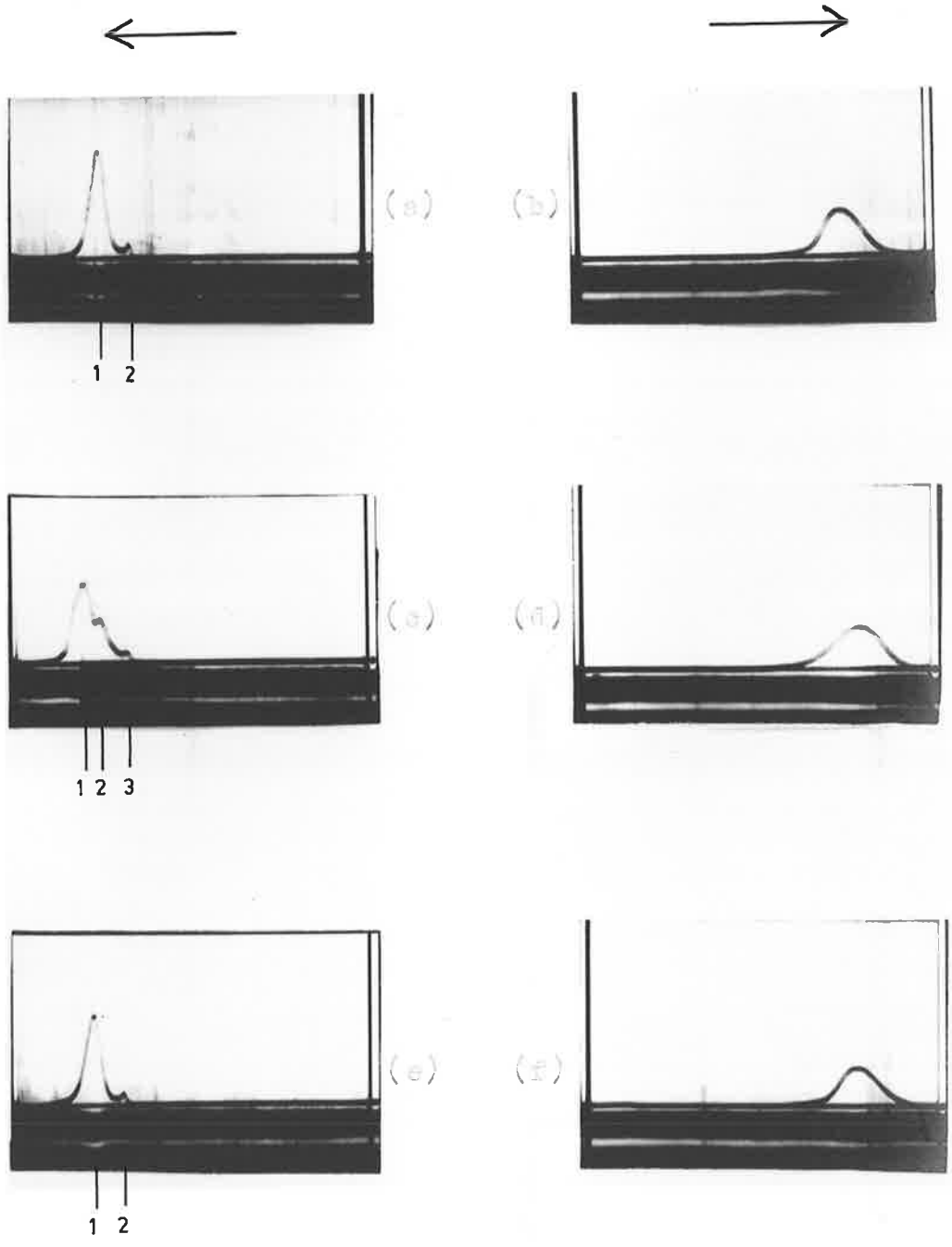


Fig. III-1

Fig. III-2 Electrophoresis of Papain and PCMS-Papain at pH 5.

Top Row

Papain in acetate buffer pH 5.01, ionic strength 0.1. Electrophoretic patterns after migration for 396 minutes at a field strength of 5.9 volt/cm.
(a) Ascending limb; (b) Descending limb.

Middle Row

PCMS-Papain in acetate buffer pH 5.02, ionic strength 0.1. Electrophoretic patterns after migration for 400 minutes at a field strength of 6.1 volt/cm.
(c) Ascending limb; (d) Descending limb.

Bottom Row

PCMS-Papain, prepared without prior treatment of papain with cysteine or EDTA, in acetate buffer pH 4.99, ionic strength 0.1. Electrophoretic patterns after migration for 409 minutes at a field strength of 5.8 volt/cm.
(e) Ascending limb; (f) Descending limb.

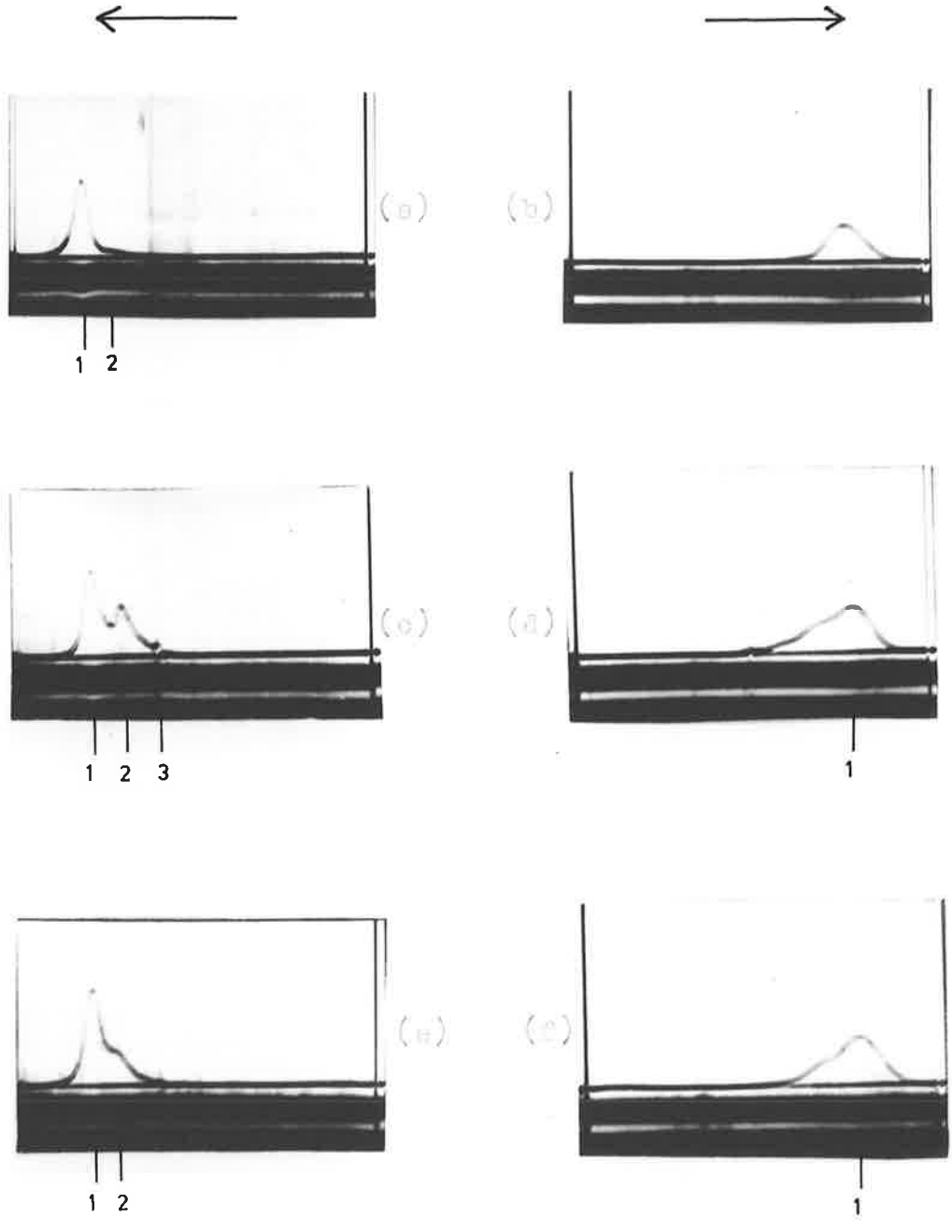


Fig. III-2

Fig. III-3 Electrophoresis of Papain and PCMS-Papain at pH 6.

Top Row

Papain in phosphate buffer pH 5.98, ionic strength 0.05. Electrophoretic patterns after migration for 400 minutes at a field strength of 5.0 volt/cm.
(a) Ascending limb; (b) Descending limb.

Bottom Row

PCMS-papain in phosphate buffer pH 6.00, ionic strength 0.05. Electrophoretic patterns after migration for 460 minutes at a field strength of 5.1 volt/cm.
(c) Ascending limb; (d) Descending limb.

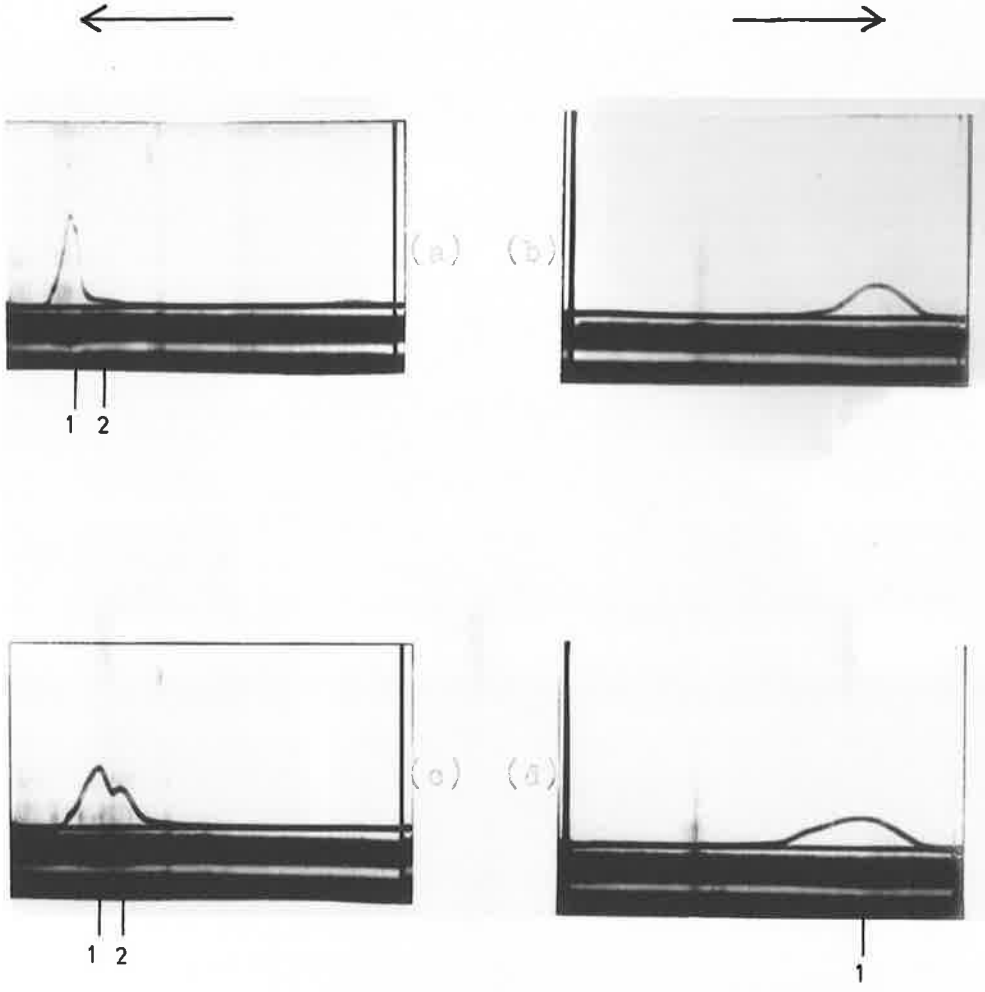


Fig. III-3

ig. III-4 Electrophoresis of Papain and PCMS-Papain at pH 7.

Top Row

Papain in phosphate buffer pH 6.98, ionic strength 0.05. Electrophoretic patterns after migration for 380 minutes at a field strength of 6.5 volt/cm.
(a) Ascending limb; (b) Descending limb.

Bottom Row

PCMS-Papain in phosphate buffer pH 6.99, ionic strength 0.05. Electrophoretic pattern after migration for 446 minutes at a field strength of 6.5 volt/cm.
(c) Ascending limb; (d) Descending limb.

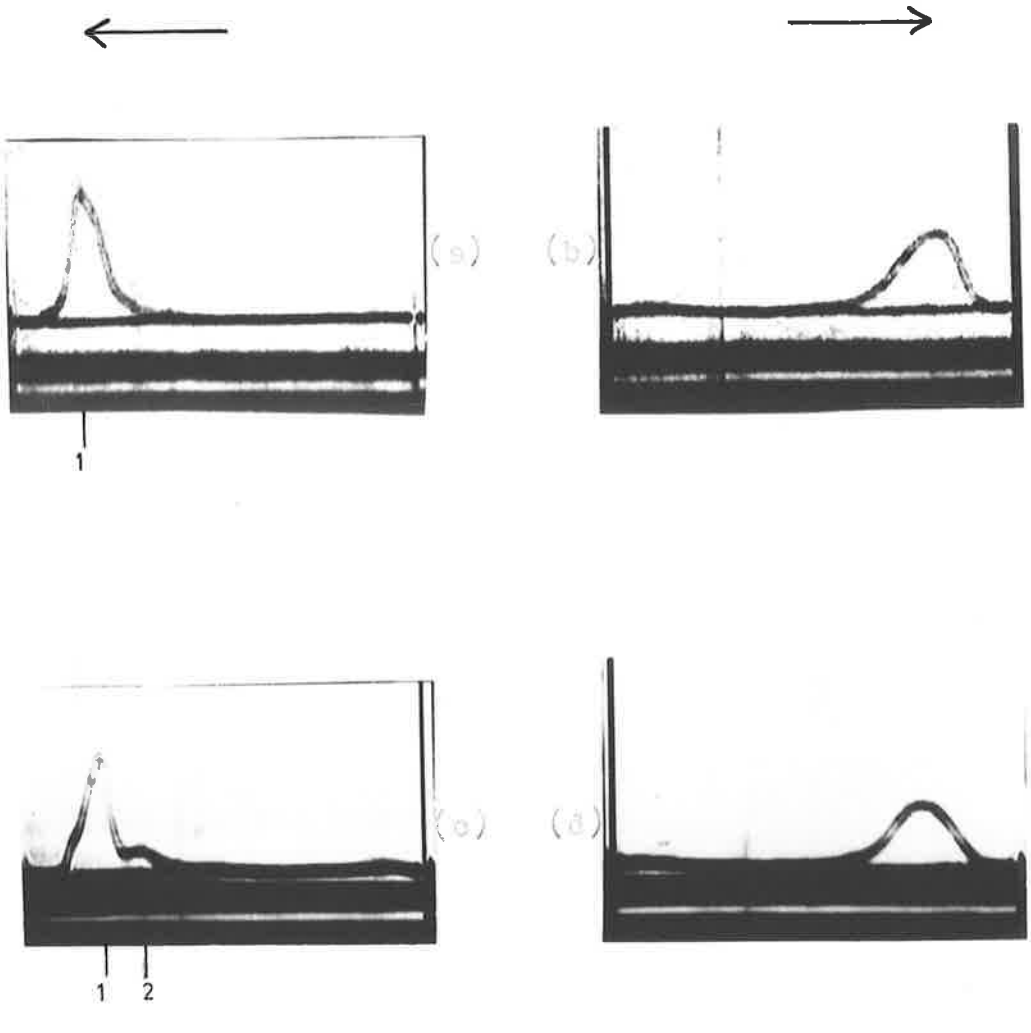


Fig. III-4

TABLE III-1

ELECTROPHORETIC DATA FOR PAPAIN AND PCMS-PAPAIN

Buffer[a]	Material and Run No.	Conc. (g/100 ml)	$\mu \times 10^5$ cm^2 sec^{-1} volt^{-1}		Other data	Boundary Shape Shown
			Ascending[b]	Descending		
Acetate pH 4.00 ± .01 I = 0.1	Papain E/20	0.45	1. 4.6_6 2. 4.0_7	4.5_9	$S_{20,w}$ = 2.40 S at 18°C	Fig.III-1 (a) and (b)
	PCMS-Papain E/23	0.48	1. 4.4_8 2. 4.4_2 3. 3.7_8	4.4_1	$S_{20,w}$ = 2.49 S at 18°C. Bound PCMS = 0.40 molar equ.	(c) and (d)
	Papain[c] E/24	0.48	1. 4.4_8 2. 3.9_7	4.4_9	-	(e) and (b)
Acetate pH 5.00 ± .02 I = 0.1	Papain E/27	0.37	1. 4.2_4 2. 3.7_7	4.0_8	-	Fig.III-2 (a) and (b)
	PCMS-Papain E/26	0.43	1. 3.9_7 2. 3.5_0 3. 2.9_7	1. 3.9_6	Bound PCMS = 0.56 molar equ.	(c) and (d)

(continued) ...

Table III-1 (continued)

Buffer[a]	Material and Run No.	Conc. (g/100 ml)	$\mu \times 10^5$ cm^2 sec^{-1} volt^{-1}		Other data	Boundary Shape Shown
			Ascending[b]	Descending		
pH 5.00 (continued)	PCMS-Papain[d] E/36	0.48	1. 4.0_6 2. 3.6_8	1. 3.9_1	-	Fig.III-2 (e) and (f)
Phosphate pH 6.00 ± .02 I = 0.05	Papain E/29	0.46	1. 5.0_3 2. 4.5_0	4.6_4	-	Fig.III-3 (a) and (b)
	PCMS-Papain E/30	0.49	1. 4.0_2 2. 3.7_2	1. 3.9_1	-	(c) and (d)
Phosphate pH 7.00 ± .02 I = 0.05	Papain E/9	0.29	1. 4.1_0	3.8_6	-	Fig.III-4 (a) and (b)
	PCMS-Papain[e] E/17	0.19	1. 3.2_8 2. 2.8_6	3.1_4	Bound PCMS = 0.61 molar equ.	(c) and (d)

μ: Apparent electrophoretic mobility I: Ionic strength

a See Table II-1 for buffer composition
b Peaks are numbered in order of decreasing mobility (see Figs.III-1 to 4)
c Control experiment: Papain activated but not modified; activators removed by dialysis.
d Control experiment: Papain modified without prior activation by adding a 30-fold molar excess of PCMS and dialysing.
e Modification procedure - (Papain + 20 moles cysteine) treated with 30 moles PCMS.

The apparent electrophoretic mobilities recorded in Table III-1 are obtained from the rates of movement of maxima in the schlieren patterns. Since the theoretically correct position to use is that of the centroidal ordinate[70], the apparent mobility figures obtained are for hypothetical symmetrical peaks with maxima in the same positions as those of the observed peaks. The specific conductance used in the calculation of electrophoretic mobilities[71] was that of the protein solution placed in the electrophoresis cell. Usually this solution consisted of the dialysed protein solution diluted with water, or in the case of acetate buffers dilute acetic acid, in order to reduce the size of the S-boundary. This procedure enables back-compensation (see section (d)) to be applied, but also has the effect of making the apparent mobility figures from the ascending and descending limbs more similar than would otherwise be the case (i.e. if the specific conductance of the dialysed protein solution were used)[72].

(ii) Discussion

(1) Boundary Shapes

It can be seen from Figs. III 1-4 that PCMS modification of papain produces an extra peak in the electrophoretic pattern on the trailing side of the main

peak. This is in accordance with the formation of the expected product, consisting of two differently charged species, by combination of the sulphydryl form of papain with the negatively charged reagent. Resolution of components can be seen more clearly in the ascending patterns than in the descending ones owing to the usual gradients of conductivity which produce sharper peaks in the ascending limb of the electrophoresis cell[73].

Since a change in electrophoretic mobility can arise from a change in either effective charge or frictional coefficient, it was necessary to eliminate the latter possibility. Accordingly, sedimentation velocity runs were conducted on solutions of papain and PCMS-papain at pH 4, since a difference in frictional coefficient can reasonably be expected to produce a change in sedimentation coefficient. It was found that both solutions gave single peaks in the ultracentrifuge and, as shown in Table III-1, the sedimentation coefficients were nearly the same. Thus the difference in electrophoretic behaviour must arise from a difference in charge.

Since the PCMS-modification of papain as described in section (i) is a two stage reaction, control experiments were carried out to ascertain the effect of each step individually. Fig. III-1(e) shows that activation of papain, not followed by PCMS-modification, does

not produce the extra peak in the electrophoretic pattern. Fig. III-2(e) shows that when PCMS-modification is carried out without prior activation, the extra peak produced is not as large as that formed by the usual method (Fig. III-2(c)). This is consistent with evidence cited in Chapter I for an increase in the sulphydryl content of papain upon activation. The extent of modification as judged from the shape of the peak was also found to be less when using a papain preparation that had been stored for some time. As papain is known to lose activity on prolonged storage[8], it is not surprising to find an apparent decrease in its free sulphydryl content.

The appearance of a double peak in electrophoresis is not conclusive proof of heterogeneity since interaction equilibria in solution can give rise to a bimodal reaction boundary from only a single macromolecule. Systems of this type are discussed in Part B of this chapter which also describes some tests for reaction boundaries which were applied to the present system. It is also of interest that the ascending boundary of unmodified papain has a small trailing peak which is apparently retained as a small third peak in the PCMS-modified preparation. In some experiments, especially at pH 4, this peak developed a spiky appearance (Fig. III-1(a)), typical of convection, after several hours of

electrophoresis. This occurred even though the heat output of the electric current was always less than 0.15 watts/c.c, and therefore unlikely to cause thermal convection[74]. In addition it was observed that the small trailing peak never completely separated from the main peak, even after 24 hours of electrophoresis (see section (d)), and that a further recrystallization of papain had no effect on the size of this peak when the sample was subjected to electrophoresis. It is therefore unlikely that this small peak represents an impurity in papain. However, as will be seen in Part B, the above observations can be interpreted in terms of an interacting system.

(2) Electrophoretic Mobilities

At pH 4 and pH 5, the apparent electrophoretic mobility of the leading peak in the electrophoretic pattern of PCMS-papain is only slightly less than that of unmodified papain. This suggests that a large proportion of the papain molecules are unaffected by PCMS. The analytical figures for bound PCMS, although subject to considerable error for reasons explained in Chapter VI, also indicate the binding of only a fraction of a molar equivalent of PCMS. Although the mobility figures at pH's 6 and 7 show a decrease in the mobility of the main peak of PCMS-papain relative to that of papain, it is

possible that association reactions occur in these solutions. In Chapter II it was shown that association of papain was favoured by an increase of pH between 4 and 8. Furthermore, the electrophoretic patterns of unmodified papain at pH's 6 and 7 show obvious asymmetry which could be due to self-association of papain. In view of the possibility of such complications at pH's 6 and 7, only data obtained at pH's 4 and 5 has been used for the approximate charge calculations made in the next section.

(iii) Charge Calculations

Assuming that each bound PCMS molecule carries a unit negative charge at pH's down to 4, the difference in charge between the two molecular species apparently present in PCMS-papain should be a measure of the number of PCMS molecules bound to each molecule of modified papain. This in turn would show the number of enzyme SH groups liberated in the activation of a papain molecule. In Chapter I analytical evidence was cited which indicated that only one SH group was liberated under these conditions. However, analytical methods cannot distinguish between, for example, a protein sample in which 90% of the molecules contain one SH group, and a sample in which 45% of the molecules contain two. Since this information is potentially available from the electro-

phoretic results, it is of interest to determine the difference in charge which would give rise to the observed difference in mobilities between the two peaks in the electrophoretic pattern of PCMS-papain. Calculation of charge from mobility has been made on the basis of two theories, since neither is free from assumptions.

(1) Gorin's Method

Gorin's method of charge calculation[75] requires that a shape for the protein be envisaged in order to make approximate allowance for the retardation of the protein ion by its own ion atmosphere. The most general form of Gorin's equation, as expressed by Vellic[76] is

$$\frac{q}{\mu} = \frac{6\pi\eta r\,(1 + \kappa r + \kappa r_i)}{f(\kappa r)(1 + \kappa r_i)} \cdot f\left(\kappa, \frac{a}{b}\right) \cdot e \qquad \ldots\ldots \text{III-(1)}$$

where q is the net charge on the protein

μ is the electrophoretic mobility

η is the viscosity of the solvent

r is the radius of a sphere having the same volume as the molecular volume of the protein in solution

r_i is the average radius of the buffer ions

κ is the Debye-Huckel constant defined by equation (19) of reference 75

$f(\kappa r)$ is a function which corrects for distortion of the electric field by the protein ion which is assumed to be non-conducting. Values of $f(\kappa r)$ are tabulated in reference 75

$f(\kappa, \frac{a}{b})$ is a function, also tabulated in reference 75 which corrects for asymmetry of the protein as expressed by the axial ratio $\frac{a}{b}$ of a cylindrical, or rod-like, molecule. For spherical proteins $f(\kappa, \frac{a}{b}) = 1$ and r is the actual radius of the molecule

and c is a dimensional constant chosen to give q in units of positive electronic charges.

In order to find the necessary molecular dimensions for the papain molecule, the frictional ratio (f/f_o) was calculated[77] from the sedimentation coefficient at infinite dilution (2.46 S), the molecular weight (21,000), and the partial specific volume (0.724 ml/g). A frictional ratio of 1.13 was obtained, which, from the contour lines calculated by Oncley[78] could correspond to either a 35% hydrated spherical molecule, or an unhydrated rod-like molecule with axial ratio $(\frac{a}{b}) = 3.4$. Calculations were made for these two extreme forms but it should

be realized that the papain molecule may have a shape intermediate between these extremes or may possibly be disc-like. The limitations of this method are therefore apparent.

For a spherical, hydrated papain molecule, the molecular radius r is 20.6 $\mathring{A}$, and $(^{q}/_{\mu}) \times 10^{-5}$ in an acetate buffer of ionic strength 0.1 was calculated to be 1.15 at pH 4, and 1.11 at pH 5. (The slight difference is due to different viscosities of the two buffers). For a rod-like, unhydrated papain molecule, the equivalent spherical radius is 18.1 $\mathring{A}$, and $f(\kappa, \frac{a}{b})$ was found by interpolation of data in Table 11 of reference 75 to be 1.45. Hence $(^{q}/_{\mu}) \times 10^{-5}$ was calculated to be 1.38 at pH 4 and 1.33 at pH 5. The charge calculations made from the $^{q}/_{\mu}$ ratios for both models are given at the end of this section.

(2) Longsworth's Method

From the Kolrausch regulating function theory for strong electrolytes[79,80] and first-order Donnan theory[81], Longsworth[82] calculated the concentration change (Δ c) across the ε, or buffer concentration boundary. His equation is

$$\Delta c = \left(\frac{r_B - r_R}{r_B} \cdot \frac{r_A}{r_A - r_R} - \frac{1}{2}\right) c_B^a \quad \text{.... III-(2)}$$

where r_B, r_A and r_R are the relative mobilities of the protein ion, buffer cation and buffer anion respectively, and C_B^a is the protein concentration in the initial solution. This concentration is in equivalents per litre. Hence if an independent measurement is made of c, the protein concentration in g/100 ml, it can readily be shown that the charge (q) on the protein ion is given by

$$q = \frac{1}{10}\left(\frac{M.C_B^a}{c}\right) \qquad \ldots\ldots \text{III-(3)}$$

where M is the molecular weight.

An equation analagous to III-(2), though slightly more complicated, can be derived for the concentration change across the δ-boundary. Both equations are inexact owing to the limitations of the Kolrausch theory when applied to weak electrolytes[82]. This uncertainty is greater in the case of the δ-boundary since the protein mobility may be different on either side of it as a result of differences in the pH and ionic strength of the solution. Consequently, calculations for papain were made only for the ε-boundary.

The number of fringes in the ε-boundary was measured on a photograph in which the papain peak had migrated well clear of the boundary. (This measurement was of course done for a run in which the stationary

boundaries had not been reduced by the method to be described in section (d)). The error in this measurement is considered to be less than 0.2 fringes which corresponds to a 5% error in $\triangle c$. A similar measurement on the δ-boundary would have been subject to about half this error, but the increase in experimental precision would be offset by the other factors which have been mentioned. $\triangle c$ was converted from fringes in the electrophoresis cell to refractive index increment[83], then to normality of sodium acetate using refractivities determined at 0°C by Longsworth[82]. The mobilities of the buffer ions were also found from the literature[84], while that of the protein was obtained from the electrophoresis experiment. The latter was corrected from 1°C to 0°C (the temperature at which the other mobility data were obtained[84]) assuming the only significant correction over such a small range to be that for viscosity[85]. The molecular weight of papain was taken as 21,000, and the weight concentration required in equation III-3 was found by refractometry. The ratio $(q/\mu) \times 10^{-5}$ obtained for an experiment with papain at pH 4 was 1.48; for one at pH 5 the ratio was 1.55. These ratios were then used to calculate charges which are given in Table II-2 together with charges calculated by Gorin's method.

Results

Table III-2 shows results of each of the foregoing methods for the calculation of the difference in charge between the two species in PCMS-papain. It was assumed that the q/μ ratio for papain was unchanged by PCMS-modification.

TABLE III-2

CHARGE CALCULATIONS FOR PCMS-PAPAIN

Run No.	pH	$\Delta\mu \times 10^5$ [a] $cm^2/volt/sec$	Δq		
			Longsworth's Method	Gorin's Method	
				spheres	rods
E/15	4.01	0.36	0.53	0.41	0.50
E/22	4.00	0.53	0.78	0.61	0.73
E/23	4.00	0.26	0.38	0.30	0.36
E/26	5.02	0.47	0.73	0.52	0.63
E/36	4.99	0.38	0.59	0.42	0.51
E/37	5.00	0.29	0.45	0.32	0.39
Average of 6			0.58	0.43	0.52

[a] $\Delta\mu = \mu_1 - \mu_2$ for ascending limb, since only the ascending pattern yielded two measurable peaks. Elsewhere in the calculations descending mobilities have been used, since the specific conductance is known more accurately in the descending limb[72].

It is seen from Table III-2 that the calculated difference in charge between the two apparent main components in PCMS-papain is about 0.5 valence units. The number of PCMS molecules bound to a molecule of papain must be an integer. It therefore appears that charge calculations are not a satisfactory means of determining this number although they suggest that it is unlikely to be more than one. It is possible that the binding of a PCMS molecule to papain affects the ionization of neighbouring groups in the enzyme resulting in a net charge decrease of less than one. However the calculated charges in Table III-2 are too tentative to allow any definite conclusions about such factors.

(d) Back-Compensated Electrophoresis of Papain and PCMS-Papain

As mentioned earlier, the appearance of two peaks in the electrophoretic pattern of PCMS-papain is not unambiguous proof of heterogeneity. It was therefore decided to conduct prolonged electrophoresis runs using back-compensation in the hope of being able to make a preparative fractionation. In back-compensated electrophoresis the movement of the moving boundary is counteracted by causing a slow flow of buffer through the cell in the opposite direction. In this way electrophoresis

can be continued for much longer times without the peak migrating out of the optical part of the cell. This technique, as applied to the present study, entailed three steps: (i) elimination of the stationary boundaries, (ii) compensation, and (iii) sampling.

(i) Elimination of the Stationary Boundaries

It is well known that in an electrophoresis experiment there occur, in addition to the moving boundaries, two stationary boundaries. These are the δ- and ε - boundaries (in the ascending and descending limbs respectively). They arise from differences in the regulating functions[a] of the protein and buffer solutions prior to electrophoresis, and comprise changes in concentration of all ionic species present above and below them.

The presence of stationary boundaries constitutes a problem in back-compensated electrophoresis, since a flow of buffer in the direction opposite to that of electrophoretic migration brings the δ-boundary into

---

[a] The regulating function B of a solution is defined by

$$B = \sum_i \left(\frac{c_i}{\mu_i}\right)$$

where c_i: equivalent concentration of ionic species i

μ_i: ionic mobility of ionic species i

and the summation extends over all ions in the solution. B has the property that it remains constant at any horizontal level in the electrophoresis cell throughout the run[79,80].

the bottom section of the cell. Convection then ensues due to there being two solutions of different density at the same level in the cell. Before attempting back-compensated electrophoresis it is therefore necessary to eliminate the δ-boundary by making the regulating functions of the protein solution and diffusate equal. In the present study this was achieved by diluting the protein solution with the unionized or weakly ionizing portion of the buffer (cf. Longsworth and MacInnes[72]).

The necessary dilution factor was deduced from a measurement of the δ-boundary in a previous run together with a measurement of the refractive index increment ($\triangle n$) of all the ions in the protein solution. The latter quantity was obtained by differential refractometry (see Chapter VI) of the protein solution using the weakly ionizing portion of the buffer (dilute acetic acid solutions in the present cases) as the reference solution. With the assumption of constant relative ion mobilities, it can be shown that all ionic species are diluted at the δ-boundary by the same factor, which is the ratio of the regulating function of the initial protein solution to that of the diffusate[73]. Hence this factor (f) must be given by

$$f = \frac{(\triangle n)_{\text{All ions}}}{(\triangle n)_{\text{All ions}} - (\triangle n)_{\delta\text{-boundary}}} \quad \ldots \text{III-(4)}$$

Dilution of the initial protein solution by this factor should therefore make the regulating functions of the protein solution and diffusate equal, thereby eliminating the δ- and ε - boundaries in a second run.

The above method was found to be quite successful in removing the stationary boundaries. However, since a slight error leading to over-dilution produces convection in the second run, it proved more practicable to use a dilution factor calculated to leave a δ-boundary of about 0.5 to 1.0 fringes. (To do this, the required refractive index decrement is substituted in equation III-(4) in place of $(\Delta n)_{\delta\text{-boundary}}$). A δ-boundary of this size was found to cause no perceptible convection in the ascending limb during the usual 24 hour duration of an experiment. The descending limb however always convected after about 12 hours.

(ii) Compensation

When the peaks had moved the entire optical length of the electrophoresis cell, back-compensation was commenced by slowly withdrawing buffer from the compartment above the descending limb. In some experiments a siphon was used for this purpose, in others a mechanical syringe. The back-compensation was applied intermittently.

Fig. III-5 shows electrophoretic patterns

obtained with papain and PCMS-papain after prolonged electrophoresis with back-compensation. Fig. III-6 shows two photographs obtained during the same electrophoresis run using PCMS-papain; (a) was obtained before compensation was commenced, (b) was obtained after 18 hours of intermittent back-compensation. It is clear that the distance between the two main peaks increases during back-compensated electrophoresis. However it can be seen from Figs. III-5(b) and 6(b) that even after a total electrophoresis time of 24 hours there is still no region of constant concentration between the peaks. As will be seen from part B of this chapter, this is a possible sign of an interacting protein system.

(iii) Sampling

Although back-compensated electrophoresis of PCMS-papain did not produce complete resolution of the two main peaks, the separation was sufficient to enable the withdrawal of a sample representing essentially the species disappearing across the leading peak. Of the buffers used for back-compensated electrophoresis, acetate of pH 5 and ionic strength 0.1 gave the best resolution and was therefore used for sampling experiments. Fig. III-6(b) was obtained from a run in which sampling was carried out. After the distribution shown

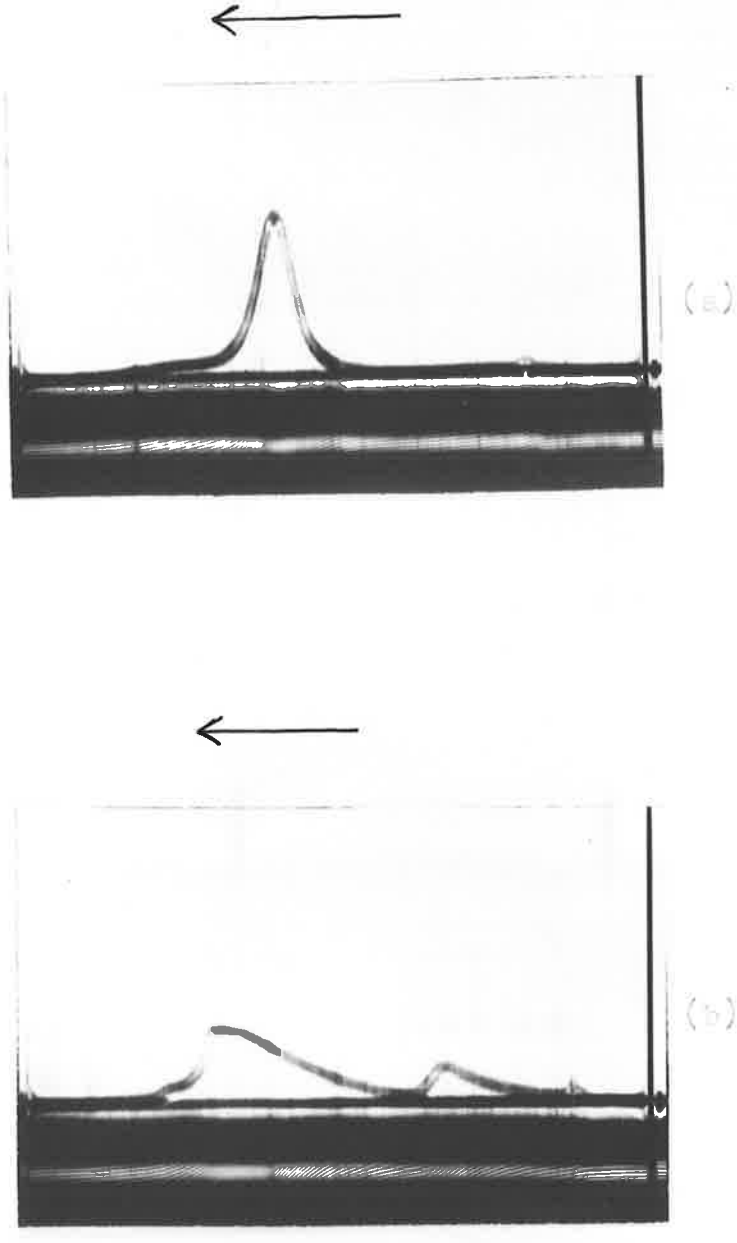


Fig. III-5 Electrophoretic patterns (ascending limb) of (a) papain, and (b) PCMS-papain, in acetate buffer pH 5.00, ionic strength 0.1, after prolonged electrophoresis with back compensation. Both photographs were obtained after a total migration time of 1440 minutes at a field strength of 6 volt/cm.

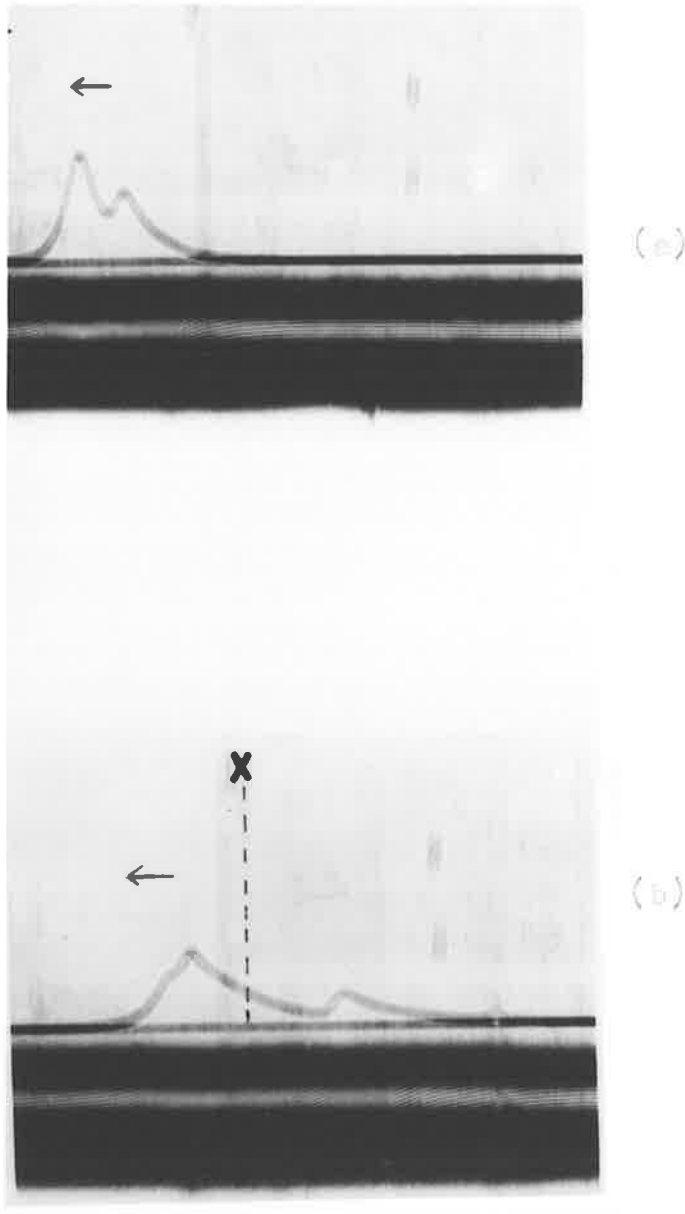


Fig. III-6 Electrophoretic patterns (ascending limb) of PCMS-papain in acetate buffer pH 5.00, ionic strength 0.1, (a) after 404 minutes and before commencing compensation, (b) after back-compensated electrophoresis and a total migration time of 1440 minutes. The field strength was 5.9 volt/cm.

had been attained, the current was turned off, the bottom section of the cell isolated from the middle section to reduce convection, and a syringe needle lowered to the level denoted X in Fig. III-6(b). This operation was monitored by means of the schlieren optical system with the cylindrical lens removed. (The needle can be seen more clearly when this is done). The cylindrical lens was then replaced and the schlieren pattern observed while a sample of the solution making up the front peak was withdrawn through the needle by a mechanical syringe, and collected in a trap (see Fig. III-7).

A sample of the unfractionated initial solution was recovered from the bottom section of the cell. Both samples were then analysed for bound PCMS in the spectrophotometer and were assayed in the usual way (see Chapter VI). The results of these analyses in two fractionation experiments are shown in Table III-3 which also records the apparent percentage composition calculated from the number of Rayleigh fringes under each peak.

It can be seen from Table III-3 that in both experiments a separation of activity occurred and was in the expected direction i.e. indicating a lower specific activity for the species, believed to be non-SH papain, disappearing across the front peak. It should be noted

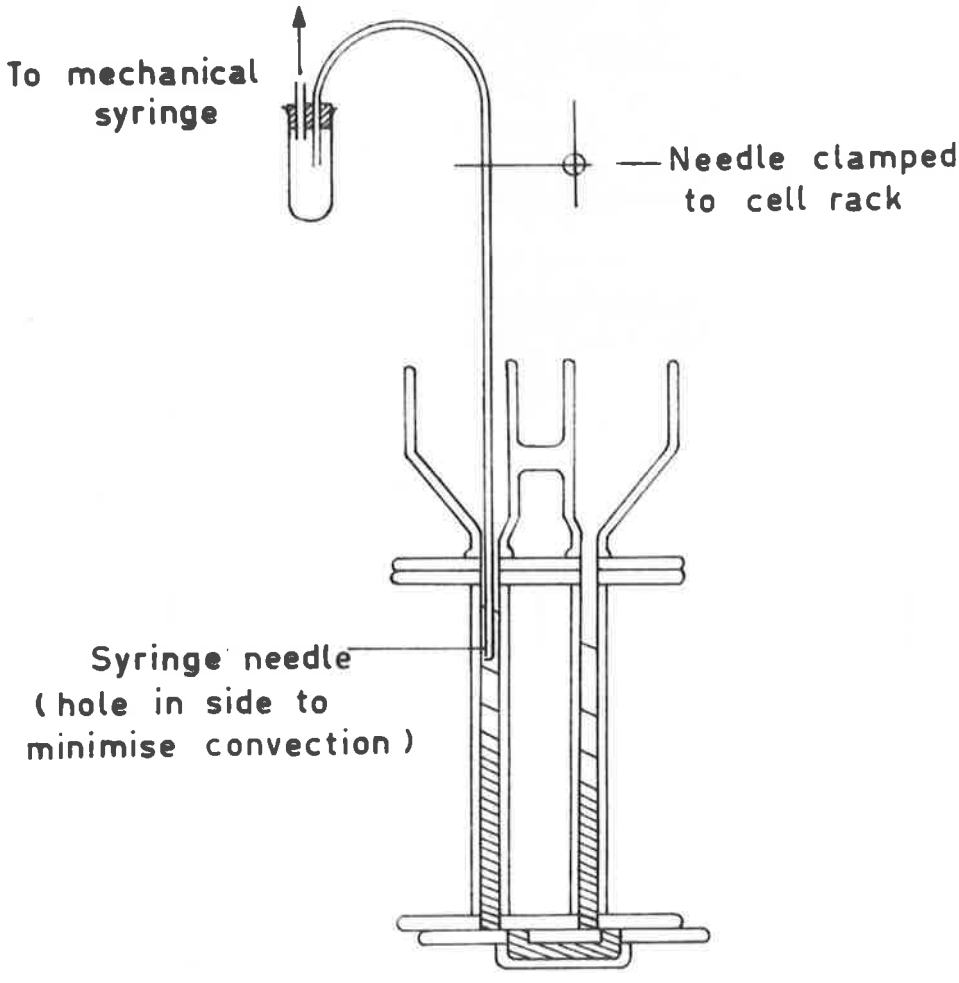


Fig. III-7 Method of withdrawing samples from the electrophoresis cell.

TABLE III-3

ELECTROPHORETIC FRACTIONATION EXPERIMENTS ON PCMS-PAPAIN

Run No.	E/33	E/37
Unfractionated Solution		
Bound PCMS (moles/mole papain)	0.69	0.63
Specific activity[a]	0.69	1.06
Front peak		
Area (per cent)	72	49
Bound PCMS (moles/mole papain)	0.49	0.96
Specific activity	0.34	0.13

[a] Specific activity is expressed as the proteolytic coefficient (c_1) (defined in Chapter VI) for the hydrolysis of α-benzoyl arginine amide (BAA).

that activities were measured in the presence of 0.005 M cysteine and 0.001 M EDTA. Under these conditions the PCMS inhibited enzyme is re-activated.

This separation of activity, persisting after completion of the electrophoresis experiment, is strong and apparently unambiguous evidence for heterogeneity. However, the detection of some residual activity in the front peak suggests that the leading component may not be simply an inactive, unmodified form of papain. Undue emphasis should not be placed on the differences in bound

PCMS between fractions since the accuracy of these determinations is probably poor (see Chapter VI). Nevertheless it seems clear that both fractions contain bound PCMS. This is also difficult to reconcile with the hypothesis of a simple electrophoretic separation between an active papain species inhibited with PCMS and an inactive unmodified species.

(e) Conclusions

The main finding of the work described in this chapter is that chemical modification of papain with PCMS gives a product comprising two species with different charges. It has been shown by sampling after prolonged electrophoresis that electrophoretic separation is accompanied by a separation in enzymic activity. These results are consistent with the hypothesis that papain, as normally isolated[41], contains an active, SH-containing species and an inactive, non-SH form.

Some observations have however been made which suggest that heterogeneity may not be the only factor responsible for the electrophoretic behaviour of PCMS-papain. Accordingly, Part B of this chapter comprises a discussion of the effects of various types of interaction on the electrophoretic behaviour of proteins. It will be seen that some forms of interaction, if super-

imposed on heterogeneity effects, could account for the observed electrophoretic behaviour of PCMS-papain. However it is difficult to show whether any of these interactions definitely occur in the present system.

It seems unlikely that a preparative fractionation of PCMS-papain can be achieved by electrophoresis in free solution since only incomplete separations have been obtained, and the method has the additional disadvantage that only small samples can be recovered. It was therefore decided to attempt a fractionation by ion-exchange chromatography. This technique also gives a separation of proteins having different charge properties, and is readily adaptable to large scale operation. An account of attempted fractionations by ion-exchange chromatography is given in Chapter IV.

CHAPTER III - PART B

SECONDARY EFFECTS IN THE ELECTROPHORETIC ANALYSIS OF PAPAIN AND PCMS-PAPAIN

(a) Introduction

(b) Non-Ideal Electrophoresis

(c) Isomerization and Protein-Buffer Interactions

  (i) Theory

  (ii) Application

(d) Protein-Protein Interactions

  (i) Theory

  (ii) Application

(e) Conclusions

(a) Introduction

It has been emphasized in recent papers and reviews[1,2,86] that a single macromolecule, if involved in certain types of equilibria in solution, can give rise to more than one peak or zone in transport experiments. Thus in any electrophoretic investigation of heterogeneity it is important to consider the possibility of interactions. There can be little doubt that the PCMS-papain system is heterogeneous in view of the activity separation brought about by electrophoresis. However, as mentioned in Part A of this chapter, heterogeneity does not immediately account for all the experimental observations. The following, apparently anomolous, secondary effects can be listed.

(1) Prolonged electrophoresis of either papain or its PCMS-derivative gave peaks of complex shapes (see Figs. III-5 and 6).

(2) The electrophoretic patterns of both papain and PCMS-papain showed a small trailing peak which never completely separated from the main peak even after 24 hours migration. The shape of this peak was often such as to indicate a mild convective disturbance.

(3) Complete resolution of the two peaks in the electrophoretic pattern of PCMS-papain did not occur, i.e. the refractive index gradient between the peaks never

became zero.

(4) Approximate calculations of the charge difference corresponding to the difference in apparent electrophoretic mobility between the two components in PCMS-papain implied a difference of less than a single valence unit per papain molecule.

(5) Attempted electrophoretic fractionations showed that the fast component of PCMS-papain had some activity. It was expected that only the slow component would be active.

The purpose of the rest of this chapter is to consider possible causes of the above effects and to describe attempts which were made to obtain experimental evidence for some types of chemical interactions. Interactions have been divided into two groups, one in which the protein molecule undergoes an isomerization or a reaction with the solvent medium, the other in which there is an association between macromolecular ions. In theories which have been formulated to describe the electrophoretic behaviour of reacting systems, a simplification which has always been made is that electrophoresis is ideal i.e. the electric field strength is assumed to be constant across the moving boundary. This assumption is unrealistic since gradients of conductivity, and hence of field strength, occur across any moving

boundary[87]. Thus, before proceeding to the electrophoresis of interacting systems, a brief outline will be given of effects arising from non-ideal electrophoresis in a heterogeneous but non-interacting protein system.

(b) Non-Ideal Electrophoresis

The Dole theory[80] for strong electrolyte electrophoresis can with reasonable justification be applied to well-buffered protein systems where variations in pH and ionic strength are small[88]. Alberty[73] has applied this theory to a hypothetical mixture of two proteins, and calculated the nature and magnitude of the deviations of electrophoresis from the usually assumed ideal case. As well as the formation of stationary boundaries in accordance with the Kohlrausch regulating function theory, deviations from ideal behaviour were manifested in three ways.

(1) Volumes swept through by the moving boundaries

The ascending boundaries swept through larger volumes than did the descending boundaries because of the greater field strength between the δ-boundary and the ascending boundary. The mobilities of the two proteins were in the ratio 2:1, but on the ascending side the ratio of the volumes swept through by the boundaries was less than 2:1 while on the descending side it was more

than 2:1.

(2) Areas in the Electrophoretic Pattern

Because of superimposed concentration gradients of buffer and protein ions the refractive index charge across the faster moving boundary was too great in both the ascending and descending patterns. The error was calculated to become less at high ionic strengths but greater with buffer ions of high mobility.

(3) Shapes of the Peaks

No quantitative assessment of the shapes of the peaks could be made. It was however evident from the variation in the calculated field strength that both peaks would be expected to spread more in the descending limb and less in the ascending limb than if diffusion alone were responsible for boundary spreading. Alberty suggested that gradients of conductivity and pH might cause the moving boundaries to deviate from the Gaussian shape produced by diffusion alone.

These non-ideality deviations will all exert some influence on the electrophoretic behaviour of PCMS-papain. Hence calculations of relative mobilities and percentage areas from measurements on the ascending pattern are unreliable. In addition, although it is unlikely that deviations of type (3) can account for the unusual shapes of individual peaks, it certainly cannot

be expected that peaks will remain Gaussian over long periods of electrophoresis.

(c) Isomerization and Protein-Buffer Interactions

(i) Theory

A simple type of chemical reaction which can complicate the electrophoresis of a protein A is an isomerization.

i.e. $A \rightleftharpoons C$

where the electrophoretic mobilities of A and C are different.

The system

$A + B \rightleftharpoons C$

is mathematically equivalent to the former case provided that the concentration of B is sufficiently constant throughout the system to ensure that the forward reaction remains essentially first-order[89]. This condition is fulfilled if, for example, B is H^+ in a buffered system or if the concentration of B is large compared to that of A and C.

Any protein in an aqueous buffered medium is involved in a variety of rapid equilibria of this type and in this sense all moving boundaries in transport experiments are reaction boundaries[90]. In most cases,

however, such reactions are rapid; thus A and C each travel with the same average or constituent velocity and only a single maximum will appear in the schlieren pattern[89,91]. The case where interconversion of A and C is very slow is also simple in that the behaviour will not differ much from that of a non-interacting, two-component system.

The intermediate case where the duration of the experiment is of the same order of magnitude as the half-time of the reaction is more involved, but useful predictions have been made from numerical[89,91,92] and approximate analytic[93] solutions of the appropriate differential equations. It has been proposed that in such systems the initial sharp Gaussian boundary may first split into two peaks which, as electrophoresis proceeds, gradually decrease in size with the concomitant growth of a central peak. Thus the schlieren pattern may show one, two or three peaks depending on the rates of reaction and the time from the start of electrophoresis. Ascending and descending patterns need not necessarily be enantiographic, even for ideal electrophoresis, but in the cases so far solved, the essential features are the same in both limbs.

Another case requiring special consideration occurs when the reaction with a constituent of the buffer

is such as to significantly change the concentration of this constituent. This reaction is no longer formally analogous to an isomerization. Cann and Goad[94] have recently discussed the electrophoresis of proteins which reversibly react with small, uncharged molecules such as undissociated buffer acid. It was shown that, with properly chosen values for parameters such as the number of bound acid molecules and the equilibrium constants, the theory could predict resolution into two moving peaks despite instantaneous re-establishment of equilibrium. Exploratory calculations illustrated how the whole spectrum of experimentally recognized types of moving boundary electrophoretic patterns, both enantiographic and non-enantiographic, may in principle arise from the rapid, reversible interaction of a single macromolecule with an uncharged constituent of the solvent medium.

(ii) Application

On the basis of the following observations it is considered that protein-buffer interactions of the type described probably do not play an important role in the electrophoresis of papain and PCMS-papain.

(1) Similar patterns were obtained in buffers of different pH between 4 and 6, and even in buffers of different composition, viz. acetate or phosphate (see

Figs. III-1 to 3). Thus the patterns are not very sensitive to the concentration of undissociated acetic acid.

(2) Apart from greater spreading in the descending limb as a result of non-ideal electrophoresis, the ascending and descending boundaries have similar shapes at pH's below 6. The Cann-Goad theory predicts non-enantiographic boundaries except when the protein binds large numbers (of the order of 300 per molecule) of uncharged molecules. However it should be realized that deviations from enantiography in the electrophoretic pattern of PCMS-papain might be hard to detect when superimposed on an already heterogeneous boundary. Furthermore, isomerization reactions are quite compatible with enantiographic boundaries.

(3) Electrophoresis of unmodified papain in the same buffers as were used for PCMS-papain gave essentially unimodal patterns. It is unlikely that the capacity of the papain molecule to bind acetic acid would be much altered by combination with a single PCMS molecule, especially since this combination appears not to be accompanied by any gross configurational change in the papain molecule. However features which appear in the electrophoretic patterns of both, such as the small trailing peak, could be due to protein-buffer interactions.

Such a mechanism might be able to account for mild convective disturbances since this effect has been predicted for ovalbumin and bovine serum albumin in acetate buffers[94].

Cann and Goad[86,94] have pointed out that an unambiguous method of distinguishing between interactions and true heterogeneity is to isolate the material constituting a particular boundary; this protein is then subjected to electrophoresis under conditions identical with those used in the initial separation. Particular care must be taken to ensure the same concentration of protein and the same specific conductance of the buffer. A single peak under these conditions would show that the original separation was due to heterogeneity. It was however not practicable to apply this test to PCMS-papain since an inordinate number of preparative runs would have been necessary in order to isolate enough protein from the front peak to use at the same concentration in an analytical electrophoresis run.

A form of experiment devised by Cann and Phelps[95] in their work on bovine serum albumin was used to test for the occurrence of an isomerization reaction in PCMS-papain. The above authors conducted prolonged electrophoresis with back-compensation to give a reasonable

separation of the two peaks. The current was then turned off for several hours during which time the only changes apparent in the boundary shapes were those expected from diffusion. On re-applying the electric field however, one or two new peaks were observed between the existing ones. It was proposed that the separated components underwent an isomeric interconversion during the interruption. Although Cann[96] later discarded this interpretation in favour of the acetic acid binding theory, there seems no reason to doubt that the above behaviour would also be observed for a protein undergoing an isomerization reaction with a half-time of the same order as the duration of electrophoresis.

Tests of this type were carried out after back-compensated electrophoresis of papain at pH 5 and PCMS-papain at pH's 4 and 5, using interruption periods of about 24 hours. No new peaks were observed when the electric field was again applied for 3 hours. Thus isomerization is not indicated. However, the sensitivity of this test was probably poor in view of the considerable broadening of main peaks during 24 hours of electrophoresis and a similar period of diffusion. Small new peaks would therefore have been difficult to detect.

To summarize, it seems clear that the bimodal boundary of PCMS-papain is not due to isomerization or

protein-buffer interactions. Assuming however that the main features of the boundary can be attributed to heterogeneity, it is possible that interactions of these types could contribute to the unusual shapes of the individual peaks after prolonged electrophoresis. In particular, interactions may be responsible for small trailing shoulders which probably hinder resolution between the main peaks. Unfortunately, experimental proof of interaction phenomena is difficult to obtain in a system such as the present one where any interaction effects are superimposed on and largely masked by effects of heterogeneity.

(d) Protein-Protein Interactions

(i) Theory

In a heterogeneous system such as PCMS-papain, there are two types of association which must be considered:

(1) those between like molecules

$$A + A \rightleftharpoons A_2 \quad \text{(or higher aggregates)},$$

and (2) those between unlike molecules

$$A + B \rightleftharpoons C$$

Such a reaction differs from the protein-buffer type discussed in the last section in that both reactants are non-dialysable and hence will be initially confined to

the same part of the electrophoresis cell.

As with isomerizations, the fundamental factor which determines the nature of the pattern is the rate of production of a species by re-equilibration compared to the difference in transport rates between species[1]. Again the simplest case is that in which the forward and reverse reactions are very slow; then each species migrates with its own mobility and the concentration gradient becomes nearly zero between the peaks. The other extreme case exists when the equilibrium is maintained by rapid reactions. This has been the subject of most theoretical exploration and the present discussion will be confined to this case. The problem of deducing boundary shapes for the case of intermediate reaction rates is very complicated. Bethune and Kegeles[61] have suggested that the inclusion of kinetic terms can be expected to broaden the calculated schlieren curve and improve resolution. This seems to be supported by numerical calculations made by Belford and Belford[97] for the sedimentation of a dimerizing system. These authors predicted that one, two, or three schlieren peaks might be observable depending on the rate of the reaction. It seems well established that a rapid dimerization gives only one peak[43,60].

As mentioned in Chapter II, the theory of

sedimentation in rapidly, reversibly associating systems has been extensively investigated. However, caution is necessary in applying the same theories to electrophoresis, first because sedimentation is formally analogous to electrophoresis only in the descending limb[2], and secondly because the assumption of a greater migration velocity for the aggregated species which is justifiably made for sedimentation is unlikely to be true in electrophoresis.

Gilbert[43] and Gilbert and Jenkins[98] have calculated theoretical ascending and descending boundary shapes for the electrophoresis of systems in which associations between either like or unlike macromolecules occur. Their theory, which neglects diffusion and non-ideal electrophoresis, predicts that for the self-association of a protein A, the boundary in one limb should broaden rapidly while becoming hypersharp in the other limb. For the bimolecular association of two unlike molecules A and B, a variety of boundary shapes can be predicted depending on the relative velocities of A, B and C, the relative concentrations of A and B, and the equilibrium constant. Two boundaries usually appear in each limb, though one may be bimodal giving the appearance of a total of three. As would be expected for a concentration-dependent reaction, the boundaries are always non-

enantiographic since in the ascending limb the direction of migration is towards a more dilute region while in the descending limb it is towards a region of higher concentration. For example in one quite simple case the pure fast component tends to give a separate peak in the ascending limb, while the pure slow component tends to separate out in the descending limb.

(ii) Application

There is sufficient similarity between the boundary features predicted by the Gilbert-Jenkins theory and those observed with PCMS-papain to warrant some consideration. In particular, if an interaction were to occur between unmodified papain and PCMS-papain, giving rise to a reaction boundary, the difference in apparent mobilities of the two observed peaks might well be less than the difference in the intrinsic mobilities of the two components. Furthermore the concentration gradient between the two peaks could not be expected to become zero, and one or both of the moving boundaries would not represent a pure component. The latter factor could account for the residual activity in the front peak.

The apparent absence of hypersharp boundaries, which are predicted in the systems considered by Gilbert

and Jenkins may not be too significant considering that their theory neglects boundary spreading due to non-ideal electrophoresis or diffusion. Of possibly more significance is the absence of the pronounced non-enantiography predicted for Gilbert-Jenkins systems. Further evidence against the occurrence of bimolecular association in the present system is provided by the sedimentation behaviour of PCMS-papain reported in Part A of this chapter. The boundary shape and sedimentation coefficient observed with PCMS-papain were nearly the same as with papain, which precludes the possibility of any great change in the extent of association. However, this sedimentation result was obtained only under one set of conditions (pH 4, 18°C). Before discarding the association hypothesis, it would be advisable to study the sedimentation of PCMS-papain at other pH's and at the same temperature as was used for electrophoresis, viz. 1°C.

One other experiment was done to test for the occurrence of interaction effects in the electrophoretic analyses; a different inhibitor, iodoacetic acid (IAA), was used and the papain derivative subjected to electrophoresis. A different papain derivative could reasonably be expected to have different association behaviour. Furthermore, IAA has the advantage of being an irrevers-

ible sulphydryl reagent thus making it unnecessary to maintain a small concentration of free inhibitor in the buffer solution. The use of 3×10^{-5} M PCMS in the buffer for the electrophoresis of PCMS-papain was in some ways undesirable since it is difficult to predict what effects might arise if it were to interact weakly with other groups in papain. The Gilbert-Jenkins theory does not seem to be applicable in this case because the PCMS is uniformly distributed through the cell, while the Cann-Goad theory has been developed only for interactions with uncharged buffer constituents whose gradients of concentration do not move in the electric field.

Electrophoresis of IAA-papain was conducted at pH 7 where the carboxylic acid group could be expected to exist in the anionic form. Modification was carried out as described in Chapter II, and an assay revealed no activity in the modified enzyme. The results of the electrophoresis are shown in Fig. III-8. Comparison with Fig. III-4 shows that the boundary shapes resemble those of PCMS-papain at pH 7 except that the slow component in the ascending pattern of the latter is hardly discernable in the IAA derivative. The apparent mobilities of the peaks are, however, only slightly less than those of unmodified papain and significantly higher than those of PCMS-papain.

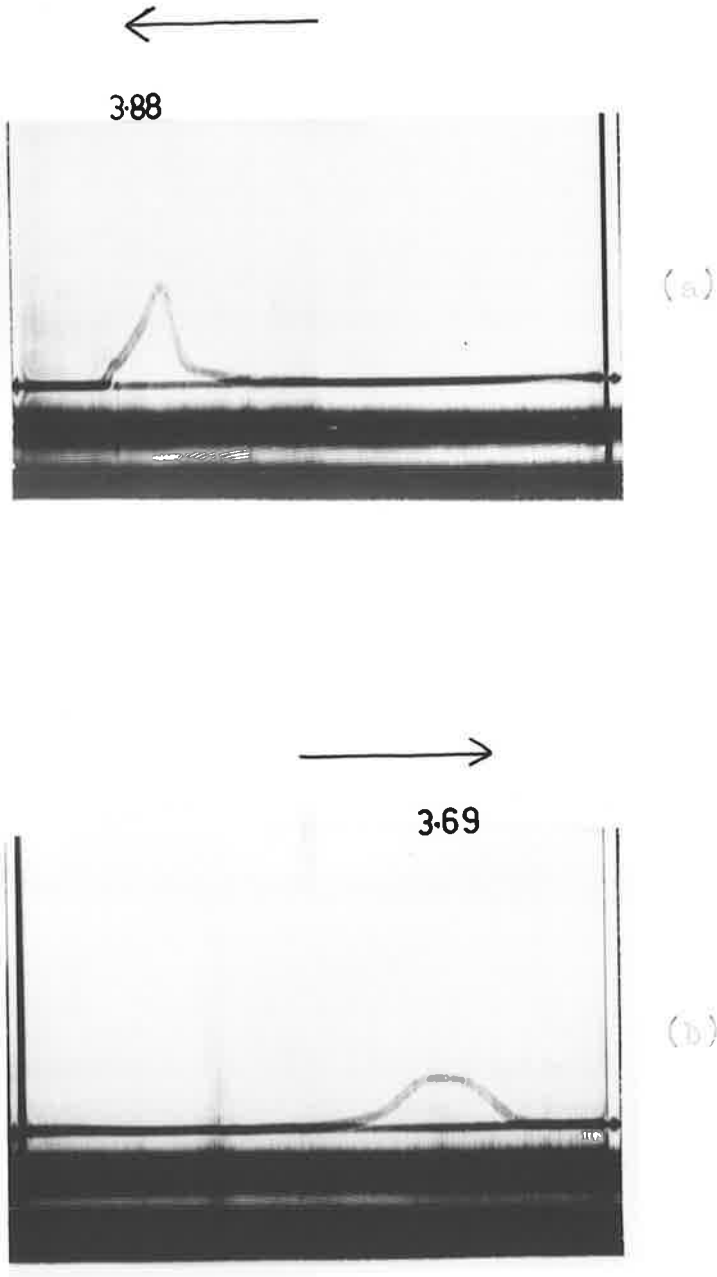


Fig. III-8 Electrophoretic patterns of iodoacetic acid-modified papain in phosphate buffer pH 7.04, ionic strength 0.05, (a) ascending limb, (b) descending limb. Photographs obtained after electrophoresis for 372 minutes at a field strength of 6.4 volt/cm. Apparent mobilities x 10^5 in cm^2 $volt^{-1}$ sec^{-1} are shown above the peaks.

It thus seems doubtful whether IAA-modification was successful in introducing a negative charge on to a significant proportion of the papain molecules at pH 7. In these circumstances the experiment is of little value for comparison between the IAA- and PCMS- inhibited forms of papain. Since carboxyl groups of proteins are usually completely ionized at pH's above 5.5[99], it is difficult to explain the electrophoretic behaviour of IAA-papain. A possible rationalization could be that the ionized S-carboxymethyl group weakens the acid dissociation of a neighbouring group, such as a histidine residue which would be expected to dissociate between pH 5.5 and 8[99].

(e) Conclusions

In general, the foregoing discussions support the conclusion expressed in Part A of this chapter that PCMS modification of papain yields a product with charge heterogeneity. The major differences in the electrophoretic behaviour of papain and PCMS-papain are evidently brought about by the existence in the latter of two differently charged forms, one of which carries a slightly lower positive charge in weakly acidic solution than does papain. Although electrophoresis of both papain and PCMS-papain reveals some other peculiarities, it is apparent that mechanisms in terms of interactions or

deviations from ideal electrophoresis or both can be suggested to account for all of the experimental observations. However, it is difficult to obtain experimental measures of the magnitude of such factors, except in the case of protein-protein associations where sedimentation velocity experiments at 1°C would be useful.

Another potentially useful method which has not been used in the present work would be to conduct electrophoresis at various concentrations of solute as far as permitted by solubility and the sensitivity of the optical systems of the apparatus. The interaction effects which have been discussed are all concentration dependent by virtue of either equilibrium or kinetic considerations. Thus it might be possible to interpret any changes in the electrophoretic behaviour. However, deviations from ideal electrophoresis become less at low protein concentrations and allowance must be made for this.

One principle which is well illustrated by the discussion of interactions is the importance of fractionation experiments and tests for chemical or enzymic differences in conjunction with any electrophoretic separation. This is the clearest way of showing whether a separation of peaks results from heterogeneity or from interactions.

CHAPTER IV

CHROMATOGRAPHY OF PAPAIN AND PCMS-PAPAIN

(a) Introduction

(b) Principles of Ion-Exchange Chromatography of Proteins

(c) Determination of Suitable Conditions for Chromatography

(d) Results of Ion-Exchange Chromatography of Papain and PCMS-Papain

  (i) Gradient Elution

  (ii) Use of a Single Eluant

(e) Discussion

  (i) Evidence for Heterogeneity

  (ii) Analogies Between Results of Ion-Exchange Chromatography and Electrophoresis

(f) Preparative Considerations and Conclusions

(g) Use of Organomercurial-Polysaccharide

(a) Introduction

The electrophoresis results presented in Chapter III provide strong evidence that PCMS modification of papain yields a product consisting of two different charged species. Thus, as mentioned at the end of Part A of that chapter, it was thought that a separation of components might be achieved by ion-exchange chromatography. This is a method for the fractionation of proteins with different charge characteristics[100], and separations analogous to those observed in electrophoresis have been reported[101,102]. Proteins of lower electrophoretic mobility were eluted more easily from a column material of opposite charge.

Previous chromatographic work on papain also gives some basis for attempting the fractionation of a suitable papain derivative. Finkle and Smith[8] carried out chromatography of papain using the polymethacrylate resin IRC-50. Papain and mercuripapain were each eluted as a single peak at pH 7. At pH 6.1, however, papain was eluted as a very broad peak and in some fractions had a higher specific activity than that of the sample applied to the column. This implied some separation of active from inactive enzyme. However it was also found that considerable autolysis (i.e. self-catalysed hydrolysis) of papain took place during the experiment. In fact the

amino acid compositions of fractions eluted at different stages were also significantly different. Thus the enrichment in specific activity in some fractions may only have been due to separation of intact papain molecules from autolysed fragments. Nevertheless the results suggest that a successful separation might be obtained by chromatography of papain in the presence of an inhibitor.

More recently, Sluyterman[20] conducted chromatography of papain on carboxymethyl cellulose (CM-cellulose). The technique was used as a criterion for the purity of a papain preparation to be used for kinetic studies. Papain was eluted by a stepwise change in buffer concentration at a constant pH (5.0), and a single peak chromatogram was obtained in which total protein and enzymic activity were superimposable.

This chapter deals mainly with attempts to fractionate papain into active and inactive species by cation-exchange chromatography on CM-cellulose columns. The use of a sulphydryl-specific column is also described. The next section is an account of the principles of ion-exchange chromatography and the ways in which the best possible resolution can be achieved.

(b) Principles of Ion-Exchange Chromatography of Proteins

Chromatography of proteins on ion-exchange

materials usually requires the establishment of electrostatic bonds between charged sites on the adsorbent phase and oppositely charged sites on the protein molecules. In some cases non-electrostatic forces may assume importance. The cellulosic ion-exchangers are particularly suitable for the chromatography of proteins since, by virtue of their low charge density and hydrophilic nature, they have little tendency to cause denaturation[100].

Diffential elution is generally achieved either by reducing the charge on the protein molecule by a pH change, or by decreasing the effectiveness of existing bonds by increasing the salt concentration[101]. Proteins of different charge, charge density or charge distribution may differ in their requirements for elution. Ideally an elution procedure should allow adsorption equilibrium at all times to achieve the maximum potential resolving power. However, in view of the range of affinities usually encountered in protein mixtures some means of progressively increasing the eluting power is usually employed. Furthermore such a technique helps to prevent the "tailing" of bands usually observed when a single eluant is used for the chromatography of a substance having a Freundlich or Langmuir type of adsorption isotherm[103]. The elution method most free from artifacts[100]

is that of gradient elution in which the composition of the buffer is increased in eluting power gradually and smoothly.

A suitably designed gradient brings the bound proteins gradually into adsorption equilibrium. The faster the increase in eluting power the sooner is the attainment of conditions that prevent re-adsorption and nullify the effectiveness of the remaining portion of the column. Thus if the gradient is made more gradual to permit the use of a longer portion of the column in multi-stage adsorption, an improvement in resolution can be expected.

(c) Determination of Suitable Conditions for Chromatography

In electrophoresis the best resolution of PCMS-papain was achieved when using acetate buffer of pH 5, a solvent in which papain is stable and quite soluble. It was therefore decided to attempt chromatography at this pH using a gradient of ionic strength. Since both papain and its PCMS derivative are positively charged at pH 5, the cation exchanger CM-cellulose was chosen as the adsorbent.

The ionic strengths required for adsorption and elution were determined by batchwise adsorption experiments. The following solutions were prepared:

(1) four acetate buffers of pH 5.00 having ionic strengths (all sodium acetate) of 0.01, 0.1, 0.25 and 0.5,

(2) an equilibrated suspension[100] of CM-cellulose (12 mg/ml) in each of the above buffers,

and (3) a concentrated (ca. 1.2%) papain solution in distilled water against which it was dialysed for 24 hours.

Mixtures of adsorbent suspension (5 ml), the corresponding buffer (5 ml), and papain solution (0.2 ml) were stirred at room temperature for 2-3 minutes then centrifuged to settle the adsorbent. The optical densities of the supernatant solutions were measured at 278 mμ, the appropriate buffer being used as the reference solution. Optical density measurements were also made on blank solutions from which the adsorbent suspension was omitted, an equal volume of buffer being added instead.

The binding capacity of the CM-cellulose for papain in each buffer was then calculated in terms of the distribution coefficient (C) which is defined by the relation[103]

$$C = \frac{\text{Fraction of solute in a certain mass of adsorbent}}{\text{Fraction of solute in the liquid phase in contact with that adsorbent}}$$

Calculation of C requires a knowledge of the void volume, or volume of liquid phase held between the particles, in

a given mass of adsorbent. For the present purpose, the manufacturer's data[104] (i.e. that 1 gram of dry cellulose yields 8 ml of settled bed volume of which 40% is void volume) was assumed to be sufficiently accurate. From this it follows that the 60 mg of added adsorbent occupies a volume of 0.48 ml, of which 0.19 ml is void volume. Thus, the total volume of liquid phase in the reaction mixture is (10.2 - 0.48 + 0.19), or 9.9 ml. Hence ...

Wt. of papain in liquid phase in contact with adsorbent particles = 0.19 D (arbitrary units)

where D is the optical density of the supernatant.

And ...

Wt. of papain in adsorbent phase = $10.2\ D^{o} - 9.9\ D$ (same units)

where D^{o} is the optical density of the 10.2 ml of blank solution.

Therefore $$C = \frac{10.2\ D^{o} - 9.9\ D}{0.19\ D}$$

Table IV-1 shows the optical density measurements and approximate distribution coefficients in each of the buffers.

TABLE IV-1

EFFECT OF IONIC STRENGTH ON THE BINDING OF PAPAIN TO CM-CELLULOSE IN ACETATE BUFFERS OF pH 5.00

Ionic Strength	$D^{1\ cm}_{278}$		Distribution Coefficient[b]
	Supernatant	Blank[a]	
0.01	0.025	0.401	810
0.1	0.061	0.397	300
0.25	0.343	0.436	16
0.5	0.419	0.433	3.4

[a] Variation in the figures in this column is mainly due to the use of 2 different papain solutions on different days.

[b] If, instead of assuming a void volume of 40%, a figure of 70% (as found by Thompson and O'Donnell[105] for diethylaminoethyl-cellulose) is used, the distribution coefficients are 25% to 60% lower. For the present purpose such a difference is not important.

Distribution coefficients between 2 and 5 are the most favourable for elution chromatography[103]. However, to achieve high resolution the protein should be applied to the column in a buffer which allows strong binding; this reserves a longer portion of the column for multi-stage re-adsorption during elution[100]. Thus in the initial experiments with papain, the enzyme was applied to the column in acetate buffer of pH 5 and ionic strength

0.1, and eluted by a gradual increase in ionic strength (as sodium acetate) at this pH. A check on the solubility of papain in such buffers at 2°C (a slightly lower temperature than was used in chromatography - see Chapter VI) showed it to be about 0.1% in the 0.25 ionic strength buffer and about 0.05% at ionic strength 0.5. This solubility was considered satisfactory for chromatography since the method for detecting papain in the eluate (i.e. ultraviolet absorption) is applicable at low concentrations. The elution conditions found in the above manner are similar to those since reported by Sluyterman[20], who eluted papain from CM-cellulose by a stepwise change from 0.1 to 0.7 ionic strength in acetate buffer of pH 5.

(d) Results of Ion-Exchange Chromatography of Papain and PCMS-Papain

(1) Gradient Elution

Fig. IV-1 shows gradient elution chromatograms for both papain and PCMS-papain. A CM-cellulose column (12 cm x 1 cm diameter) was used in both experiments; a 50 mg load of enzyme was applied as 0.2% solution in acetate buffer of pH 5, ionic strength 0.1, and eluted by an approximately linear gradient to 0.4 ionic strength buffer over a volume of 200 ml. The gradient is illustrated in the chromatogram by measurements of

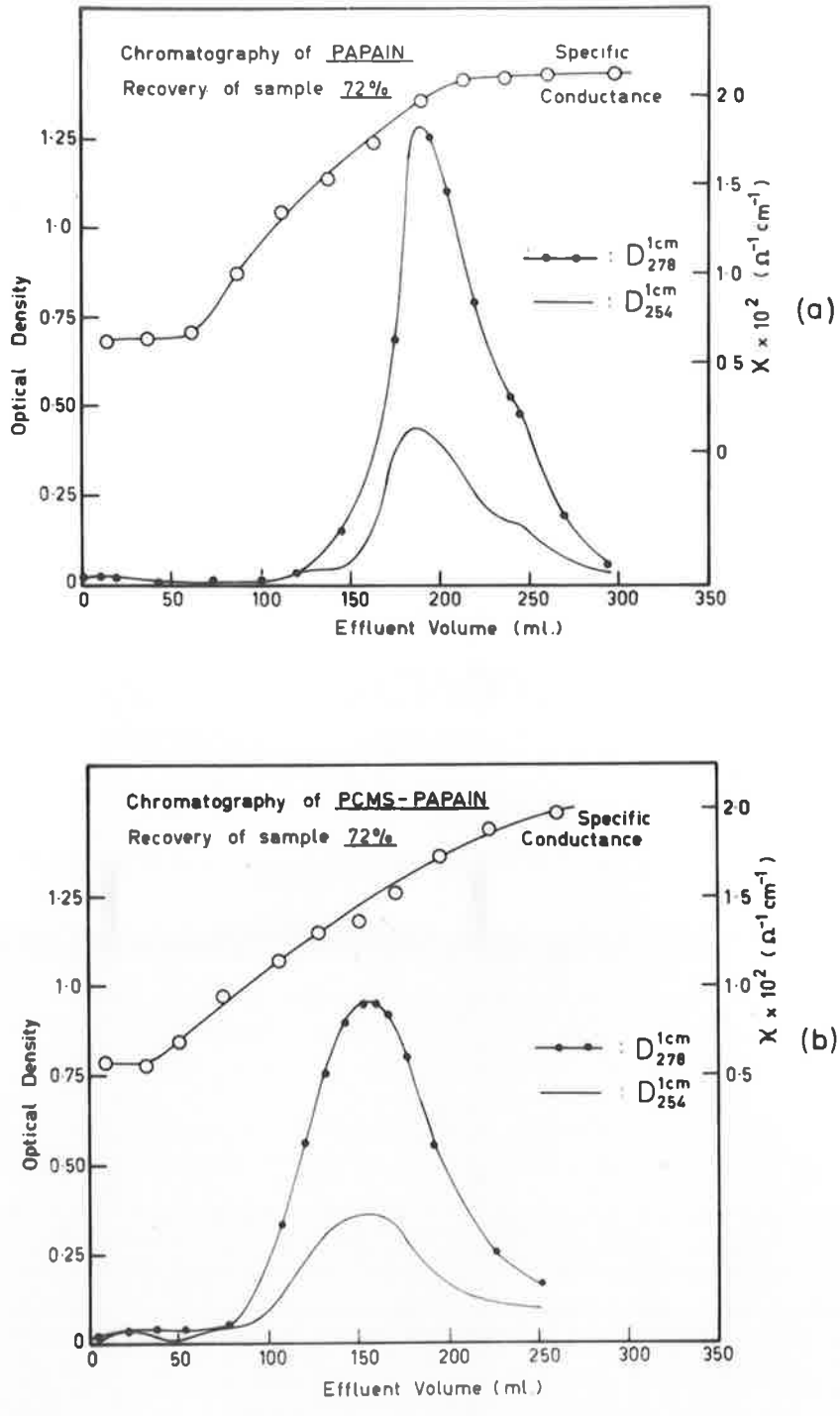


Fig. IV-1 Gradient elution chromatograms on CM-cellulose of (a) papain, and (b) PCMS-papain in acetate buffer pH 5.0, ionic strength 0.1 → 0.4. Optical density at 278 mμ was measured on eluate fractions; optical density at 254 mμ was measured with a flow stream analyzer at the base of the column. The ionic strength gradient is shown by the specific conductance (K) of the effluent.

specific conductance which were made on the eluate fractions. The protein peaks are shown by optical density measurements both at 254 mμ, recorded by a flow analyzer attached to the base of the column, and at 278 mμ (the absorption maximum for papain) measured on the fractions. Details of the chromatographic techniques and instrumentation are given in Chapter VI.

It can be seen from Fig. IV-1 that no apparent separation of PCMS-papain was achieved, since it gave a chromatographic profile only slightly broader than that of papain. The small shoulder on the trailing edge of the latter is almost certainly due to the levelling out of the ionic strength gradient. With PCMS-papain, protein begins to appear in the eluate earlier (i.e. at smaller elution volumes) than with papain under the same conditions, which is in accordance with the hypothesis that the average positive charge on the molecules is less. However, PCMS-papain is eluted in a single peak which gives no indication of heterogeneity.

When a more gradual gradient, from 0.2 to 0.3 ionic strength, was used for the chromatography of PCMS-papain the eluted peak was slightly broader but still showed no apparent resolution into components. However, assays of a few widely separated fractions revealed a slight fractionation of enzymic activity. The specific

activity of PCMS-papain (measured in the presence of 0.005 M cysteine and 0.001 M EDTA) was higher by about 25% in an early fraction, and lower by about 20% in a fraction coming after the optical density maximum, than that of an unfractionated sample. In view of this indication of separation it was decided to compare the chromatographic profiles, both for total protein and for enzymic activity, of papain and PCMS-papain under conditions of maximum resolving power, i.e. using a single eluant rather than a gradient.

(ii) Use of a Single Eluant

Figs. IV-2 and 3 show the results of chromatography of papain and PCMS-papain respectively on similar CM-cellulose columns (11-12 cm long x 1 cm diameter) in a single eluant, viz. a sodium acetate-acetic acid buffer of pH 5.0 and ionic strength 0.2. The solid lines show the optical density of the eluate fractions, and the circles represent enzymic activities calculated as the first order rate constant (k_1) for the hydrolysis of BAA under the usual conditions (see Chapter VI). In the inset graphs above the chromatographic profiles, the crosses represent specific enzymic activities of the fractions assayed and the horizontal broken line represents the specific activity of the unfractionated sample.

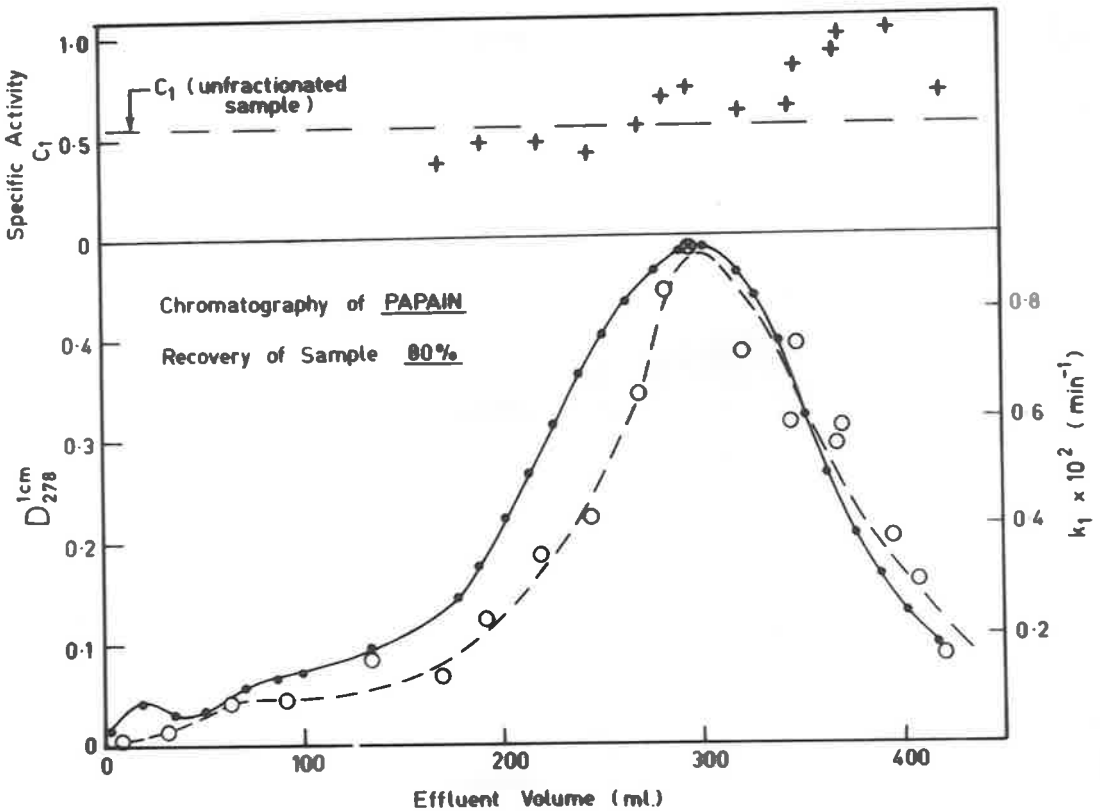


Fig. IV-2 Chromatography of papain on CM-cellulose in acetate buffer pH 5.0, ionic strength 0.2. Points denote optical density at 278 mμ; circles denote enzymic activity (k_1). The scale for k_1 has been chosen to make k_1 and D_{278}^{1cm} coincide on the graph at the point denoted ⊗.

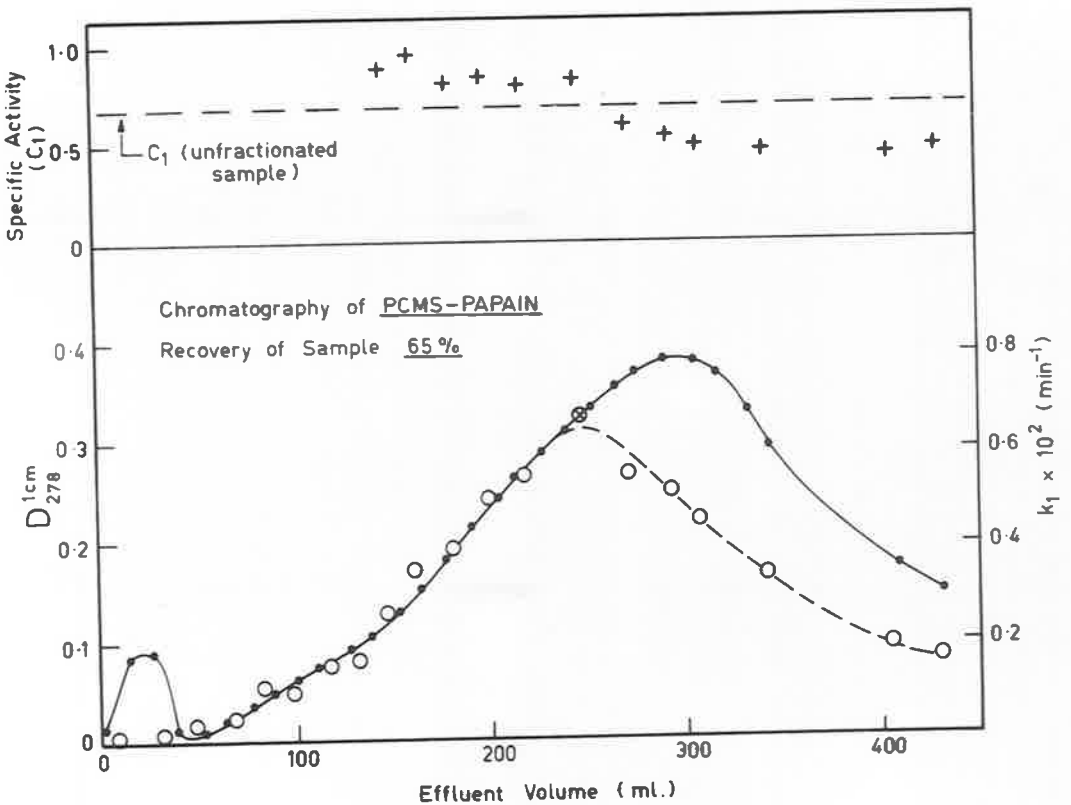


Fig. IV-3 Chromatography of PCMS-papain on CM-cellulose in acetate buffer pH 5.0, ionic strength 0.2. Points denote optical density at 278 mμ; circles denote enzymic activity (k_1). The scale for k_1 has been chosen to make k_1 and $D^{1\,cm}_{278}$ coincide on the graph at the point denoted ⊗

In neither case does the optical density curve indicate a resolution of the protein into two components. The small peak eluting ahead of the main peak in each chromatogram corresponds to a component which was not retarded by the column and which differed from papain in that its absorption spectrum had no maximum at 278 mμ, instead showing a steady increase in extinction below this wavelength. The main optical density peaks in both chromatograms are similar, although that for PCMS-papain is flatter and slightly less symmetrical. A marked difference is however observed in the activity graphs. With papain the maxima in the activity and total protein curves approximately coincide, but the early fractions have lower <u>specific</u> activity than the later fractions. The opposite trend is shown by PCMS-papain, the specific activity being higher in fractions constituting the leading edge of the peak. Furthermore the maximum in the activity curve occurs before the maximum in the total protein curve. With both samples, the specific activity figures obtained at very low protein concentrations (optical density below 0.1) have not been plotted. Their uncertainty is considered to be too large owing to the high relative error in the determination of low activities and low protein concentrations.

(c) Discussion

(i) Evidence for Heterogeneity

The chromatogram for unmodified papain on CM-cellulose (Fig. IV-2) is in some respects similar to that obtained by Finkle and Smith[8] using a polymethacrylate resin column and phosphate buffer of pH 6.1. These investigators also observed a slight fractionation of activity, the early fractions showing depleted specific activity and the later fractions greater specific activity compared with the unfractionated solution. As mentioned in section (a), it was considered that the activity fractionation was at least partly due to the separation of autolysed fragments from intact papain molecules. It is possible that similar behaviour may have occurred in the present study, especially since the papain sample had been stored for several months (in the form of a crystallized suspension - see Chapter VI) before use in the experiments described in the last section.

It is however significant that the activity fractionation observed with PCMS-papain is in the opposite direction. The specific activity was highest in the fractions constituting the front of the peak where, by analogy with Finkle and Smith's experiments[8], autolysed fragments of papain might occur, and lower at the protein maximum where there should be a greater predominance of

intact molecules. This result is consistent with the hypothesis that in PCMS modification the positive charge on active papain molecules is made less than that on inactive molecules. The former would then be retarded less by the CM-cellulose column.

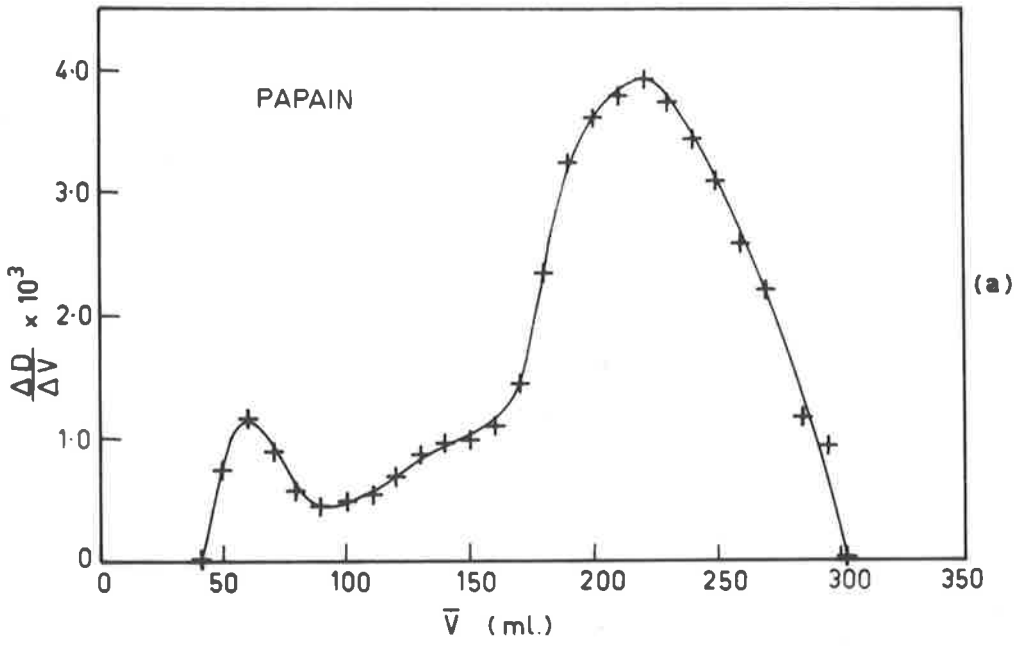
(ii) Analogies Between Results of Ion-Exchange Chromatography and Electrophoresis

Assuming that protein cations of higher electrophoretic mobility should be retarded more by the cation-exchange cellulose, the apparent transference of activity towards earlier fractions in the chromatography of PCMS-papain compared to papain is analogous to the results of preparative electrophoresis which showed that most of the activity of PCMS-papain was associated with the component constituting the slower electrophoretic peak. The elution volume at which the optical density maximum was reached was approximately the same in the chromatography of papain and PCMS-papain. This seems analogous to the observation that the electrophoretic mobility of the main peak was approximately the same for both papain and PCMS-papain at this pH (Table III-1). The explanation in both cases is believed to be that a large proportion of both modified and unmodified papain consists of the same component, a non-SH form of papain.

The shape of the chromatographic profile of

PCMS-papain (Fig. IV-3) provides a much less convincing demonstration of heterogeneity than does its electrophoretic pattern (e.g. Fig. III-2). It must be appreciated however that the electrophoretic patterns are differential plots ($^{dc}/_{dx}$ v. x) which are always more sensitive to changes in shape than are plots of concentration v. distance, or volume in the case of chromatography. Accordingly, in Figs. IV-4(a) and (b) are plotted first derivative chromatograms for papain and PCMS-papain, obtained by graphical differentiation of the optical density curves in Figs. IV-2 and 3 respectively. The change in optical density (ΔD) was calculated for each 20 ml increment in effluent volume (ΔV), and the quantity $\frac{\Delta D}{\Delta V}$ has been plotted against $\bar{V}$, the mean value of the effluent volume in the interval ΔV. Differentiation was carried out in this way for the leading edge of the main peak in each case, i.e. from the point at which elution of the main peak commences up to the optical density maximum.

The derivative curves so obtained show some similarity to the electrophoretic patterns of papain and PCMS-papain at this pH (Fig. III-2). A small leading peak is seen in both elution derivative curves analogous to the trailing one noticed in electrophoresis. In addition, the main derivative peak, though not symmetrical



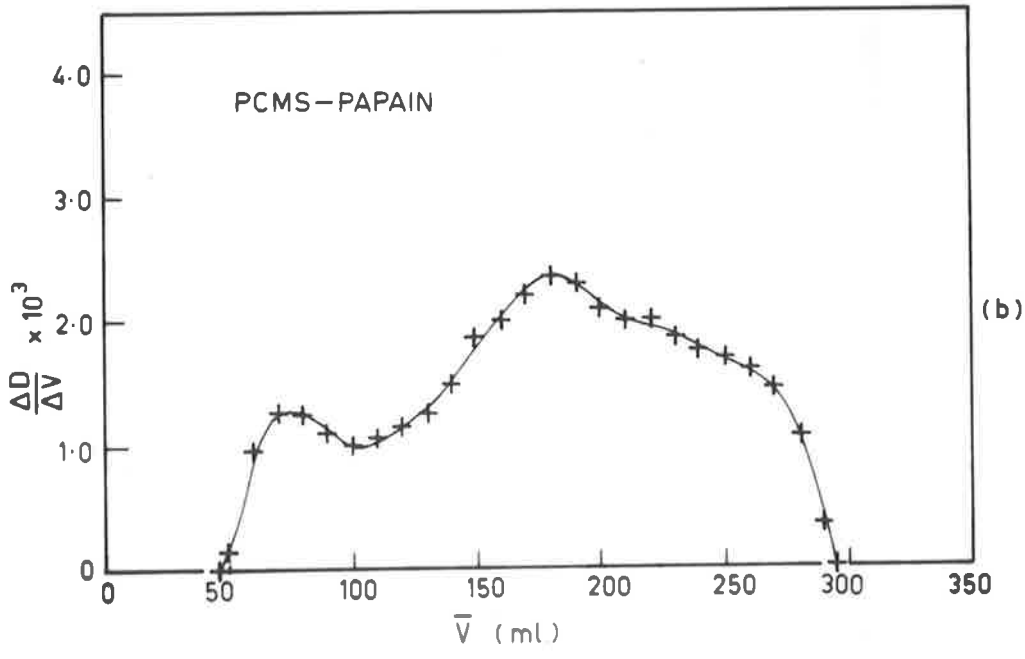


Fig. IV-4 Elution derivative curves for (a) papain, and (b) PCMS-papain, obtained by graphical differentiation of the leading sides of the chromatograms in Figs. IV-2 and IV-3 respectively.

in either case, shows more tendency to split into two peaks in the case of PCMS-papain (Fig. IV-4(b)). Thus, heterogeneity of PCMS-papain is tentatively implied by the elution derivative curves.

Winzor and Scheraga[106], and Winzor and Nichol[107] have observed, both for associating and non-associating protein systems, distinct analogies between derivative curves of Sephadex gel filtration chromatograms and ultracentrifuge schlieren patterns. Briefly, the similarities arise because both techniques tend to separate proteins of different molecular size. It therefore seems reasonable to expect an analogy between ion-exchange chromatography and electrophoresis both of which methods tend to separate protein molecules of different charge density. It should be noted however, that in the gel filtration work mentioned, the technique of frontal analysis was used, i.e. the protein sample was fed continuously on to the column until a constant concentration of protein in the effluent was attained. This technique is necessary for any quantitative theoretical interpretation of chromatographic behaviour since it is the only way in which the concentration of protein in the liquid phase inside the column can be controlled[2,107].

(f) Preparative Considerations and Conclusions

It can be seen from Figs. IV-2 and 3 that higher specific activities of papain were obtained in the later fractions of unmodified papain than in the early fractions of PCMS-papain. Thus, as a method of separating active papain from inactive molecules and possibly autolysed fragments, chromatography appears to be more successful with unmodified papain. In neither case however is the separation sufficiently clear-cut to provide much hope of using ion-exchange chromatography to obtain samples of active and inactive papain.

The conclusions to be drawn from the ion-exchange chromatography described in this chapter are that confirmatory evidence has been obtained for the existence of two different charged species in PCMS-papain, but that the separation of these species was less distinct than in electrophoresis. The latter occurrence may be associated with the nature of groups in the region of the protein surface which is affected by PCMS-modification, i.e. the active site. If this region is negatively charged in unmodified papain, as may well be the case in view of evidence cited in Chapter I for the existence of an ionized carboxyl group in the active site, it probably has little tendency to bind to the cation exchanger. In this event the chromatographic behaviour

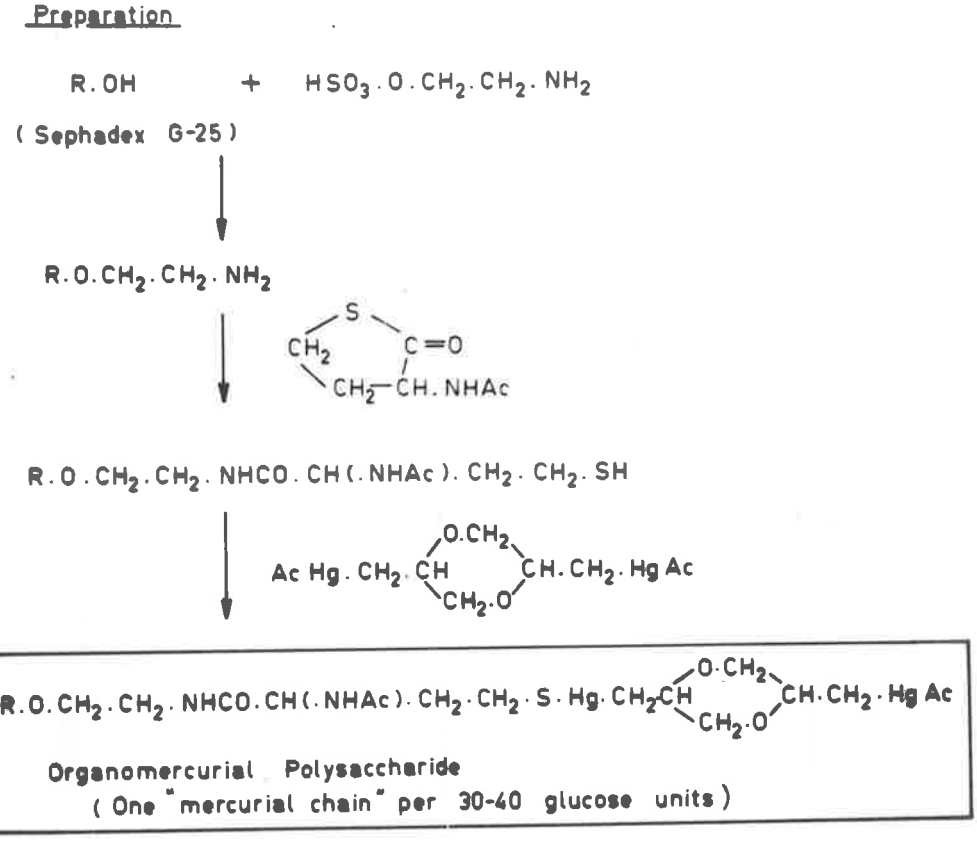
of papain might not be much altered by PCMS-modification.

One disadvantage of both electrophoresis and ion-exchange chromatography in the present case is that separation is determined by a difference in charge which is small in comparison with the total charge on the papain molecule. More success might be achieved by a separation method based on chemical differences. Thus the next section describes attempts to separate the sulphydryl and non-sulphydryl forms of papain by the use of an adsorbent having an affinity for sulphydryl compounds.

(g) Use of Organomercurial-Polysaccharide

Eldjarn and Jellum[108] have recently reported the preparation of organomercurial-polysaccharide, a column material with a specific affinity for sulphydryl compounds, and have used it to separate sulphydryl from non-sulphydryl proteins. The function of organomercurial-polysaccharide is based on the reversible reaction between SH proteins and an organomercurial grouping firmly anchored to a cross-linked dextran. The structure of organomercurial-polysaccharide is illustrated in Fig. IV-5 which also shows the method of synthesis and mode of action of this material.

The above authors found that proteins containing SH-groups were, in contrast to non-SH proteins, readily



Mode of Action

$R.O.CH_2$ ------ $CH_2.HgAc$ + HS.Protein

$R.O.CH_2$ ------ $CH_2.Hg.S.$Protein

Eluant (Mercury complexing agent e.g. EDTA)

$R.O.CH_2$ ------- $CH_2.HgAc$ + HS.Protein

Fig. IV-5 Preparation, structure and function of organomercurial-polysaccharide.

bound to columns of this material and could be eluted in an active, unchanged state by small molecular thiols or other complexing agents having affinity for mercury. A number of protein mixtures were fractionated by the use of organomercurial-polysaccharide. Of particular interest were the fractionations of, (1) human serum albumin originally containing 0.58 molar equivalents of SH-groups into mercaptalbumin with 1.0 SH-groups per molecule and an albumin without titratable SH-groups, and (2) a crude sample of urease originally containing 15.6 molar equivalents of SH-groups into two fractions, one without SH-groups and the other containing active enzyme with an SH-titre of 25.2 groups per molecule. Since the problem of separating papain into sulphydryl and non-sulphydryl forms appears to bear a close analogy to the above separations, it was decided to attempt chromatography of papain on organomercurial-polysaccharide.

Experimental and Results

Organomercurial-polysaccharide was synthesised from Sephadex G-25 by alkylation, thiolation, and combination with 3,6 bis-acetatomercurimethyl-dioxane as described by Eldjarn and Jellum[108]. The product was characterised by means of haemoglobin which, in accordance with the results of the above authors, was shown to be extracted

from solution in 0.01 M phosphate buffer of pH 7.6, and released in the presence of 0.05 M cysteine.

For experiments with papain it was considered necessary to first activate the enzyme in order to free the SH-groups for binding to the column material. Papain solutions were therefore treated with cysteine (20 moles, concentration .005 M in the activation mixture) and EDTA (4 moles) just before use; these compounds were then removed by gel filtration on a column of Sephadex G-25 (coarse grade) equilibrated with the appropriate buffer.

Preliminary batchwise experiments on the binding of papain to organomercurial-polysaccharide showed a partial binding of papain from solution in 0.01 M phosphate buffers between pH 6.0 and 7.6. The bound papain was completely liberated by cysteine or EDTA at a concentration of 0.05 M, and substantially liberated in 0.01 M acetate buffer at pH 4.0. These results seemed consistent with the binding of a fraction of the papain molecules to the adsorbent in a similar manner to other sulphydryl proteins[108].

Chromatography however proved quite unsuccessful. The column retained all but a slight trace of papain samples applied in 0.01 M phosphate buffers of

pH 7.6 or 6.0. Stronger eluants such as acetate buffer of pH 4, EDTA, or cysteine solutions each removed some protein but the total recovery was still less than 50% of the initial sample. No explanation can be offered for this apparently unspecific binding of papain to organomercurial-polysaccharide. Eldjarn and Jellum[108] observed retention of non-SH albumin on columns of this material which had been equilibrated with distilled water only, but this apparently unspecific adsorption was not observed in 0.01 M phosphate buffers.

In view of the lack of success with chromatography, attempts were made to fractionate papain by batchwise binding and elution on organomercurial-polysaccharide. It was found that no difference in specific activity could be detected between papain which was not bound to the adsorbent at pH 6.0 or 7.6 and papain which became bound and was later eluted at pH 4.0. Further investigation showed that papain which had not been subjected to any activating procedure was bound to the same extent as was activated papain. In addition, papain inhibited with iodoacetic acid was also extracted from solution to the same extent.

It thus appears that the binding of papain to organomercurial-polysaccharide is not through the active SH-group but is probably unspecific. Consequently no

separation of the sulphydryl and non-sulphydryl forms of papain can be expected from the use of this adsorbent.

CHAPTER V

CONCLUDING DISCUSSION:

SOME FEATURES OF THE ACTIVE SITE OF PAPAIN

(a) Mechanism of Activation

(b) Concept of a Sterically Hindered Active Site

(c) Consideration of Possible Oxidation States other than Disulphide for the Blocked SH Group

  (i) Reversibly Inactivated Papain

  (ii) Irreversibly Inactivated Papain

The principal conclusions arrived at in previous chapters can be listed as follows.

(1) Conditions for the occurrence of reversible association of papain in solutions of pH's below its isoelectric point have been ascertained.

(2) There is no reason to believe that association of papain requires the formation of intermolecular disulphide or metal to protein bonds.

(3) Papain, as usually isolated, comprises at least two similar molecular species which apparently differ in the chemical state of one sulphur atom and in enzymic activity.

Further details of these and some other conclusions have been given in the appropriate chapters. However, since much of this work has been concerned with derivatives of papain in which the active SH group was combined with inhibiting reagents, the results also provide some insight into the chemistry of the active site. It was mentioned in the introductory chapter that there is still some doubt over the possible chemical states of the active SH group, and the chemical sequences of events which bring about activation and inactivation of papain. The purpose of this concluding chapter is to consider what information about these problems can be deduced from the present study.

(a) Mechanism of Activation

It was shown in the course of electrophoretic analyses described in Chapter III that the proportion of papain molecules reacting with PCMS, as judged from the size of the slow peak, was increased when papain was treated with cysteine and EDTA before modification. This observation supports previous evidence cited in Chapter I that the usual activators of papain liberate reversibly "blocked" SH groups present in some of the enzyme molecules. The blocked SH groups are apparently in an oxidized state since the enzymic activity of papain is increased by reducing procedures and decreased by oxidation[8,14,29].

Objections have been pointed out in Chapter I to the possibility of any known form of disulphide bonding in proteins, i.e. intramolecular, intermolecular, or of the mixed (Protein-S-S-R) type, constituting the reversibly oxidized form of papain. Further evidence against the existence of the first two types is provided by results of the present study. Activation by reduction of an intramolecular disulphide bond to two SH groups seems unlikely on the basis of approximate calculations made in Chapter III for the difference in charge between the two main electrophoretic components of PCMS-papain. These results, though indefinite, were more consistent

with the incorporation of one PCMS molecule than two into the active papain molecule. With regard to intermolecular disulphide bonding, the results of Chapter II confirm previous findings that papain exists predominantly as a monomer at the concentrations and pH's used for enzymic studies, and show also that under conditions where aggregation does occur this type of bonding is not the cause.

(b) Concept of a Sterically Hindered Active Site

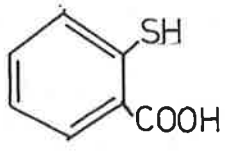
Since the active SH group of papain is readily oxidized, it is surprising that papain does not form disulphide-linked aggregates as do some other proteins such as urease[109] and a soybean protein[110]. This behaviour could be explained however if steric hindrance or electrostatic repulsion by the surrounding protein surface were to prevent formation of a disulphide bond between the active sites on two neighbouring molecules. Steric hindrance of the active site of papain has previously been suggested to occur by Kirschenbaum[111]. From studies of steric and sequential substrate specificity in papain-catalysed peptide syntheses, he postulated that the active site may be situated in a cavity or invagation on the enzyme into which the substrate must fit.

This concept seems capable of accounting for

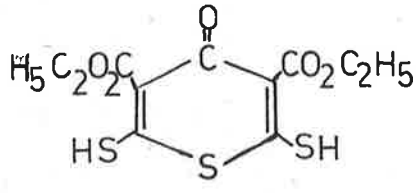
other observations made during the present study. Specifically, it might explain why the ion-exchange chromatographic behaviour of papain was not greatly altered by PCMS-modification, and why the active SH group of papain appeared not to bind to organomercurial polysaccharide (see Chapter IV).

(c) Consideration of Possible Oxidation States other than Disulphide for the Blocked SH Group

The proposal mentioned in Chapter I that the active SH group of papain may undergo oxidation to a state such as a sulphenic acid or sulphinic acid now assumes greater significance. There is evidence that some small molecules containing sterically hindered SH groups are more likely to undergo oxidation to products other than dimeric disulphides. For example the compounds



and



can be oxidized in high yields to the sulphinic acid[112] and the di-sulphinic acid[113] respectively. In addition, high yields of sulphonic acids can be obtained by hydrogen peroxide oxidation of tertiary mercaptans[114,115]. It is

by no means certain that steric hindrance is an important factor in all of the above reactions. Nevertheless, if it could be shown that organic oxyacid states of sulphur exist in the inactivated forms of papain, there would be strong grounds for believing that the active SH group is unfavourably situated for intermolecular disulphide bonding.

Of the above mentioned groups, viz. sulphenic acid (-SOH), sulphinic acid (-SO.OH), and sulphonic acid ($-SO_2.OH$), the first is probably the only one which could constitute a <u>reversibly</u> oxidized form of papain. Little is known of the properties of free sulphenic acids since only one such compound is sufficiently stable to be isolated, viz. 1-anthraquinone sulphenic acid, and this probably owes its stability to a substantially rearranged type of structure[116]. However, other compounds corresponding to the sulphenic oxidation state can be reduced to disulphides by mercaptans[117] and would therefore probably give rise to an SH group in the presence of a large molar excess of the mercaptan. According to Ferdinand <u>et al</u>[38], a sulphinic acid could also give rise to an SH group under these conditions, but Reid's monograph[117], although giving several methods for the reduction of sulphinic acids, makes no reference to this reaction. For the present, sulphinic acid will be

considered a possible oxidation state in both reversibly and irreversibly inactivated papain. It is generally accepted that reduction of a sulphonic acid group to an SH group requires the use of more powerful reducing agents than mercaptans in aqueous solution[118].

From the results of the present study it is possible to speculate about the magnitude of charges carried at various pH's by groups in the active site of modified and unmodified papain. Only tentative suggestions about the nature of groups can be made on this basis since interactions with neighbouring groups often cause the ionization characteristics of groups in proteins to differ from those in simpler molecules[99,119]. However, as there are no established methods for the detection of sulphenic or sulphinic acids in proteins[38], some speculation is probably worthwhile.

(1) Reversibly Inactivated Papain

The following points are noted which suggest that the acid dissociation of the reversibly oxidized SH group in papain is not greatly altered by reduction to the sulphydryl state.

(1) When papain, before electrophoresis at pH 4, was treated with cysteine and EDTA, these activators then being removed by dialysis, its electrophoretic mobility

showed only a very slight decrease compared with unreduced papain (Table III-1).

(2) It was shown in Chapter II that the extent of association of some papain derivatives at pH 8 was in the order

$$\text{IAA-papain} > \begin{matrix}\text{unreduced}\\ \text{papain}\end{matrix} \simeq \begin{matrix}\text{cysteine}\\ \text{reduced papain}\end{matrix} > \text{IAM-papain}$$

If, as appears likely, association is enhanced by the presence of an anionic group in the active site, the above sequence implies that the charge carried by a papain molecule at this pH is not substantially affected by activation. However, the spatial positions of charged groups may also be important in which case a comparison of papain with a derivative into which a carboxymethylene group has been substituted would be unjustified. Moreover it is difficult to decide how much charge difference exists between the above species; electrophoresis of IAA-papain at pH 7 surprisingly indicated very little charge difference from papain at this pH (see Chapter III, Part B).

If the reversibly oxidized form of the active SH group does have similar ionization characteristics to the free SH group then it is unlikely to be a sulphinic acid. Simple sulphinic acids are stronger than corres-

ponding carboxylic acids[120]. Nevertheless the presence of a compound such as an internal ester of a sulphinic acid might still be possible. Similarly the sulphenic state, though almost unknown as the free acid in simple molecules, might exist in a protein in the form of an internal ester. The formation of the sulphenyl oxidation state in some proteins has been postulated[121-124] to account for the results of oxidation with iodine. Fraenkel-Conrat[121,122] found evidence for this state in native, but not in denatured, tobacco mosaic virus protein after iodine oxidation of the SH groups. This could again be a case of steric hindrance preventing the formation of disulphide bonds. It would be interesting to find whether denatured papain would form disulphide-linked aggregates on oxidation with, for instance, iodine or hydrogen peroxide.

(ii) Irreversibly Inactivated Papain

The electrophoretic results of the present study confirm previous findings cited in Chapter I that most papain preparations contain a substantial proportion of molecules which do not react with sulphydryl reagents such as PCMS even after activation. The results also indicate that the charges carried by all unmodified forms of papain are very similar. For example, electrophoresis of

cysteine activated papain at pH 4 showed no apparent separation of active and inactive forms (Fig. III-1(c)). Separation became apparent however when the active form was combined with the reagent PCMS which carries an ionized sulphonic acid group. These observations suggest that, like the reversibly inactivated form, irreversibly inactivated papain does not contain a strongly acidic derivative (such as a sulphinic or sulphonic acid) of the sulphur atom in the active site.

To summarize this discussion it can be said that the present study provides further evidence that activation of papain liberates, in some molecules, free SH groups from bonding which is not of the intramolecular or intermolecular disulphide type. However the actual nature of both the reversibly and irreversibly inactivated states remains obscure. If the different molecular forms of papain could be separated it might be possible to decide from comparative studies what differences exist. Determination of titration curves and spectral characteristics might be useful in this respect. Unfortunately however, the fractionation of papain into different species has been found to be an awkward problem.

CHAPTER VI

MATERIALS AND METHODS

(a) Papain

(i) Commercial Samples
(ii) Preparation
(iii) Assay

(b) Other Materials

(c) pH Measurements

(d) Preparation of Papain Solutions

(e) Determination of Papain Concentrations

(f) Estimation of Protein-Bound PCMS

(g) Sedimentation

(i) Experimental
(ii) Calculations

(h) Viscosities and Densities

(i) Electrophoresis

(j) Conductance Measurements

(k) Chromatography

(i) Preparation of Columns
(ii) Loading and Eluting Conditions
(iii) Monitoring and Collection of Effluent

(a) Papain

(i) Commercial Samples

In the initial stages of this investigation, twice recrystallized papain was obtained from Sigma Chemical Co.[55] Lot number P41B-60 was used for investigating the effect of cysteine on the sedimentation of papain at pH 4, and lot number P52B-59 was used for some of the experiments on the sedimentation of papain at pH 7. Papain for all other experiments was prepared as reported in part (ii) of this section.

The above commercial samples were found to have specific activities of 1.0 and 0.9 respectively (expressed as the proteolytic coefficient (c_1), determined in the manner described later in this section). Electrophoresis showed batch number P52B-59 to contain 2-3% of impurities with mobility at pH 4 different from that of the main component.

(ii) Preparation

Following the discovery of impurities in a commercial papain preparation, all further samples were prepared from dried papaya latex (a gift from Wallerstein Laboratories, New York) by the method of Kimmel and Smith[41]. The samples were recrystallized three times from dilute sodium chloride solution as described by the

above authors. As a precaution against contamination by heavy metal ions, the second of these recrystallizations was from a solution containing 0.001 M EDTA (di-sodium salt). Papain was stored at 0-4°C as a crystallized suspension in 0.05 M sodium acetate solution the pH of which had been adjusted to 4.5 by addition of hydrochloric acid.

Freshly prepared papain samples had c_1 values of 1.1 to 1.3. Activity was lost slowly during storage under the above conditions; samples which had been stored for several months had c_1 values of 0.5 to 0.6. Electrophoretic analyses of several batches of papain prepared from latex revealed no trace of the impurities detected in the commercial sample.

(iii) Assay

Since the specific activity of papain samples is variable and related to the sulphydryl content, it is clearly necessary to determine the specific activity of any sample as a measure of the proportion of active enzyme present. Thus, from the results of Finkle and Smith[8], the c_1 values of 1.1 to 1.3 indicate that about 50-60% of the papain molecules in each preparation were able to be activated to the sulphydryl form.

Papain was assayed by measuring the rate of

hydrolysis of α-benzoyl-L-arginine amide (BAA) under the conditions described by Kimmel and Smith[41]. The liberated ammonium ion was estimated by titration with alkali in 90% ethanol solution as described by Davis and Smith[125]. The proteolytic coefficient (c_1) is defined as the first order rate constant for the hydrolysis, expressed in decimal logarithms, divided by the enzyme concentration in milligrams of protein nitrogen per millilitre of reaction mixture.

5 ml volumetric flasks were used as the reaction vessels instead of the 2.5 ml size recommended by Davis and Smith. Although this requires the use of double quantities of all reagents, it has the advantage that twice as many 0.2 ml samples can be taken for analysis during the reaction. This permits the construction of more precise rate curves. Usually about 15-20 samples were titrated during the course of the hydrolysis, and duplicate assays by this method always gave the same specific activity to within 2%.

In determining the activities of chromatographic fractions, recorded in section (d),(ii) of Chapter IV, some sacrifice in precision was necessary in order to assay such a large number of samples. Here 1 ml reaction mixtures were made up and only 4 samples were taken for titration over the reaction period of 3 hours.

(b) Other Materials

α-Benzoyl-L-arginine amide was an "A" grade Calbiochem product stated by the manufacturer to be free of ammonium salts. Lot numbers 660281 and 30233 were used during the course of this work. p-Chloromercuribenzene sulphonic acid was obtained as the lithium salt from L. Light and Co. Iodoacetic acid, iodoacetamide, and cysteine hydrochloride were B.D.H. laboratory reagents. Carboxymethyl cellulose was a product of Bio-Rad Laboratories and was supplied by Calbiochem, supplier's lot number 105410. All buffer salts were of analytical reagent grade and solutions were made up in glass-distilled water.

(c) pH Measurements

pH measurements were made at 25°C using a Radiometer pH meter, type pHM4. Glass electrodes were standardized by means of 0.05 M potassium hydrogen phthalate solution, the pH of which was taken to be 4.005[126].

For some of the preparative work a direct reading Philips pH meter, type PR9400, was used and measurements were made at room temperature.

(d) Preparation of Papain Solutions

Papain solutions were prepared by adding the

required amount of crystallized enzyme suspension, the concentration of which was known approximately, to a buffer solution, and dialysing overnight at 4°C against a large excess of the appropriate buffer. 18/32 Visking tubing was used for all dialyses; this tubing has been shown to be impermeable to insulin molecules of molecular weight 6000[127].

When using buffers in which papain was difficultly soluble, the enzyme was first dissolved by dialysis against water at 4°C. The resulting solution was then dialysed against buffer for at least 36 hours with a change of diffusate after 18 hours. Any precipitate was then removed by centrifuging at about 2500 x g for 10 minutes at 5-10°C.

(e) Determination of Papain Concentrations

Two methods were routinely employed for the determination of papain concentrations, each being useful in a certain concentration range. Refractometry was useful for protein concentrations above 0.1 g/100 ml and up to 2 g/100 ml, and spectrophotometry for protein concentrations below 0.05 g/100 ml and down to 0.005 g/100 ml.

Refractometry

A differential refractometer of the type

described by Cecil and Ogston[128] was used. The instrument was calibrated[129] using standard solutions for which accurate refractive index data is available, viz. sodium chloride[130] and sucrose[131].

Spectrophotometry

Optical densities were measured in 1 cm silica cells with either a Unicam SP500 or a Beckman model DU spectrophotometer, the latter having a photomultiplier attachment. The ultraviolet absorption spectrum of papain was found to have an extinction maximum at 278 mμ, the same wavelength as that reported by Glazer and Smith[132]. Accordingly measurements for concentration determination were made at this wavelength.

Both of the above methods required standardization in order to obtain papain concentrations in absolute units. Micro-scale Kjeldahl nitrogen analysis was used for this purpose, the technique being a slight modification of McKenzie and Wallace's procedure[133]. The technique was practised with standard amino acid and protein solutions until the results were reproducible and accurate to within 2% of the theoretical figures.

For standardization of the routine methods, an approximately 1.2% papain solution in acetate buffer of

pH 4, ionic strength 0.1 was analysed in triplicate for nitrogen, with appropriate diffusate blanks. The refractive index increment of the same solution was measured in the refractometer, and optical density measurements were made on other solutions diluted volumetrically from the original one. Using Kimmel and Smith's[13] figure of 16.1% for the nitrogen content of papain, the refractive increment of a 1% papain solution was estimated to be 0.00190. (Smith *et al*[7] used a value of 0.00184 for the corresponding quantity, but as in the present case this was based on nitrogen analyses with an estimated error of 2%). The specific extinction coefficient, $D^{1\,cm}_{278}$ (1% solution), was found to be 23.4. Previous reported determinations have given figures of 25.0 at 278 mμ[132], and 24 at 280 mμ[134].

(f) Estimation of Protein-Bound PCMS

The reagent PHMB, which gives rise to the p-mercuribenzoate ion in solution, is commonly used for the estimation of SH groups in proteins by utilizing methods described by Boyer[67] or Sela *et al*[135]. These methods depend on the increase in absorbance of the organomercurial at 250-255 mμ on binding to an SH group. Since proteins and the free organomercurial also absorb significantly at these wavelengths the preparation of

appropriate blanks is essential.

The use of an analogous method to estimate the protein-bound PCMS in a solution of PCMS-modified papain however introduces the following complications.

(1) The increase in extinction at 250 mμ by PCMS on forming a mercaptide is only about one third that of PHMB[67]. A greater increase is obtained with PCMS at 240 mμ, but at this wavelength absorption by papain is proportionately higher. Benesch and Benesch[136] have commented that PHMB is the only mercurial which gives rise to an adequate absorbance increase in a useful spectral region (250-255 mμ is a region of minimum extinction for many proteins).

(2) In the present study it was necessary to estimate bound PCMS in a protein solution after dialysis or even after a fractionation procedure. This is more difficult than the spectrophotometric titration of SH groups because appropriate blank solutions cannot readily be prepared.

(3) It was desired to estimate the <u>fraction</u> of a mole of bound PCMS per mole of papain. Clearly this requires greater accuracy than does the estimation to the nearest integer of SH groups in proteins with several such groups.

The following method was employed to avoid the use of blank solutions. The optical density of the modified papain solution was measured at 278 mμ and at 240 mμ with diffusate as the reference solution. Then, neglecting the absorption by bound PCMS at 278 mμ[67], the theoretical optical density of papain at 240 mμ if no binding had occurred was read from a previously determined calibration graph of D_{240} v. D_{278} for unmodified papain (Fig. VI-1). The absorbance change of papain at 240 mμ was thus ascertained. The molar concentration of bound PCMS was calculated from this by using the increment in molar extinction coefficient for cysteine (6.8×10^{-3} at 240 mμ) determined by spectrophotometric titration of a standard cysteine solution with PCMS. The molar concentration of papain was computed from its optical density at 278 mμ by using the previously determined specific extinction coefficient and a molecular weight of 21,000.

Since the increase in optical density due to the binding of a fraction of a molar equivalent of PCMS is small relative to the optical density of papain at 240 mμ, it is very difficult to obtain accurate results. Assuming an uncertainty of 0.005 in an optical density measurement of the order of 0.80, the estimated error of the method is about ± 0.1 in a $\frac{[\text{Bound PCMS}]}{[\text{Papain}]}$ ratio of 0.5.

This is certainly an underestimate as several other sources of error have been neglected.

(1) It has been assumed that no change occurs in the relationship between D_{278} and D_{240} for papain other than that due to reaction with PCMS. However, Fig. VI-1 shows that slight changes occurred when a papain solution was stored for two weeks at 4°C in the absence of PCMS. A similar graph of D_{250} v. D_{278} showed much more pronounced changes with time. This is probably not surprising since Myers and Abernethy[137] have observed marked changes in the ratio of optical density at 250 mμ to that at 280 mμ for papain as a result of γ-ray irradiation. It was suggested that these changes were due mainly to destruction of tryptophan, a reaction believed to be an oxidation[138]. Similar changes might therefore occur slowly in papain solutions by way of atmospheric oxidation. The observed spectral changes, together with the smaller extinction of bound PCMS at 250 mμ precluded use of optical density measurements at this wavelength for estimation of bound PCMS.

(2) If there were any form of binding of PCMS to papain other than mercaptide formation, high results would be obtained. The usual methods[67,135] measure only the amount of mercaptide since the total concentration of mercurial is made the same in both the unknown and the blank solutions. The increase in absorbance can then be

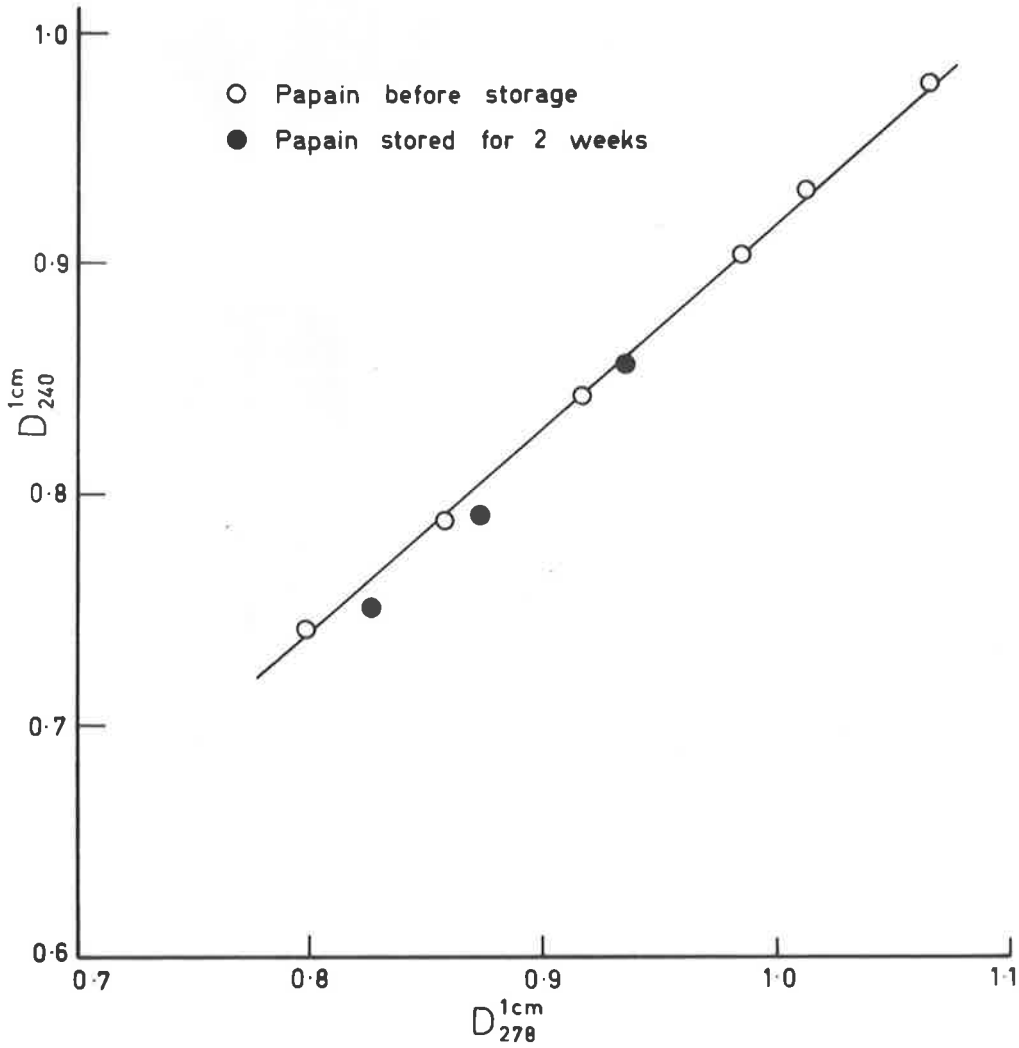


Fig. VI-1 Changes occurring in the ratio of optical densities at 240 mμ and 278 mμ on storage of papain at 4°C in acetate buffer pH 4, ionic strength 0.1.

attributed to organomercurial which is bound in the protein solution but free in the blank solution.

(3) Since papain solutions were activated with cysteine before PCMS-modification (Chapter III), the solutions before dialysis contained appreciable amounts of a cysteine-PCMS complex. If this complex were not completely removed by dialysis, it would add to the optical density of the bound PCMS giving high results.

(g) Sedimentation

(i) Experimental

A Spinco Model E ultracentrifuge equipped with a phase plate as the schlieren diaphragm was used for all sedimentation velocity experiments. When the protein concentration was above 0.5 g/100 ml, solutions were placed in a 12 mm light path cell and centrifuged at 59780 r.p.m. in an "An-D" rotor. To improve the definition of optical patterns at lower protein concentrations a 30 mm light path cell in an "An-E" rotor was used. The latter experiments were mostly conducted at a rotor speed of 44770 r.p.m. which is slightly below the rated maximum for the rotor, because sapphire windows had to be used to bring the weight of the cell up to that of the counterbalance. Slight differences in the measured sedimentation coefficients were found between the two

types of runs, and some possible reasons are discussed in the appendix.

Double sector cells were used to give a base-line in the schlieren pattern in case detailed measurements of the boundary shape were required. The rotor temperature was controlled by means of the manufacturer's "Rotor Temperature Indicator and Control Unit" at close to 20°C except for the two experiments done at 5°C (Chapter II, section (d), (i)). Temperature was recorded several times during each run; variations were never greater than ± 0.1°C.

Photography was commenced as soon as the schlieren peak was completely visible, and ten or twelve photographs were taken at 8 minute intervals. Kodak type II-G plates were used, the optical system being fitted with a Kodak Wratten filter no. 77A, (green). All optical patterns were measured on a toolmaker's microscope[139] equipped with a projection viewer and a stage having 2-dimensional movement. Photographs were aligned along the meniscus image which was assumed to be perpendicular to the direction of sedimentation. The radial position of the meniscus was determined for the first and last photographs of a run to ensure that no leakage had occurred from the cell.

(ii) Calculations

Sedimentation coefficients were evaluated from the slope of the line relating the logarithm of the boundary position to time. This procedure is based on the equation[140]

$$S = \frac{1}{\omega^2} \frac{d \ln \bar{x}}{dt} \qquad \ldots\ldots\ldots \text{VI-(1)}$$

where S is the sedimentation coefficient, ω is the angular velocity of the rotor in radians/sec, x is the distance of the maximum ordinate of the boundary (see Chapter II) from the axis of rotation, and t is the time in seconds. The sedimentation coefficient obtained from equation VI-(1) has the units seconds but is usually converted to Svedberg units (S), where 1 S = 10^{-13} sec.

The slope of each log $\bar{x}$ y. t line was calculated from a least squares regression[51] of log $\bar{x}$ on t. In order to distinguish between random fluctuations and systematic trends it was necessary to have some estimate of the error in the determination of sedimentation coefficients. Therefore the standard error of each slope was calculated by the routine statistical method, and multiplied by the appropriate "t" factor to give the 95% fiducial limit[51]. This latter quantity represents the $\pm$ limits within which 95% of an infinite number of deter-

minations of ($d \log \bar{x}/dt$) could be expected to lie. This error was then used to compute the errors in $s_{20,w}$ shown on the graphs in Chapter II. The relative errors in ω (calculated by timing with a stop watch the period for 500 or more revolutions of the odometer), and in the correction factors used in equation II-(1) are small in comparison with that in ($d \log \bar{x}/dt$) determined by comparator measurements.

(h) Viscosities and Densities

Viscosities and densities of buffer solutions were required for the correction of sedimentation coefficients to standard conditions by means of equation II-(1). The viscosity correction factor can be split into two parts (i.e. $\frac{\eta}{\eta_{20,w}} = \frac{\eta}{\eta_w} \cdot \frac{\eta_w}{\eta_{20,w}}$), and since the factor $\frac{\eta}{\eta_w}$ varies little with temperature for aqueous salt solutions between 20°C and 40°C[141], it was considered sufficient to know this factor at one temperature only. For the acetate and phosphate buffers used, $(\frac{\eta}{\eta_w} - 1)$ and $(\rho - \rho_w)$ were calculated by summation of figures for the individual components as described by Svedberg and Pedersen[141]. The required viscosity and density data for water at various temperatures were also obtained from Svedberg and Pedersen's book[50]. However, for experiments at 5°C where a larger temperature correction was

required, a more up to date source was used[142].

Since the necessary data for tris buffers and solutions of cysteine could not be found in the literature, the densities and relative viscosities for these solutions were determined experimentally by use of pycnometers and an Ostwald viscometer respectively. These experiments were carried out at 25°C. The viscometer was of the type BS 188 for which the kinetic energy correction could be neglected for the purposes of this investigation.

The tris buffer solution (pH 8.00, ionic strength 0.02) had the composition shown in Table II-1. Cysteine solutions were prepared by mixing equivalent amounts of cysteine hydrochloride and sodium hydroxide, and thus contained equal molar concentrations of cysteine and sodium chloride. Since cysteine was added in this form whenever used in the present study, corrections for sodium chloride have not been subtracted. The experimentally obtained density increments ($\Delta\rho$) and relative viscosities ($\frac{\eta}{\eta_w}$) are set out in Table VI-1.

TABLE VI-1

EXPERIMENTALLY DETERMINED VISCOSITY AND DENSITY DATA

Solution Composition (molarities)	$\Delta\rho$ (25°C) (g/ml)	$\frac{\eta}{\eta_w}$ 25°C)
.0347 Tris + .02 HCl	.0015	1.012
.025 Cy + .025 NaCl	.0021	1.005_5
.05 Cy + .05 NaCl	.0043	1.012
.10 Cy + .10 NaCl	.0086	1.026

Cy: cysteine, Tris: tris-hydroxymethyl-aminomethane

(1) Electrophoresis

Electrophoresis was conducted in a Spinco Model H Electrophoresis-Diffusion instrument. In order to allow simultaneous photography of usable patterns from both the Rayleigh interference and schlieren optical systems, the manufacturer's channel selector flag was replaced by one which did not obscure the reference fringes (see Fig. VI-2).

Experiments were performed at 1°C in the standard size (11 ml) cell. All compartments of the cell assembly were open to the atmosphere. The field strength applied was usually about 6 volt/cm, and the cell assembly was always tested for leaks as described in the manufacturer's

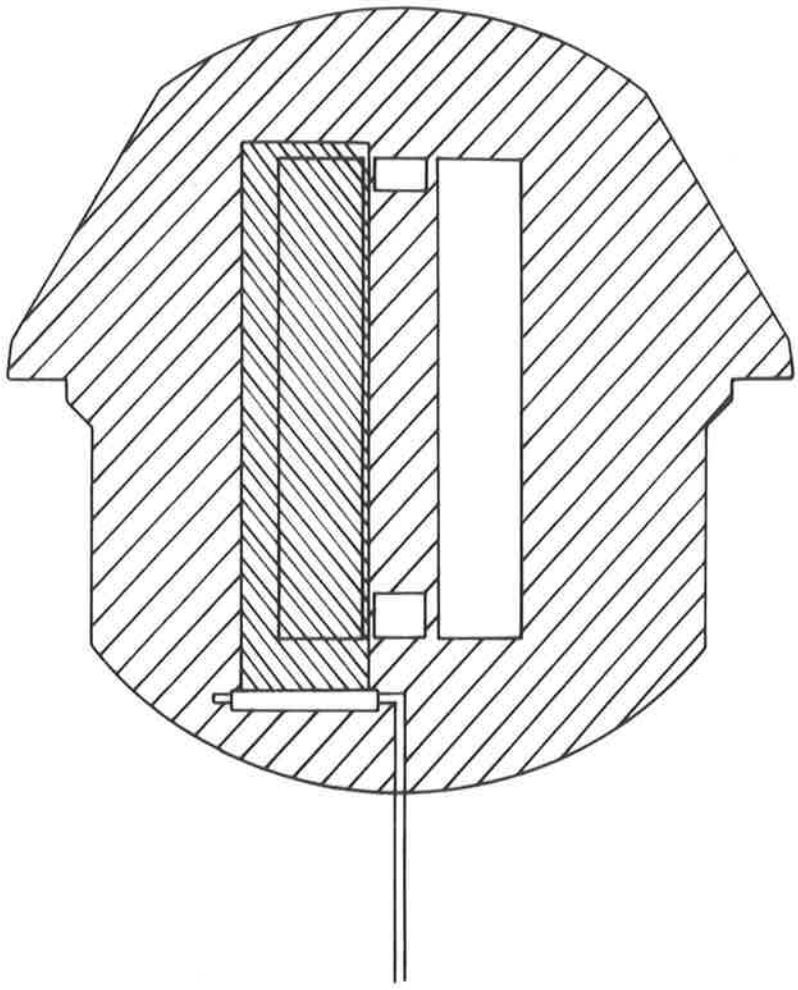


Fig. VI-2 Diagram showing the mask situated in front of the schlieren lens of the electrophoresis instrument, and the channel selector flag. The existing mechanical mechanism can be used to block either lateral aperture while leaving exposed the small central apertures through which light must pass if reference fringes are to be obtained.

instruction manual immediately after formation of the boundaries. The boundaries, formed at the ends of the centre section of the cell, were brought into the optical path by slowly withdrawing buffer from the compartment above the ascending limb with a mechanical syringe. The syringe was then removed from the solution, the initial boundary photographs taken, and the electric current turned on and adjusted.

The photographic system was operated manually, 5 or 6 photographs from each cell channel being obtained while the peak moved from one end of the viewing screen to the other. Photographs were taken on sheet film, either Kodak Process Pan (now out of production), Kodak Royal Pan or Kodalith Pan. Optical patterns were measured on the previously described toolmaker's microscope, the reference fringes being used for alignment of both the Rayleigh interference and schlieren patterns.

(j) Conductance Measurements

The specific conductance of a protein solution is required for the evaluation of electrophoretic mobilities[71]. Conductance measurements were made in the electrophoresis water bath at 1°C, with a cell similar to that described by Mysels[143] for measurements on small volumes of liquids. Only 4 ml of solution was

required to fill the cell which had a cell constant of 3.31 cm^{-1}, determined at 0°C with the standard potassium chloride solutions recommended by Jones and Bradshaw[144].

Resistances were measured at 5 frequencies ranging from 0.5 to 10 kc with a 4-decade resistance and capacitance bridge[145]. In accordance with the recommended procedure[146] the true resistance was found by extrapolation to infinite frequency. Graphs of R v. $1/\sqrt{f}$ and R v. $1/f$ were drawn for this purpose, the more nearly linear curve (usually the latter) being extrapolated.

For conductance measurements on chromatographic fractions during gradient elution experiments, less solution was available but less precision was required. Hence a Philips conductivity bridge, type GM 4249/01 was used in conjunction with a Philips type PR 9512/01 cell having dipping electrodes and requiring only 2 ml of solution for a measurement. The cell constant was 0.77_5 cm^{-1}, and measurements were made at only one frequency (1.0 kc).

(k) Chromatography

(i) Preparation of Columns

CM-cellulose was washed with alkali and with acid, then equilibrated with the appropriate buffer before use[100]. For experiments with PCMS-papain this buffer

contained 3×10^{-5} M PCMS, as did all solutions used in the elution, for the purpose of suppressing possible dissociation of the protein-PCMS complex. Organomercurial polysaccharide was thoroughly washed with the appropriate buffer before use as recommended by Eldjarn and Jellum[108].

A water-jacketed chromatography column of about 1 cm internal diameter was used. The column was cooled by circulation of water through the water-jacket from a refrigerated water bath kept at 2°C. The columns were packed at room temperature, a slight pressure (2-5 lb/sq.in.) being applied in the case of the CM-cellulose, then cooled and washed with several hundred millilitres of buffer to dissolve air bubbles.

(ii) Loading and Eluting Conditions

Papain was applied to the top of the column as a dialysed solution (ca. 0.2 g/100 ml) and allowed to drain into the column without application of pressure. The load was never greater than 50 mg of papain on an average column containing about 1.5 g of dry CM-cellulose. The column was immediately connected to a buffer reservoir and feed line, and as soon as the cellulose was covered to a depth of 1 cm (i.e. sufficient to just keep immersed the delivery tip of the feed line) an air-tight joint at the top of the column was sealed.

The flow rate was adjusted to 6-9 ml/hr for experiments with CM-cellulose. Sufficient pressure to maintain this flow rate was provided by mounting the buffer reservoir approximately 2 ft above the top of the column. When gradient elution was required, the buffer reservoir was replaced by a simple linear gradient forming device comprising 2 identical flasks mounted at the same level and connected by tubing to maintain hydrostatic equilibrium. Concentrated buffer was placed in one flask and an equal quantity of starting buffer in the other. Liquid withdrawn from the latter (i.e. the mixing chamber), which was stirred continuously by means of a magnetic stirrer, should then increase linearly in concentration[100].

(iii) Monitoring and Collection of Effluent

The column effluent was continuously monitored by passage through the flow cell of an Isco Model UA recording ultraviolet analyzer. This instrument measures extinction at 254 mμ. A check with the Beckman spectrophotometer showed the results to be reasonably accurate provided that allowance is made for a gradually increasing "baseline" absorption due mainly to air bubbles coming out of solution in the flow cell. The flow analyzer was particularly useful for ascertaining elution conditions when a careful analysis of the fractions was not required.

Equal fractions of approximately 2.5 ml were collected in test tubes using a drop counting device attached to a fraction collector. This collector was kept in a refrigerator of the showcase cabinet type, and by use of a fan to circulate the air in the cabinet the fractions were kept at about 6-8°C. The effluent volume was determined by weighing each batch of 10 fractions and subtracting an average tare for this number of test tubes (determined from the weight of 100 tubes). To minimise random errors a linear graph of effluent volume y. fraction number was drawn and used to find the effluent volume for any given fraction.

Optical density measurements were made on pooled samples of two fractions in the Beckman spectrophotometer. The chromatogram obtained in this way is more reliable than that from the flow analyzer because the absorbance of papain is nearly three times as high at 278 mμ as at 254 mμ. In addition, impurities absorb less and no corrections have to be made for the changing baseline.

APPENDIX

CORRECTIONS TO SCHLIEREN DATA

(a) Experimental Evidence for an Effect of Ultracentrifuge Cell and Rotor on the Measured Sedimentation Coefficient

(b) Possible Causes of Differences between Sedimentation Coefficients Determined in Different Cells and Rotors

- (i) Rotor Temperature Calibration
- (ii) Reference Edge Positions
- (iii) Angular Velocity of Rotor
- (iv) Effect of Cell Thickness
  - (1) Magnification Factor of Camera Lens
  - (2) Distortion of Optical Patterns

(c) Theory of Corrections to Schlieren Data

- (i) Geometrical Effects of Light Bending and Refraction
- (ii) The Equivalent Level

(d) Evaluation of Corrections

- (i) Geometrical Effects of Light Bending and Refraction
- (ii) The Equivalent Level
- (iii) Results and Discussion

(a) Experimental Evidence for an Effect of Ultracentrifuge Cell and Rotor on the Measured Sedimentation Coefficient

In Chapter II it was noted that discontinuities appeared in graphs of $s_{20,w}$ v. initial solute concentration at the point where a change was made to the longer light path cell. Even though the same type of discontinuity was observed both at pH 4 and in two separate series of runs at pH 7, it was at first suspected that this effect was a random error. To ascertain therefore whether the calculated 95% fiducial limits were realistic estimates of experimental error, a number of sedimentation coefficient determinations were carried out on a single papain stock solution. Table A-1 shows results of such sets of runs for papain at pH 4 and at pH 7.

TABLE A-1

Reproducibility of Sedimentation Coefficient Determinations

Papain (0.68 g/100 ml) in Acetate buffer pH 4, I = 0.1		Papain (0.72 g/100 ml) in Phosphate buffer pH 7, I = 0.05	
Time[a] (days)	$s_{20,w}$ ± 95% f.l.	Time[a] (days)	$s_{20,w}$ ± 95% f.l.
0	$2.42 \pm .03_3$	0	$2.78 \pm .02_7$
0	$2.45 \pm .03_0$	0	$2.77 \pm .02_9$
1	$2.45 \pm .01_6$	1	$2.80 \pm .02_6$
3	$2.46 \pm .02_5$	4	$2.73 \pm .02_0$
7	$2.40 \pm .03_2$	8	$2.72 \pm .03_8$

[a] Time from date of first run in series.

It appears from Table A-1 that day to day differences between $S_{20,w}$ figures can be accounted for in terms of the 95% fiducial limits, each of these being computed from a graph in which the scatter of points is presumably due to experimental error in measuring the photographs (see Chapter VI). The results also demonstrate the stability of papain with regard to changes in sedimentation coefficient over the period required for a series of runs. The observed changes in $S_{20,w}$ are all less than the 0.1 S discontinuity which occurred on changing from one cell and rotor to the other.

To detect a dependence of $S_{20,w}$ on the cell and rotor, identical solutions were centrifuged in different cells (12 mm and 30 mm light path) on the same or successive days. Three such pairs of experiments were done, one each with papain at pH 4 and pH 7, and one with bovine serum albumin (BSA) at pH 7.3. The results are shown in Table A-2; the results for papain have also been included in the appropriate graphs in Chapter II (Figs. II-2 and II-4).

It is seen that in each pair of experiments the standard sedimentation coefficient is about 0.1 S higher in the E than in the D rotor. The difference appears to be outside the estimated limits of experimental

TABLE A-2

Sedimentation Coefficients Obtained with Identical Solutions in Different Cells and Rotors

Solution	Run No.	Cell Thickness (mm)	Rotor	$s_{20,w}$ ± 95% f.l.
Papain (0.50%) in acetate pH 4, I = 0.1	UC/35	12	D	$2.38 \pm .01_6$
	UC/36	30	E	$2.51 \pm .03_1$
Papain (0.42%) in phosphate pH 7, I = 0.05	UC/30	12	D	$2.65 \pm .03_5$
	UC/31	30	E	$2.75 \pm .03_6$
BSA (0.3%) in phosphate[a] pH 7.3, I = 0.2	UC/28	12	D	$4.23 \pm .03_3$
	UC/29	30	E	$4.33 \pm .05_1$

[a] Buffer composition: 0.004 M KH_2PO_4, 0.02 M Na_2HPO_4, 0.142 M NaCl.

error in each case. The next section comprises a consideration of possible causes of this difference; one such cause, viz. the optical effects of cell thickness, is discussed in more detail in the rest of this appendix.

(b) Possible Causes of Differences between Sedimentation Coefficients Determined in Different Cells and Rotors

The observed discrepancies might be imagined to arise from any of the following causes: (1) incorrect

temperature calibration of either rotor, (ii) incorrect positions of the reference edges in either counterbalance when positioned in the rotor, (iii) an effect of changing the angular velocity of the rotor, and (iv) an effect of the thickness or optical light path of the cell. Each of these possibilities was therefore considered.

(i) Rotor Temperature Calibration

Both rotors were temperature calibrated, using the same thermometer, during the course of the experiments described in section (a). This was considered advisable as the rotor thermistor characteristics are liable to change with time. It is essential that this calibration be accurate to within 0.05°C over the entire temperature range used since the viscosity of aqueous solutions varies by about 2% per centigrade degree[147] and the sedimentation coefficient is therefore subject to the same variation.

(ii) Reference Edge Positions

The positions of the reference edges in the counterbalance when positioned in each rotor were determined as follows. The longest axis of the rotor was measured to 0.001 in. with calipers and the centre to edge distance was assumed to be half of this. The distance from the edge of the rotor to each reference edge was then measured

on the toolmaker's microscope (Chapter VI). From these data the distance of each reference edge from the centre of rotation was calculated. For the D rotor the average distance of the inner and outer reference edges from the centre was 6.490 cm; for the E rotor it was 6.503 cm. The discrepancies between these and the manufacturer's stated distance (6.500 cm) could not cause any significant error in the sedimentation coefficient.

Stretching of the rotor at high speeds is known to be a factor affecting the positions of reference edges. Schachman[148] has found that the reference edges move outwards about 0.04 cm when a type An-D rotor accelerates from 5000 to 59780 r.p.m. The estimated centrifugal field in the region of the boundary would therefore be in error by about 0.7%, leading to an error of less than 0.02 S in a sedimentation coefficient of 2.58. The resulting discrepancy between $S_{20,w}$ figures in the two rotors would certainly be less than this, since the An-E rotor will also be stretched to some extent when rotating at a speed of 44770 r.p.m.

(iii) Angular Velocity of Rotor

A rotor speed of 59780 r.p.m. was used for the D rotor, and 44770 r.p.m. for the E rotor. The apparent sedimentation coefficient, determined as described in

Chapter VI, might vary with the angular velocity of the rotor if it were pressure dependent or so strongly concentration dependent that it could be affected by differences in radial dilution at different angular velocities. In either case a variation of sedimentation coefficient should be detectable during a particular run, because as the boundary moves down the cell, radial dilution occurs and the average pressure on the sedimenting molecules increases.

Taking as an example the sedimentation of papain (0.5 g/100 ml) at pH 4 in the D rotor (see Table A-2, run no. UC/35), the variation of sedimentation coefficient during the experiment was therefore computed. The method used was similar to that described by Alberty[149] who calculated an average sedimentation coefficient (S_t) up to the time of each photograph by use of the equation

$$S_t = \frac{1}{\omega^2 t} \ln \left(\frac{\bar{x}}{x_m}\right) \qquad \text{.... A-(1)}$$

where t is the total time of sedimentation (corrected for sedimentation occurring while the rotor was being accelerated), x_m is the distance of the meniscus from the centre of rotation and the other terms are as defined in Chapter VI. In the present case, the boundary position on the first photograph was used instead of the meniscus position since the time before the first photograph was taken was

not known. The so calculated sedimentation coefficients varied randomly with time by the order of 1% or less. There is thus no reason to believe that the determination of sedimentation coefficients of papain is significantly affected by pressure or concentration dependence.

(iv) Effect of Cell Thickness

(1) Magnification Factor of Camera Lens

Since the schlieren optical system when focussed on the mid plane of the 12 mm cell is focussed on a slightly different plane in the 30 mm cell (see next section and Fig. A-2), the possibility of a difference in the camera lens magnification factor was considered. However, when the E rotor was positioned in the rotor chamber with a ruled disc and holder on the spacer ring supplied by the manufacturer, the optics were still observed to be in a good focus. A determination of the magnification factor gave the same figure within 1 part in 750 as that found previously with the same disc and holder, but no spacer, in the D rotor.

(2) Distortion of Optical Patterns

In two recent papers Ford and Ford[150,151] have deduced certain corrections which should be applied to ultracentrifuge schlieren patterns. Calculated sedimenta-

tion or diffusion coefficients are affected. No experimental verification was reported by the above authors, but their calculations for a hypothetical BSA solution (of concentration 1.22 g/100 ml) predicted an observed sedimentation coefficient 1.38% higher in a 30 mm cell than in a 12 mm cell. Furthermore it appeared likely that the corrections might be larger for a faster diffusing, slower sedimenting protein like papain.

This appendix therefore deals with the explanation of these corrections and their evaluation for the data in Table A-2. It will be seen that the predicted discrepancies are of the same sign as those found experimentally but are only one third to one half the magnitude.

(c) Theory of Corrections to Schlieren Data

Ford and Ford showed that two types of distortion are present in camera recorded schlieren patterns of refractive index gradients in the ultracentrifuge cell: (i) geometrical effects of light bending and refraction[150], and (ii) the "equivalent level" phenomenon[151].

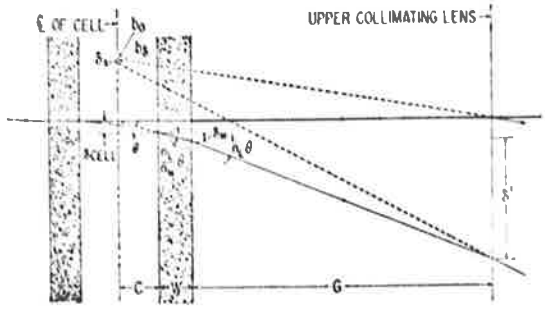
(i) Geometrical Effects of Light Bending and Refraction

This first effect arises because rays of light entering normal to the lower cell window are bent by the refractive index gradient in the cell and then are

refracted on entering and leaving the upper cell window (Fig. A-1(a)). Ford and Ford showed by geometrical construction that the apparent radial position (x) in the cell image recorded by the camera is slightly different from that which would be recorded for an undeviated ray. The apparent displacement is proportional to the refractive index gradient ($\frac{dn}{dx}$) and hence results in tilting of the photographed schlieren peaks. The direction of the displacement, and hence of the peak tilt, depends on the thickness of the cell. For the examples considered[150], the peak was calculated to tilt forwards (i.e. away from the centre of rotation) in a 12 mm cell, and backwards in a 30 mm cell. During a sedimentation velocity experiment, in either type of cell, the peaks will not only broaden as the refractive index gradients decrease by diffusion, but also tilt less. Therefore, experimentally obtained sedimentation coefficients will be slightly low if determined in a 12 mm cell and slightly high in a 30 mm cell.

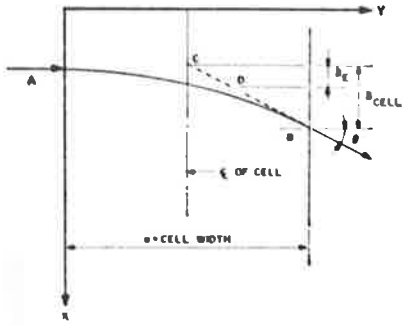
(ii) <u>The Equivalent Level</u>

Since a light ray traverses a finite x-coordinate interval in passing through the cell, the camera-recorded deflection from the schlieren baseline is determined by the refractive index gradient in the whole coordinate



(a)

Diagram illustrating the bending and refraction for deflected rays.



(b)

A slightly modified version of the diagram used by Svensson in his analysis of the effect of light curvature on the positions of schlieren center lines.

Fig. A-1 Origin of two kinds of distortion of schlieren patterns (from Ford and Ford[150,151]).

(a) Due to geometrical effects of light bending and refraction, the correction δ_c has to be applied to the cell level. Detailed diagrams of the entire optical system are necessary to derive this correction.

(b) Since the light ray curves and travels through regions of different refractive index in the cell, the resulting deviation corresponds to the refractive index gradient not at the camera-recorded entry level C, but at the "equivalent level" D.

interval between the entrance and exit levels. Svensson[152, 153] recognized the need to deduce the position of a single level whose refractive index gradient would give the same deflection as that given by the x-coordinate interval traversed. By approximate methods he deduced that this "equivalent level" was situated below the camera-recorded entry level by a distance δ_E equal to about one third of δ_{cell}, the x coordinate interval between the exit level of a ray and its camera-recorded entry level (see Fig. A-1(b)). δ_E thus represents the distance between the camera-recorded and the actual cell level where a particular refractive index gradient exists. Since δ_{cell} is clearly greatest for regions of high refractive gradient, δ_E is greatest at the tops of peaks and less at other levels. This leads to a skewing of schlieren peaks referred to as Weiner skewness[154].

The distinction between this type of distortion and that described in (i) may not be immediately apparent. The equivalent level effect arises because the light ray, in passing through the cell, curves and therefore travels through levels of varying refractive index gradient. The geometrical optical bending and refraction effect takes no account of the path followed by a light ray in the cell, only of the resulting deviation. So long as a ray is deviated there is likely to be an error in its camera-recorded entry level.

One way of appreciating the difference is to imagine a given deviation, in terms of the angle between the paths of a ray on entering and leaving the cell, taking place in cells of gradually decreasing thickness. The equivalent level displacement, being approximately proportional to δ_{cell}, decreases steadily with decreasing cell thickness. However, this is not the case for the displacement, δ_x, due to geometrical effects, which is proportional to the displacement of the light ray at the schlieren diaphragm. Ford and Ford[150] have shown that the ratio δ_x/δ_{cell} increases sharply for very thin cells (of less than 10 mm light path).

Svensson[153] has shown that Weiner skewness is minimised if the camera lens is focussed not on the mid plane of the cell but on a plane situated one third of the cell thickness below the lower surface of the upper window. However, in order to record the true position of the meniscus, the camera lens has to be focussed on the mid plane of the cell[155]. Since this latter condition is critical in Archibald and equilibrium work, mid plane focussing is employed in most ultracentrifuges including that used in the present study. The camera lens was focussed by means of the ruled disc mentioned in the previous section. The thickness of this disc and its position in the holder are so designed that when it is

installed in an An-D rotor attached to the drive shaft, the rulings are at a position optically equivalent to the mid plane of a 12 mm cell filled with water and having quartz windows.

Since Ford and Ford's optical corrections have been formulated only for optical systems employing mid plane focussing, it was important to ascertain the position of focus for the 30 mm cell. The distance was measured (see next section) from the upper collimating lens to the top surface of the cell window for each of the two cell and rotor assemblies when installed in the rotor chamber. This distance was 6.2 mm shorter for the long light path cell and rotor. The approximate geometrical analysis in Fig. A-2 indicates that the focussing is therefore also very close to the mid plane of a 30 mm cell. It will be assumed for the purpose of evaluating corrections in the next section that the camera lens was focussed on the mid plane of each of the cells.

Ford and Ford[151] have pointed out the limitations of Svensson's approximate analysis, and have devised a numerical method for computing more exact equivalent level corrections for Gaussian schlieren curves. They have shown that the curves are tilted towards the axis of rotation and are distorted. The corrections become smaller with successive photographs in a sedimentation

$\overrightarrow{ABCD}$ and $\overrightarrow{APQR}$ represent an on-axis and off-axis light ray respectively. Both pass through the mid plane of a water-filled 12 mm cell at A, and after emerging from the quartz window at C and Q respectively both are focussed to the same point by the camera lens. Vertical distances (in mms) measured from a horizontal plane through A are shown at the left of the diagram. (Thickness of cell window = 5.1 mm).

Now if the 12 mm cell is replaced by a 30 mm cell, a new plane through the point A' will be focussed. The problem is to find a relation between the increase in height (x) of the top window and the lowering (y) of the plane of focus.

The paths of the rays will now be $\overrightarrow{A'B'C'D}$ and $\overrightarrow{A'P'Q'R}$ (i.e. still emerging along the same directions in order to be focussed).

Consider the horizontal projections of each ray on the line DR. Clearly

$$DK + KL + LN = DM + MN$$

$$6 \tan \angle BAP + 5.1 \tan \angle KPQ + x \tan \angle LQR = (x + y + 6) \tan \angle B'A'P' + 5.1 \tan \angle MP'Q'$$

If both cells have quartz windows of the same refractive index then $\angle MP'Q' = \angle KPQ$, and $\angle B'A'P' = \angle BAP$

$$\therefore (x + y) \tan \angle BAP = x \tan \angle LQR$$

$$\frac{\sin \angle BAP}{\sin \angle LQR} = \frac{x}{x+y} \cdot \frac{\cos \angle BAP}{\cos \angle LQR}$$

Provided $\angle BAP$ and $\angle LQR$ are less than 10°, their cosines can be taken as unity with an error of only 1.5%. The error in assuming the ratio of the two cosines to be unity is certainly less than this. The ratio of the sines can be equated to the ratio of the refractive indices of air and water ($1.0/1.3$) by Snell's law. Hence,

$$y \simeq \frac{x}{3}$$

---

x was measured to be 6.2 mm (see Text), thus y is 2.1 mm, and the distance of A' from the lower surface of the quartz window is $(2.1 + 6.2 + 6) = 14.3$ mm. This is only 0.7 mm from the mid plane.

If the upper window for the 30 mm cell is made of sapphire which has a refractive index of 1.71, compared to 1.54 for quartz, it is no longer possible to put $\angle MP'Q' = \angle KPQ$. Instead ...

$$1.71 \sin \angle MP'Q' = 1.54 \sin \angle KPQ = 1.33 \sin \angle B'A'P = 1.33 \sin \angle BAP$$

and the same ratios are assumed for the tangents. From this it can be shown that $y \simeq \frac{x + 1.3}{3}$ showing that the point D' is only 0.3 mm from the mid plane.

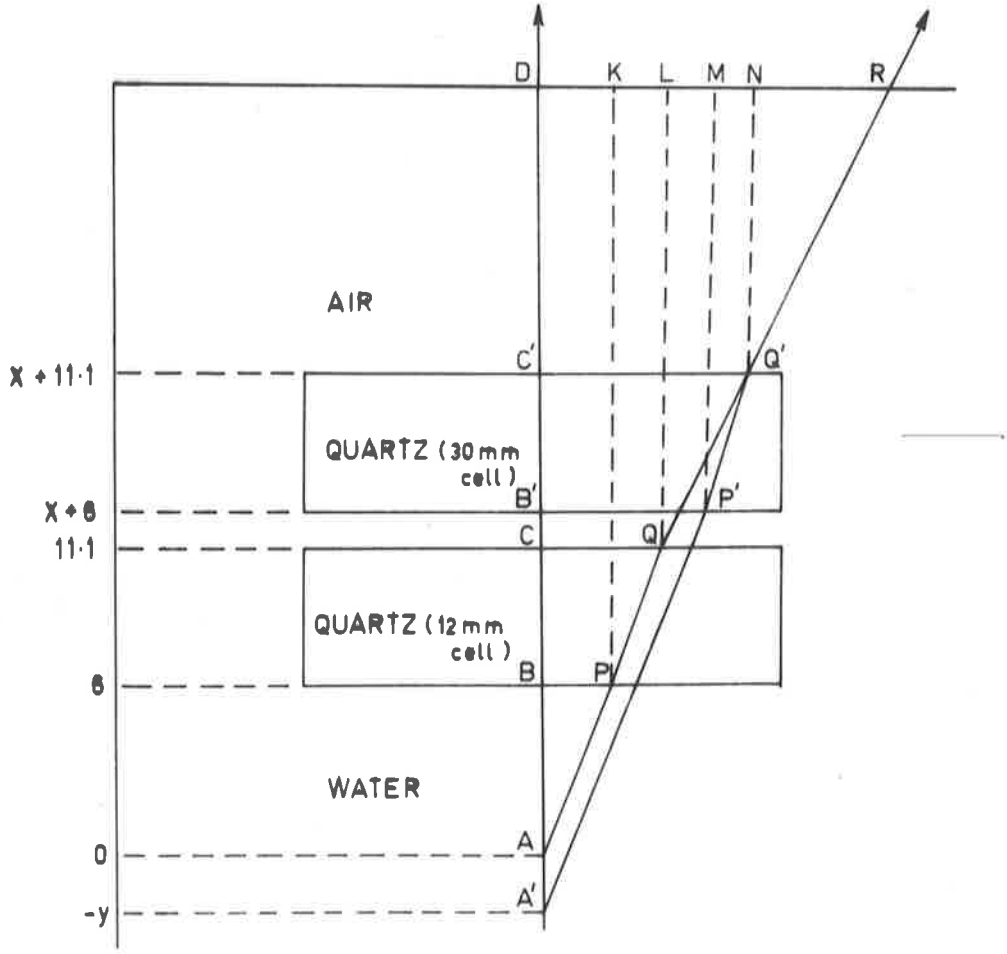


Fig. A-2 Effect of cell thickness on plane focussed by camera lens of ultracentrifuge, see derivation opposite.

velocity run as the peak spreads. Thus, experimentally obtained sedimentation coefficients are too high. For 12 mm cells these corrections tend to cancel those due to geometrical optical bending and refraction, but for 30 mm cells the two corrections are usually in the same direction.

(d) Evaluation of Corrections

(1) Geometrical Effects of Light Bending and Refraction

Ford and Ford[150] have shown that the displacement, δ_x, (away from the axis of rotation) of a point in the camera-recorded schlieren pattern is given by the relation

$$\frac{\delta_x}{\delta} = 1 - \left(1 - \frac{G}{F} - \frac{W}{F}\frac{1}{n_W} - \frac{C}{F}\frac{1}{n_L}\right)\frac{T}{F} \qquad \text{.... A-(2)}$$

where δ is the deviation of the light sheet at the schlieren diaphragm (corrected for deviation due to the solvent).

G is the distance from the top of the upper cell window to the principal plane of the upper collimating lens.

F is the focal length of the upper collimating lens, and can be equated to the optical lever arm for a correctly focussed optical system.

C is half of the inside thickness of the cell.

W is the thickness of the upper cell window.

$T = C + W + G + F$

n_W is the refractive index of the cell window material. and n_L is the refractive index of the liquid.

The required constants in equation A-(2) were determined as follows.

G:

This distance was found by stretching a cotton thread between the upper cell window and the upper collimating lens. The thread was marked and measured with a ruler. The chamber side of the upper collimating lens can, to a close approximation, be taken as the principal plane[156]. In this way G was found to be 4.72 for the 12 mm cell in the D rotor, and 4.10 cm for the 30 mm cell in the E rotor.

F:

The spacing (w) of Rayleigh fringes obtained with an empty cell was used to give a value of $F \times M_c$, where M_c is the magnification of the cylindrical lens, using the equation[157]

$$F \times M_c = \frac{wh}{\lambda} \qquad \text{.... A-(3)}$$

where h is the distance between the slits in the lower window holder

and λ is the wavelength of the light used.

Then the cylindrical lens magnification was found by photography of a ruled plate at the plane of the schlieren diaphragm (c.f. Van Holde and Baldwin[158]).

A fringe spacing of $(2.79 \pm .02) \times 10^{-2}$ cm was found, giving a figure of (203.5 ± 1.5) cm for $F \times M_c$. The factor M_c was found to be $3.567 \pm .007$. Hence F is $(57.0_5 \pm 0.5)$ cm. The errors quoted are standard errors obtained from linear regressions which were used to obtain the distances between fringes or rulings.

C:

The manufacturer's stated distances were considered to be sufficiently accurate. Thus C = 0.60 cm or 1.50 cm depending on the cell used.

W:

The thickness of the cell wall was measured with a micrometer and found to be 0.51 cm.

n_W:

The refractive indices of quartz and sapphire were taken as 1.544 and 1.709 respectively[159].

n_L:

An average figure of 1.335 was used for the refractive index of the liquid in the cell. This represents the refractive index of water at 20°C (1.334) to which has been added half the refractive increment for a 0.5% papain solution in acetate buffer of pH 4 and

ionic strength 0.1.

In this way the following values of $^{\delta x}/_{\delta}$ were obtained from equation A-(2)

(1) $^{\delta x}/_{\delta} = (+4.0 \pm 0.1) \times 10^{-3}$ for a 12 mm cell with quartz windows, in an An-D rotor.

(2) $^{\delta x}/_{\delta} = (0.0 \pm 0.1) \times 10^{-3}$ for a 30 mm cell with sapphire windows, in an An-E rotor.

Similar figures were obtained by Ford and Ford[150]; the particular figure depends of course on the constants of the optical system.

To obtain the correction δ_x it was then necessary to know δ, the deviation at the schlieren diaphragm of the light sheet passing through a particular cell level. δ varies with cell level (x), but can be calculated for any x-coordinate from the height (z) of the schlieren curve above the baseline. The equation used[160] is

$$z = \delta M_o \cot \theta \qquad \ldots\ldots \text{A-(4)}$$

where M_o is the previously determined magnification factor of the cylindrical lens, and θ is the diaphragm angle.

Since all sedimentation coefficients were obtained from measurements of the movement of the maximum ordinate of the schlieren curve, it was sufficient for the present

purpose, to calculate δ_x only for the maximum ordinate. Furthermore, to reduce the amount of computation, measurements were made only on the first and last photographs of a particular run. The difference in δ_x values was then expressed as a fraction of the distance moved by the peak to deduce the correction to be applied to the sedimentation coefficient. Ford and Ford[150,151] adopted the same procedure.

(ii) The Equivalent Level

Ford and Ford[151] have shown that the distortion of Gaussian schlieren peaks due to the equivalent level effect may to a close approximation be calculated in terms of one dimensionless parameter, L.

$$L = \frac{a}{2\, u_{0.6065}} \sqrt{\frac{\Delta n}{n}} \qquad \text{.... A-(4)}$$

where a is the cell thickness in cm.

$2\, u_{0.6065}$ is the Gaussian inflection leg in cm. (i.e. the distance across the Gaussian curve at the apparent inflection level).

Δn is the total refractive index change due to concentration

and n is the refractive index of the solution.

The same authors have made theoretical calcula-

tions for the dependence on L of parameters required for the correction of schlieren data. These parameters include the negative (i.e. towards the centre of rotation) displacement, δ_ξ, at the apparent inflection level of the bisector or centre line of the peak. The term "apparent" is used because it is only the distorted, camera-recorded schlieren pattern which can be observed and measured. However, the distorted patterns in most cases so closely resemble the correct patterns that corrections based on the dimensions of the distorted pattern are in relative error by a lower order of magnitude[151].

The following method was therefore used to evaluate equivalent level corrections for the data in Table A-2.

a:

As before the cell thickness was taken to be that stated by the manufacturer.

$2\,u_{0.6065}$:

It was first necessary to determine the inflection level of the photographed schlieren peak, which was assumed to be Gaussian. This was accomplished by measuring the height of the maximum ordinate above the baseline and utilizing the fact that the inflection level of a Gaussian curve is found at 0.6065 times the peak height. The Gaussian inflection leg was then measured

on the photograph and converted to its value in the cell using the magnification factor of the camera lens.

$\triangle n$:

The refractive increment of the protein at the beginning of the run was known from its concentration. Radial dilution corrections were applied to find $\triangle n$ at the time of a particular photograph.

n:

The refractive index of the solution in the region of the boundary was taken to be the sum of the refractive index of water, the refractive increment of the buffer components and half the refractive increment of the protein. The first of these terms made up 99.8-100% of n.

L was thus evaluated from equation A-(4). Since the relationship between L and δ_ε is expressed in Ford and Ford's paper[151] only by a small graph, an enlarged transparency of this illustration was made. The graph was then plotted on a large scale from comparator measurements on this transparency. The value of δ_ε thus obtained was in Gaussian units[151] and was converted to centimetres by multiplying by the factor $^2 u_{0.6065/1.4142}$.

As mentioned previously δ_ε is the displacement of the schlieren centre line at the apparent inflection level. The required displacement at the maximum ordinate,

which was the point measured in all sedimentation coefficient determinations, was obtained by dividing δ_ε by 0.6065. As with the other correction, measurements were made only for the first and last photographs in a run, the difference in correction terms calculated as a percentage of the distance moved by the peak, and the same percent correction applied to the sedimentation coefficient.

(iii) Results and Discussion

The results obtained by applying the two corrections to the data in Table A-2 are shown in Table A-3.

TABLE A-3

Comparison of Corrected and Uncorrected $S_{20,w}$ Figures

Run No.	$S_{20,w}$ uncorrected	C.1 (%)	C.2 (%)	Net % Corr.	$S_{20,w}$ corrected
UC/35	$2.38 \pm .01_6$	+0.65	-0.26	+0.39	$2.39 \pm .01_6$
UC/36	$2.51 \pm .03_1$	0.0	-1.25	-1.25	$2.48 \pm .03_1$
UC/30	$2.65 \pm .03_5$	+0.30	-0.15	+0.15	$2.65 \pm .03_5$
UC/31	$2.75 \pm .03_6$	0.0	-1.22	-1.22	$2.72 \pm .03_6$
UC/28	$4.23 \pm .03_3$	+0.25	-0.07	+0.18	$4.24 \pm .03_3$
UC/29	$4.33 \pm .05_1$	0.0	-0.99	-0.99	$4.29 \pm .05_1$

C.1 : Correction for light bending and refraction.
C.2 : Equivalent level correction.

It is seen from Table A-3 that the corrections, though all less than the estimated experimental error, are in such a direction as to reduce the observed discrepancies. However the magnitude of the corrections is only one third to one half that of the observed discrepancies. Better correlation between theory and experiment might be obtained if some refinements were made to the methods for evaluating corrections. A number of criticisms can be made of the methods used in the present study.

(1) The validity of applying corrections based on measurements on only the first and last photographs is questionable. It seems reasonable, for the purpose of making a small percent correction, to assume the centrifugal field to be constant during the run, thus making the sedimentation coefficient proportional to the distance travelled by the peak in unit time. However, a larger error may be incurred in assuming that the correction terms vary linearly with time. If, for instance, most of the change in δ_x or δ_ε takes place during the time for the first few photographs the method used would be incorrect. Strictly, δ_x and δ_ε should be evaluated for every photograph and the corrected $\bar{x}$ values plotted as log $\bar{x}$ y. time.

(2) Even the corrected sedimentation coefficients may be subject to a few tenths of a percent error due to

being calculated from the rate of movement of the maximum ordinate rather than the point given by the square root of the second moment of the gradient curve.

(3) In the evaluation of the equivalent level correction, the dependence of δ_ε on L is slightly different if "background gradients" are considered. These are elevations of the schlieren baseline arising from pressure effects which produce refractive index gradients and cell distortions[151]. However, Ford and Ford have calculated this correction only for optical patterns observed with their ultracentrifuge at 59780 r.p.m. Since no data were available for the ultracentrifuge used in the present study at this speed or for either ultracentrifuge at 44770 r.p.m., it was necessary to neglect background gradients. For values of L below about 0.7, as were those for the experiments in Table A-3, the differences between δ_ε calculated with and without background gradients appear to be slight (see Fig. 7 of reference 151).

(4) Some error must be incurred in attempting to estimate δ_ε from L since the relationship between them has only been published graphically. It would be useful to have this data in some more accurate form.

(5) Ford and Ford[151] have emphasized that no account has been taken of original non-horizontal entry of the light ray into the liquid. This could be caused

by misalignment of the optical system or by pressure distortion of the ultracentrifuge cell at high speed. The light bending and refraction correction should not be affected provided a solvent baseline is obtained, but distortion due to the equivalent level effect would be increased. In view of this factor it is not surprising that any attempt to correct experimental data should yield an under-correction.

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PUBLISHED ABSTRACT

"Fractionation of Papain into Active and Inactive Species"

J.C. Swann and J.H. Coates, Aust.J.Sci., 28, 81, (1965).

Abstract of a paper presented at a meeting of the Australian Biochemical Society in Adelaide, January 1965.

Xerox copy appended.

Swann, J. C. & Coates, J. H. (1965). Fractionation of papain into active and inactive species. Australian Journal of Science, 28(2), 81.