THE UNIVERSITY OF MANITOBA

THE ROLE OF NONCOVALENT FORCES IN FOOD PROTEIN INTERACTIONS - A STUDY SYSTEM USING THE LEGUME STORAGE PROTEIN VICILIN

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ABSTRACT

Vicilin, a storage protein in the seed of the fababean, Vicia faba minor var. Diana, was selected as a study system to investigate noncovalent forces on an intramolecular and intermolecular level. The vicilin was isolated by differential solubilization of a protein concentrate in 0.2 M sodium acetate, pH 7.5 followed by gel filtration; homogeneity of the protein fraction was established with isoelectric focusing and ultracentrifugation. In terms of physicochemical parameters, the isolated vicilin was a multimer (154,000 M.W., 6.5 S) formed by the noncovalent association of four possible subunits with molecular weights of 42,000, 28,300, 19,000 and 15,700 as determined by SDS-polyacrylamide gel electrophoresis.

Step-wise chemical modification of lysine residues with maleic anhydride was used to examine the influence of electrostatic perturbation on the structural integrity of vicilin. The feasibility of differential scanning calorimetry as a probe of conformational change was established using chemical modification of cytochrome c as a model system. Changes in the relative exposure of surface hydrophobic residues (So) were also monitored for vicilin using cis-parinaric acid as a fluorescent probe. Both cytochrome c and vicilin were relatively tolerant to extensive electrostatic modification; conformational variations appeared minor up to approximately 60% maleylation. Beyond this modification level, structural changes

were more pronounced; however, nearly total maleylation did not result in complete protein denaturation. The possible existence of a critical labelling point was attributed to the cumulative effects of electrostatic repulsion and/or modification of specific key residues. Vicilin, as a multimeric protein, was more resistant to electrostatic manipulation than cytochrome c, possibly as a result of the stabilizing influence of vicilin arginine residues and/or the reduced amount of modification relative to the size of the entire molecule.

The effects of a spectrum of neutral salts on selected conformational parameters were assessed to evaluate the relative significance of hydrophobic associations in vicilin quaternary structure. As a result of the influence of pH on protein conformational properties, the pH of the various salt media was restricted to a range of values from 6.2 to 7.0. At low ionic strengths, where electrostatic salt effects predominate, there were minimal differences in the thermal properties and So values for vicilin among a variety of sodium salts. At higher salt concentrations, the thermal parameters and So values were correlated with the molal surface tension increment, a numerical reflection of the position of an anion in the Hofmeister series. These relationships were extrapolated to infer a dependence of vicilin subunit associations on hydrophobic interactions. A limited study with cationic influences on vicilin conformation only illustrated the dependence of cationic effects on the

associated anion.

The consideration of noncovalent forces was extended to intermolecular vicilin interactions; specifically the ability of vicilin to self-associate into a micelle arrangement upon sudden exposure to a defined aqueous environment. For micelle formation, a specific level of hydrophobic residue exposure appeared critical; this reinforced the implied significance of hydrophobic associations in micelle structures. Elevated So values, induced by destabilizing anions, were unfavorable due to a disturbed hydrophilic-hydrophobic balance; low So values, caused by preferential hydration, did not allow sufficent hydrophobic residue exposure for intermolecular association. The most appropriate electrolyte environment for micelle formation was one in which vicilin electrostatic repulsion was minimized to allow hydrophobic association.

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INTRODUCTION

The basis for protein functionality in a food system is often related to the capacity of protein molecules to interact with other molecular constituents. Within a food framework, a diverse spectrum of interactions may occur involving different associations between proteins and other macromolecules or frequently, among various types of proteins. In some cases, with a single or relatively few protein species present, protein-protein interactions may be described as self-association phenomena. Although the general importance for such intermolecular associations is recognized, the actual mechanisms of interaction are poorly understood(Hermansson, 1977).

Protein-protein associations within a food system may be products of either covalent and/or noncovalent reactions. Covalent interactions are exemplified by disulfide linkages; noncovalent associations mainly involve electrostatic and hydrophobic interactions. Recently, it has become apparent that these noncovalent forces may be more important in the understanding and utilization of proteins in a food application than was previously appreciated.(Murray et al., 1981). This recognition, however, does not immediately simplify the analyses of protein-protein interactions in foods. Noncovalent parameters are difficult to estimate quantitatively; moreover, they are also subject to a wide degree of variability with changing environmental conditions. In addition, the concept of a balance of forces is intrinsic to the consideration of noncovalent aspects, both from an intramolecular and an intermo-

lecular perspective. The significance of this last point is exemplified by the protein micelle, a relatively unique arrangement of protein molecules formed under specific environmental conditions. This association of proteins appears to necessitate hydrophobic interactions; however, despite an adequate exposure of surface hydrophobic residues, these interactions may be prevented if electrostatic repulsive forces are extensive(Murray et al., 1981). This specific example can be extrapolated to a general consideration; the manipulation of proteins to a desired end-point within a particular system requires recognition of the potential existence of opposing noncovalent forces.

Investigations into the role of noncovalent forces in protein-protein interactions within foods should take into account several fundamental considerations. One is the inherent complexity of most food systems; in general, the more complex the system, the more difficult it is to interpret. Consequently, studies with a single self-associating protein species may be ultimately desirable as an initial starting point. Secondly, the establishment of a simple protein system necessitates a particular protein isolation procedure. With the implementation of this separatory procedure, it is important to minimize alterations in protein structure both from the viewpoint of designing a study system, and on a larger scale, from the possible incorporation of the protein into a food product. Eventual manipulation of a protein to achieve specific functional properties is only possible with a rela-

tively native starting protein. Thirdly, a complete understanding of noncovalent forces in protein-protein interactions necessitates some appreciation for the structural characteristics of the associating molecules within the study system. Finally, a combination of molecular characteristics with defined environmental responses (in terms of types and degree of molecular association) should allow some interpretation of the noncovalent forces involved in the general interaction mechanism.

With these considerations, the purpose of this study was to provide a further understanding of some of the noncovalent forces apparently involved in the establishment of a protein micelle. The significance of protein micelle formation as a protein isolation method was initially recognized by Murray et al. (1978); subsequently, the noncovalent interactions operative in micelle formation have been suggested to be potentially significant to a food system if these forces could be controlled and predicted. In this respect, the initial step to assessing some of the noncovalent forces associated with a protein micelle was to design a study system with a single species of protein. As the original micelle studies by Murray et al. (1978) involved a mixed system of storage proteins from the seed of fababean, Vicia faba, it was decided to pursue this approach by isolating one of these storage proteins. Prior to examining inter-protein associative reactions, several investigations were performed to derive some appreciation for the structural integrity of the individual protein mol-

ecules with specific emphasis on the significance of intramolecular noncovalent forces. One study involved stressing the molecule by specific chemical modification procedures to assess the relative significance of electrostatic parameters to overall molecular stability; another involved the exposure of the molecule to various electrolyte environments to derive some appreciation for the types of conformational responses that may be observed and, in turn, to relate these responses to intramolecular associative forces. Finally, the self-association of the protein molecules into a micelle arrangement was examined in an endeavour to understand some of the molecular conformational prerequisites for micelle formation and the noncovalent associative-repulsive forces involved.

CHAPTER ONE

ISOLATION AND CHARACTERIZATION OF VICILIN

INTRODUCTION

The selection of fababean storage proteins as a study system for the investigation of specific noncovalent forces in both intramolecular protein structure and intermolecular protein associations necessitated the isolation of a single protein species. The developing fababean seed appears to be an ideal source for the extraction of a chemically pure protein as it contains a large complement of proteinaceous material of which approximately 90% is represented by two large storage proteins conventionally referred to as vicilin and legumin(Boulter, 1970). Considerable difficulty has been encountered in the isolation and characterization of these two protein fractions since their original identification in Pisum sativum by Osborne and Campbell(1898). As purification of legumin and vicilin is a fundamental prerequisite to subsequent structural investigations, much effort has been directed toward the establishment of suitable separatory methods. In the development of more recent extraction techniques, the severity of an isolation method, in addition to its specificity, has been recognized as a significant parameter. Although a purified protein is the ultimate goal of any particular isolation technique, it is important that the structural characteristics of the protein in question do not become extensively modified by the overall separatory method.

Several techniques, involving a variety of legumes, have been used to extract legumin and vicilin as a "globulin fraction" from the intact seed prior to further purification of

the individual storage proteins(Derbyshire et al., 1976). In one of the earliest studies, Danielsson(1949) established a procedure, now described as "classical", to isolate a globulin fraction from Pisum sativum using protein solubility characteristics to achieve the separation. Both legumin and vicilin exhibit the traditional characteristics attributed to globulin proteins - solubility in dilute salt solutions and insolubility in water. As a result, these proteins were initially extracted with buffered sodium chloride and then precipitated with ammonium sulfate(70% saturation) to remove nonproteinaceous material. Gradual removal of salt by dialysis resulted in solubilization of some protein components and precipitation of legumin and vicilin. Unfortunately, this approach had two main disadvantages: incomplete separation of the storage proteins from other proteinaceous components and possible enhancement of protein-protein associations with precipitation(Derbyshire et al., 1976). These disadvantages may have been at least partially overcome with an extraction procedure developed by Matta et al. (1981). In this approach, a globulin fraction was initially isolated from Vicia faba by solubilization of the proteins in a mildly alkaline buffer of low ionic strength(eg. 0.05 M sodium borate buffer, pH 8.0). This was followed by selective isoelectric precipitation of legumin and vicilin at pH 5.0.

A number of different techniques have been used in an attempt to separate legumin and vicilin after the preparation of a crude globulin fraction from a variety of

legumes(Derbyshire et al., 1976). Although these methods have met with varying degrees of success, they have served to demonstrate that legumin is the easier of the two proteins to isolate. For example, isoelectric precipitation of legumin from Pisum sativum at pH 4.7 resulted in a relatively pure protein fraction; however, the soluble vicilin portion remained contaminated with legumin(Danielsson, 1949). Similarily, zonal isoelectric precipitation of a crude globulin fraction from Vicia faba resulted in a pure legumin fraction and a contaminated vicilin fraction(Wright and Boulter, 1974). Scholz et al. (1974) successfully extended this zonal precipitation method by separating legumin from contaminated vicilin using ion exchange chromatography with DEAE cellulose.

In terms of selecting a separatory procedure for legumin and vicilin on the basis of their differing physical properties, it would appear from a theoretical viewpoint that gel filtration is a logical approach. Vicilin and legumin from various legumes exhibit significant differences in sedimentation coefficients(7S and 11S) and molecular weights (approximately 186,000 and 330,000)(Derbyshire et al., 1976). However, as a result of their similar Stokes' radii, gel filtration of a crude globulin fraction resulted in incomplete protein separation characterized by extensively overlapping elution profiles(Derbyshire et al., 1976). For example, Koshiyama(1972), applying gel filtration to a globulin fraction from Glycine max, found that only a limited amount of pure legumin could be isolated in the fractions corresponding

to the initial segment of the leading edge of a chromatographic profile.

A variety of other separatory principles have been used in a number of methods for the isolation of legumin and vicilin. In one approach, Simard and Boulet(1978) reported the extraction and purification of vicilin(7S) and legumin(11S) from several legumes, including Vicia faba, using differential precipitation of the proteins by various levels of ammonium sulfate. They found selective precipitation of legumin in solutions of 60 to 70% saturation and precipitation of vicilin in solutions of 70 to 85% saturation. Despite the apparent success of this method, the only criterion used to determine homogeneity of the isolated protein species was ultracentrifugal analysis. Single analytical techniques such as this are inadequate for the definition of purity (Phelps,1978).

Another separatory approach has involved the use of affinity chromatography with monospecific antibodies or agglutinating proteins such as concanavalin A for the selective isolation of vicilin. Despite its apparent specificity, this technique has met with limited success due to the fluctuations in vicilin carbohydrate content (Kitamura et al., 1974; Derbyshire et al., 1976). In fact, of all the separatory techniques employed, it appears that the most successful isolation of legumin and vicilin involved hydroxyapatite chromatography of a crude protein extract in a potassium phosphate buffer, pH 8.0(Gatehouse et al., 1980). In the consideration of the numerous separatory procedures available at the onset of this

project, the mechanism of hydroxyapatite chromatography had not been established. As such, it was difficult to assess the possible influence of this isolation method on protein conformational parameters. Recently, the mechanism of protein separation using this type of chromatography has been somewhat clarified. As outlined by Gorbanoff and Timasheff(1984), basic proteins seemed to bind to the column by general electrostatic interactions between amino groups and negative column residues; acidic proteins appeared to form specific complexes between protein carboxyl groups and column calcium ions. Differential removal of proteins such as legumin and vicilin seemed to be achieved successfully by variations in the elution media.

Although separation of vicilin and legumin has proven to be difficult, it is necessary to have as detailed a structural assessment of them as possible in order to establish criteria for purity and to further understand the legume storage proteins in general. As a result of isolation problems, the legumin fraction has been characterized more extensively than the elusive vicilin. In Vicia faba, Matta et al. (1981) have developed an operational definition of the legumin protein. From their viewpoint, legumin represents a single storage globular protein with a molecular weight of 300,000 to 400,000. As an additional characterization, the protein contains large acidic subunits and small basic subunits covalently linked by disulfide bonds in acidic-basic pairs. Considerable heterogeneity exists in the actual subunits and the

pairs formed.

Similar attempts to define the secondary vicilin fraction have been complicated by two main factors - possible legumin contamination and apparent protein heterogeneity. With respect to the latter, vicilin seems to be a heterogeneous protein mixture containing two or three major proteins depending upon the legume source(Derbyshire et al., 1976). For example, the vicilin fraction of Vicia faba appears to contain at least two protein components. The major component is a 7S protein having a molecular weight of 150,000 and possibly four subunits. It is also a glycoprotein containing less than 0.5% neutral sugars (w/w) (Wright and Boulter, 1972). This main 7S protein of the vicilin fraction seems to show a number of chemical similarities among the different legumes; however, precise comparisons will only be possible when more of the proteins have been extensively characterized(Derbyshire et al., 1976). Although the major 7S protein has been physically described, the characteristics of the other proteins which contribute to the heterogeneity of this fraction have not been fully established. The entire vicilin situation has been more clearly defined in Pisum sativum, although Gatehouse et al., (1981) reported that results of their preliminary investigations were indicative of a similar situation in Vicia faba. In field peas, the vicilin fraction is composed of two proteins - one with a molecular weight of 170,000, designated as "vicilin" and a second with a molecular weight of 280,000, designated as "convicilin"(Croy et al., 1980). As a complicating factor, the

vicilin(170,000) protein is suggested to actually represent a number of similar protein species(Gatehouse et al., 1981). Needless to say the source of this vicilin heterogeneity has been an area of concern. Gatehouse et al., (1981) suggested that the multiple protein species of the vicilin fraction were not a product of protein proteolytic degradation during isolation; types of proteins extracted did not change in the presence of proteinaceous inhibitors or under dissociating conditions. In order to explain the heterogeneity, it was suggested that the subunits of vicilin undergo post-translational "nicking" within the developing pea seed to yield polypeptides of varying molecular weights. This premise was developed as a result of the discrepancy between the subunit content of vicilin isolated from whole seeds and the subunits recognized as immediate vicilin translation products in both in vivo and in vitro situations. If such variations in post-translational modification occur, this may result in different subunit combinations which, in turn, may ultimately give rise to different molecules within the vicilin fraction.

Recognizing previous difficulties in protein extraction and characterization, the purpose of this study was to isolate one of the storage proteins from Vicia faba minor var. Diana in order to establish a single protein species for the investigation of specific noncovalent forces involved in molecular structure and certain interprotein associations. A different isolation approach was used from those described in preceding studies. The initial globulin fraction was extracted using a

unique mild treatment developed by Murray et al. (1978). In this procedure, the globulins were solubilized from an air-classified concentrate using 0.3 M sodium chloride to form a high salt protein extract(HSPE). The HSPE was subsequently diluted into a specific volume of cold tap water. This rapid dilution resulted in massive protein association and precipitation. The precipitated protein was characteristically in the form of microscopic spheres, or micelles, possibly as a consequence of hydrophobic interactions(Murray et al., 1978). Analytically, this protein isolate known as protein micellar mass(PMM) contained approximately 96% protein(N x 5.85) with minimal traces of ash(Murray et al., 1981).

Using PMM as the starting material, rather than a crude globulin extract, a technique was developed which would selectively isolate a single species of storage protein. In addition to specificity for a single protein, the advantages of this isolation method included minimal protein manipulation and production of relatively large protein quantities for further experimentation. Previously established techniques were judged to be unattractive due to problems relating to pre-separation treatments, heterogeneity of the isolated fraction or relatively low protein yields. The isolated storage protein was subsequently characterized in terms of molecular weight, isoelectric point, subunit structure and amino acid composition. Isoelectric focusing was used as the main technique for assessment of protein homogeneity.

MATERIALS AND METHODS

Preparation of the Protein Isolate

The initial isolation of the storage proteins, vicilin and legumin, from fababean (*Vicia faba minor*, var. *Diana*) involved the method of Murray *et al.* (1978). Air classified fababean protein concentrate(10% w/v) was suspended in 0.3 M sodium chloride and stirred for 30 min at 37°C in a water-jacketed extraction vessel. The resulting suspension was centrifuged at 4000g for 15 min at 37°C in an RC2-B Sorvall controlled temperature centrifuge. After centrifugation, the supernatant was decanted and carefully diluted into three volumes of cold tap water. This dilution resulted in massive protein precipitation. After 30 min, the precipitated protein settled into a compact mass which was collected by decantation of the supernatant. The resulting protein isolate, referred to as protein micellar mass(PMM), was lyophilized prior to further experimentation.

Isolation of Vicilin from PMM

The protein isolate PMM is predominantly composed of the two structural proteins, legumin and vicilin, with some contaminating compounds(Murray *et al.*, 1981). Preliminary attempts to separate vicilin and legumin by gel filtration using various suspending salt and detergent media were ineffective as a result of the proteins' similar Stokes' radii(132A° and 185 A°); (Murray *et al.*, 1981) and their apparent tendency to associate. However, in the course of this

study it was discovered that a method involving differential solubilization of vicilin in 0.2 M sodium acetate, pH 7.5, followed by chromatographic removal of glycosidic contaminants could result in the separation of vicilin from legumin. No evidence of legumin contamination in vicilin preparations was detected during subsequent isoelectric focusing and ultracentrifugal analyses.

The extraction of vicilin from PMM initially involved a suspension of 1.0 g aliquots of PMM in approximately 10 mL of 0.2 M sodium acetate, pH 7.5. The ionic strength of the extracting salt solution was maintained at 15.0 mS cm^{-1}; slight variations in the molarity occurred with changes in acetate moisture levels.

The resulting suspension was stirred for 3 h at room temperature and then centifuged for 10 min at 12,000g. The supernatant was applied to a Pharmacia K26-100 gel filtration column containing Sephacryl S-300. A downward flow rate of 30 mL h^{-1} of the eluting solution, 0.2 M sodium acetate, pH 7.5 with 0.02%(w/v) sodium azide, was maintained by an LKB Microperpex peristaltic pump(Model No. 2132). Fraction volumes of 80 drops per tube(approximately 3.75 mL) were collected with an LKB 2117 Redirac fraction collector. The column eluant was monitored continuously by an ISCO dualbeam monitor at a wavelength of 280 nm. The resulting chromatographic profile, as recorded by an ISCO Model VA5 recorder, was characterized by two distinct peaks. The first peak was collected and characterized by further experimentation as the storage protein

vicilin. The second broader peak contained non-proteinaceous material as determined by a modified Lowry procedure for the assay of proteins in the presence of interfering materials(Bensadoun and Weinstein, 1976). This second peak was subsequently analysed for glycoside content using the HPLC(high performance liquid chromatography) method of Marquardt and Frolich(1981).

Characterization of Vicilin

A. Determination of Amino Acid Composition

For all amino acid analyses, samples of vicilin isolated by gel filtration were dialysed extensively against distilled water containing 0.02% sodium azide to remove a significant amount of the sodium acetate. The dialysed protein was lyophilized and stored at -10°C.

1. Standard Amino Acid Procedure

A 10 mg sample of the lyophilized vicilin(approximately 98% protein) was weighed into a 20 mL glass test tube. After the addition of 2 mL of 6 N hydrochloric acid, the tube was evacuated and hermetically sealed. Hydrolysis was carried out for 16 h at 121°C. The hydrolysed sample was then cooled and evaporated to dryness under vacuum. The dried residue was shaken vigorously for 15 s with 10 mL of 0.2 N sodium citrate buffer, pH 3.25, in the presence of a few glass beads. Insoluble material was removed by filtration with Whatman No. 40 filter paper.

The resulting filtrate was used for amino acid analysis.

All analyses were performed with a Beckman Model 119C automatic amino acid analyser equipped with a Beckman Computing Integrator System AA following the Beckman Standard Method; a modification of the procedure of Spackman et al. (1958). The entire procedure was repeated using hydrolysis times of 24 and 48 h. The amino acids serine and threonine are frequently degraded by extensive acid hydrolysis. As a result, the linear relationships between amounts of each of these amino acids and hydrolysis times were established by regression analysis(Appendix I). The actual level of serine and threonine in the original protein was then estimated by a linear extrapolation of concentration to zero hydrolysis time.

2. Procedure for Cysteine, Cystine and Methionine

Standard amino acid hydrolysis results in significant loss of the sulfur-containing amino acids. In order to derive a more reliable estimation of these amino acids, cysteine and cystine were initially oxidized to cysteic acid and methionine is converted to methionine sulfone. The performic acid oxidation method used for this conversion was a modification of Hirs(1967). Performic acid(2 mL) was added to a 10 mg sample of lyophilized vicilin(approximately 98% protein). The performic acid-protein mixture was maintained in ice at 0°C for 20 h; after which 0.3 mL of 6 N hydrochloric acid was added and the hydrolysed protein was evaporated to dryness using a Vapomix under vacuum at 70°C. The dried residue was resuspended in 0.2 N sodium citrate buffer using the standard sample preparation procedure and analysed on the amino acid

analyser.

3. Determination of Tryptophan

Loss of tryptophan occurs during acid or alkaline hydrolysis prior to routine amino acid analysis. To avoid this problem, tryptophan levels in vicilin were assessed by the spectrophotometric method of Messineo and Musarro(1972) for the determination of free and bound tryptophan.

4. Determination of Amide Nitrogen

The level of amide nitrogen in vicilin was determined using an ammonia electrode(Orion model 95-10) following the procedure of Arntfield and Murray (1981).

B. Determination the Isoelectric Point

The isoelectric point of vicilin was determined using an isoelectric focusing procedure based on methods described in LKB Application Note No. 250 by Winter et al., (1977). The basic isoelectric focusing protocol necessitates that a protein sample be dissolved in a medium with as low an ionic strength as possible. As a result, prior to focusing, samples of vicilin in 0.2 M sodium acetate, pH 7.5, were dialysed extensively against the following salt solutions: 0.01 M sodium citrate, pH 8.0; 0.025 M sodium citrate, pH 8.0; 0.025 M sodium phosphate, pH 8.0, and 0.025 M sodium phosphate, pH 6.6. Vicilin solubility in most salt solutions at reduced ionic strengths is relatively low; these selected media represented environments in which an appropriate amount of vicilin could be solubilized. To determine the extent of

this solubilization, protein levels of the vicilin fraction were monitored before and after dialysis by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Isoelectric focusing of the dialysed protein samples was carried out on an LKB 2117 Multiphor apparatus with an LKB 2197 Constant Power Supply. A Haake circulating water bath was used for temperature control. Gels used for the focusing were LKB Ampholine PagPlates with a potential pH range of 4.0 to 6.5. Vicilin samples(approximately 1 mg mL^{-1}) were applied in 10 μL aliquots directly onto the surface of the gel well below the expected isoelectric point as this reduced the tendency for protein precipitation during focusing. All samples were applied near the anode. The gel was focused at a constant power of 25 W for 2 h at 10°C. Anode and cathode solutions were 0.1 M glutamic acid in 0.5 M phosphoric acid and 0.1 M β-alanine, respectively. At the end of 2 h, the pH gradient across the gel surface was determined with an Ingold surface electrode. After refocusing for 30 min, the gel was fixed, destained, stained and destained as described in Table 1.1. Gels were photographed after two days of destaining. The distance between the vicilin band and the anode was measured. The isoelectric point of the vicilin was then determined from a plot of the gel pH as a function of the distance of the pH measurement from the anode(Appendix II).

C. Subunit Analysis with SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) was carried out according to the method

TABLE 1.1. Staining procedure for isoelectric focusing.

Solution	Time	Solution composition
Fixative	1 h	17.3 g sulfosalicylic acid (Fisher Chem. Co.), 57.5 g trichloroacetic acid(Fisher Chem. Co.), 500 mL distilled water.
Destaining	30 min	500 mL ethanol, 160 mL acetic acid, distilled water to make 2000 mL.
Stain	10 min at 60°C.	0.460 g Coomassie Brilliant Blue R-250(Sigma Chem. Co.), 400 mL destaining solution. Stain filtered through Whatman No. 1 filter paper.
Destaining	1-2 days with frequent solution changes.	As given above.

described by Fehrnstrom and Moberg(1977) using an LKB 2111 Multiphor apparatus with an LKB Constant Power Supply. Cooling was achieved with a Haake circulating water bath. All gels contained 7.5% polyacrylamide(see Table 1.2 for complete composition).

Samples of vicilin(approximately 3 mg mL^{-1}) in 0.2 M sodium acetate, pH 7.5, were dialysed for 16 h against 0.01 M imidazole buffer, pH 7.0 containing 1.0% SDS(w/v). Following dialysis, the samples were heated in a 100°C water bath for 2 min. For some samples the reducing agent 2-mercaptoethanol (Aldrich Chem. Co.) was included in the original buffer at a level of 1%(w/v). After cooling, all 250 μL sample aliquots were mixed thoroughly with 10 μL of bromophenol blue tracking dye. Ten μL of the sample mixture were then pipetted into the sample slots of a horizontal slab acrylamide gel which had been pre-electrophoresed for 30 min at 80 mA. After sample application, the gel was electrophoresed at 20 mA for 10 min followed by 200 mA for 2 h at 10°C.

Two procedures used for staining the gels are outlined in Tables 1.3 and 1.4. Gels stained in Coomassie Blue were photographed after 3 to 4 days of destaining; those stained with silver exhibited banding patterns that were not sufficiently different from the background to record photographically. Molecular weight standards for SDS-PAGE were obtained from Bio-Rad(Table 1.5). Prior to electrophoresis, the markers were diluted 1:10 with 0.01 M imidazole buffer, pH 7.0, containing 0.1% SDS and then denatured by incubation at 100°C for 5 min.

TABLE 1.2. Composition of polyacrylamide gel for SDS-PAGE.

Solution	Volume of solution for a final acrylamide concentration of 7.5%
Distilled water	7.5 mL
Buffer stock solution[1]	33.0 mL
Acrylamide solution[2]	22.2 mL
Ammonium persulfate[3]	3.2 mL
N,N,N',N' tetramethyl ethylene diamine(TEMED)	0.1 mL
Final volume	66.0 mL

[1] Buffer stock solution: 34.0 g imidazole(Sigma Chem. Co., Grade I), 10.0 g SDS. Titrate with orthophosphoric acid to pH 7.0; adjust to 5000 mL with distilled water.

[2] Acrylamide solution: 22.2 g acrylamide(Eastman Kodak Co., Enzyme grade), 0.6 g bisacrylamide, adjust to 100 mL with distilled water.

[3] Ammonium persulfate: 15 mg mL^{-1} distilled water. Prepared fresh daily.

TABLE 1.3. Staining procedure for SDS-PAGE using Coomassie Brilliant Blue.

Solution	Time	Solution composition
Fixative	1 h	17.0 g sulfosalicyclic acid (Fisher Chem. Co.), 57.0 g trichloroacetic acid(Fisher Chem. Co.), 150 mL methanol, 300 mL distilled water.
Stain	2 h	1.25 g Coomassie Brilliant Blue R-250(Sigma Chem. Co.), 227 mL methanol, 227 mL distilled water, 46 mL acetic acid. Stain filtered through Whatman No. 1 filter paper.
Destaining	Several days with frequent solution changes.	1500 mL ethanol, 500 mL acetic acid, 3000 mL distilled water.

TABLE 1.4. Staining procedure for SDS-PAGE using a silver stain.

Solution	Volume used(mL)	Time(min)
Fixative[1]	400	60
Fixative[2]	400	30
Fixative[2]	400	30
Oxidizer[3]	200	10
Deionized water	400	10
Deionized water	400	10
Deionized water	400	10
Silver reagent[4]	200	30
Deionized water	400	2
Developer[5]	200	1
Developer[5]	200	5
Developer[5]	200	5
Acetic acid(5%, v/v)	400	5

[1] Fixative: 40% methanol, 10% acetic acid(v/v).

[2] Fixative: 10% methanol, 5% acetic acid(v/v).

[3],[4],[5] Oxidizer, silver stain, developer: all components of a Bio-Rad Silver Stain Kit.

TABLE 1.5. Molecular weights of protein standards used for SDS-PAGE.

Protein	Molecular weight	Reference
Lysozyme	14,400	Sophianopoulos et al. (1962)
Soybean trypsin inhibitor	21,500	Wu and Scheruga(1962)
Carbonic anhydrase	31,000	Davis(1971)
Ovalbumin	45,000	Warner(1954)
Bovine serum albumin	66,200	Brown(1975)
Phosphorylase B	92,500	Seery et al. (1967)

The relative mobility(R.M.) of all proteins was calculated from the following relationship:

$$R.M. = \frac{\text{distance of protein migration}}{\text{gel length after drying}} \times \frac{\text{gel length after fixing}}{\text{distance of dye migration}}$$

A calibration curve for the relationship between relative mobility of the molecular weight standards and log molecular weight was established by standard linear regression analysis(Appendix III). The molecular weights of the vicilin subunits were then interpolated from their relative mobilities.

D. Molecular Weight Determination

1. Gel Filtration

The molecular weight of vicilin was approximated by gel filtration using a Pharmacia K26-100 column of Sephacryl S-300 equilibrated with 0.2 M sodium acetate, pH 7.5 containing 0.02% sodium azide. The column was calibrated with a Bio-Rad Gel Filtration Standard containing a mixture of molecular weight markers(Table 1.6). Approximately 36 mg of lyophilized protein standard were dissolved in 1 mL of 0.2 M sodium acetate, pH 7.5, and chromatographed. A downward flowrate was maintained at 30 mL h^{-1}; 80 drops of eluant per tube were collected. Collection tubes were weighed before and after sample collection to determine exact elution volumes. The molecular weight of vicilin was estimated from the linear relationship between log molecular weight and elution volume of the standard proteins(Appendix IV).

TABLE 1.6. Molecular weights of protein standards for calibration of gel filtration column.

Protein	Molecular weight	Reference
Cyanocobalamin	1,350	Merck Index, 10th Edition
Myoglobin(horse)	17,000	Schellenberg(1967)
Ovalbumin(chicken)	44,000	Edsall(1953)
Gammaglobulin(bovine)	158,000	Merck Index,10th Edition
Thyroglobulin(bovine)	670,000	Steiner and Edelhoch(1961)

2. Sedimentation Diffusion

The molecular weight of vicilin was also calculated from the Svedberg equation:

$$M = (RTs)/(D(1-vp))$$

where R = gas constant

T = temperature(°K)

s = sedimentation coefficient

D = diffusion coefficient

v = partial specific volume of the protein

p = density of the solvent

The sedimentation and diffusion coefficients of vicilin in 0.2 M sodium acetate, pH 7.5, were determined by ultracentrifugal analysis according to the method described by Chervenka(1969). The partial specific volume of vicilin was calculated from the amino acid data(Rowe, 1978); the density of 0.2 M sodium acetate, pH 7.5, was assessed by means of a hygrometer.

RESULTS

Gel Filtration of PMM in 0.2 M Sodium Acetate, pH 7.5

The chromatographic profile resulting from the gel filtration of vicilin solubilized preferentially in 0.2 M sodium acetate, pH 7.5, was characterized by two distinct peaks - an initial symmetrical peak followed by a second broader slightly skewed peak(Figure 1.1). Analytical characterization of the fractions corresponding to the first peak were performed to verify the isolation of vicilin and to ascertain its physical homogeneity. The protein content of the fractions corresponding to the second peak was negligible. Analysis of these fractions by HPLC revealed the presence of two substances, identified by standard comparison as the glycosides, vicine and convicine.

Determination of Amino Acid Composition

The amino acid composition of vicilin, as collected from the initial peak of the gel filtration separation, is presented in Table 1.7. Also included in this table are amino acid data for vicilin as derived from Jackson *et al.*, (1969) and Wright and Boulter(1972). Values for average hydrophobicity($H\Phi$), positive and negative charge potential and frequency of the charged groups, were calculated from the amino acid data. The calculation procedure for these parameters, summarized in Table 1.8, is as follows:

Figure 1.1. Column elution profile from gel chromatography of PMM in 0.2 M sodium acetate, pH 7.5 on Sephacryl S-300.

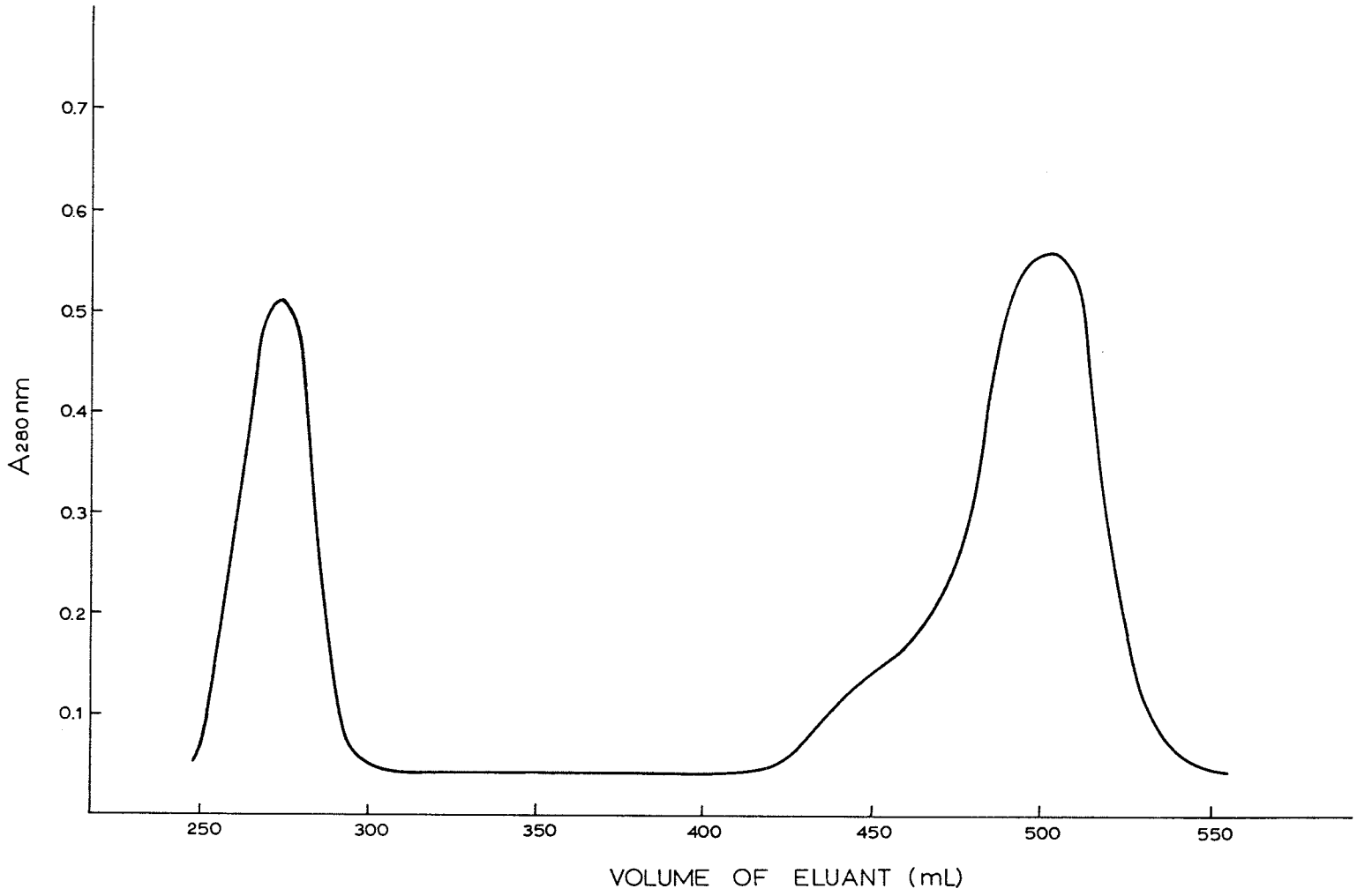
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A280 nm
250
300
350
400
450
500
550
VOLUME OF ELUANT (mL)

TABLE 1.7. Amino acid composition of vicilin isolated from *Vicia faba*.[1]

Amino acid	Present study	Jackson *et al.* (1969)	Bailey and Boulter (1972)
Glu	18.96	17.60	17.10
Asp	13.09	11.90	12.30
Ser	6.03	5.10	4.77
Thr	2.60	2.90	2.62
Arg	9.71	7.80	9.21
Lys	7.33	8.10	8.25
His	1.95	2.40	2.72
Tyr	1.09	3.80	4.10
Trp	0.08	nd[2]	nd[2]
Phe	5.82	6.80	6.83
Pro	3.64	nd	6.25
Gly	3.15	2.50	2.98
Ala	2.75	3.10	2.65
Val	5.15	4.30	5.35
Ile	5.37	5.70	5.73
Leu	9.52	9.30	9.71
Met	0.45	0.60	0.20
1/2 Cystine	0.36	0.30	0.13
Amide N_2	1.13	1.00[3]	1.00[3]

[1] Values are g AA/100g protein.

[2] Not determined.

[3] Value not determined; approximated value of 1.00 used for charge potential calculations.

TABLE 1.8. Calculated value for average hydrophobicity(HΦ) and charge potentials in vicilin.[1]

	HΦ[2]	Negative potential[3]	Positive potential[3]	Charge frequency
Present study	901.43	160.76	118.45	0.38
Jackson, et al. (1969)	930.13	150.23	115.64	0.39
Bailey and Boulter(1972)	1054.07	149.84	126.83	0.37

[1] Bigelow(1967).

[2] cal/AA residue.

[3] moles/100 Kg protein.

1. Average hydrophobicity ($H\Phi$)

$$H\Phi = \frac{\Sigma \text{ (Amino Acid) (Amino acid hydrophobicity value)}}{\text{Total moles of amino acids/100 kg protein}}$$

$$= \frac{\text{Total hydrophobicity}}{\text{Total moles of amino acids/100 kg protein}}$$

The hydrophobicity values for the individual amino acid residues used to calculate the total vicilin hydrophobicity were derived by Bigelow(1967) from the transfer free energies(ΔFt) of the amino acid side chains as determined by Tanford(1962). Transfer free energy is defined as the free energy required to transfer one mole of amino acid from water to ethanol. The ethanolic environment was considered to simulate the hydrophobic interior of a protein molecule. Tanford(1962) did not estimate ΔFt values for cysteine, cystine, histidine, glutamic acid and aspartic acid. For the latter three amino acids, Bigelow(1967) assumed that, as charged residues, these structures did not contribute to the hydrophobic stability of a protein and consequently assigned a hydrophobicity value of 0.0 kcal/residue to each. Cysteine and 1/2 cystine were assigned an estimated value of 1.0 kcal/residue, approximately 2/3 of the value for methionine.

2. Negative charge potential

$$\text{Negative potential} = \text{Total moles of Glutamic Acid + Aspartic Acid - Amide groups}$$

Negative charge potential represents the total possible negative charge for vicilin assuming that all glutamic and aspartic acid residues are in an ionized form.

3. Positive charge potential

Positive potential = Total moles of Lysine + Arginine + Histidine

Positive charge potential represents the total possible positive charge for vicilin assuming that all lysine, arginine and histidine side chains are in an ionized form.

4. Frequency of charged groups

$$\text{Charge frequency} = \frac{\text{Negative + Positive Charge Potential}}{\text{Total moles of amino acids/100kg protein}}$$

Analysis of the Vicilin Fraction by Isoelectric Focusing

The isoelectric focusing profiles of vicilin originally suspended in four different media are shown in Figure 1.2. In all situations, the major protein band had a pI of 5.0. Vicilin, originally solubilized in 0.025M phosphate buffer, pH 6.6, consistantly focused as a single protein band. However, the focusing profiles for vicilin in three different media at pH 8.0(0.025 M phosphate buffer, 0.01 M and 0.025 M sodium citrate) were characterized by a major single band with a pI of 5.0 accompanied by multiple smaller bands - two distinct bands with a pI of 5.2 and several less distinct bands ranging in pI values from 5.4 to 5.7(Figure 1.2). The multiple banding patterns were not a function of variations in protein concentrations. The amount of protein originally applied to the gel surface was similar in all situations, i.e. 10 μL of a 1 mg mL^{-1} protein solution. Loss of protein solubility during dialysis was also not a contributing factor. Percent protein recoveries after dialysis for the four media were as follows:

Figure 1.2. Isoelectric focusing patterns of vicilin solubilized in four different salt media.

Pattern	Salt Medium
A	0.01 M sodium citrate, pH 8.0
B	0.025 M sodium citrate, pH 8.0
C	0.025 M sodium phosphate, pH 8.0
D	0.025 M sodium phosphate pH 6.6

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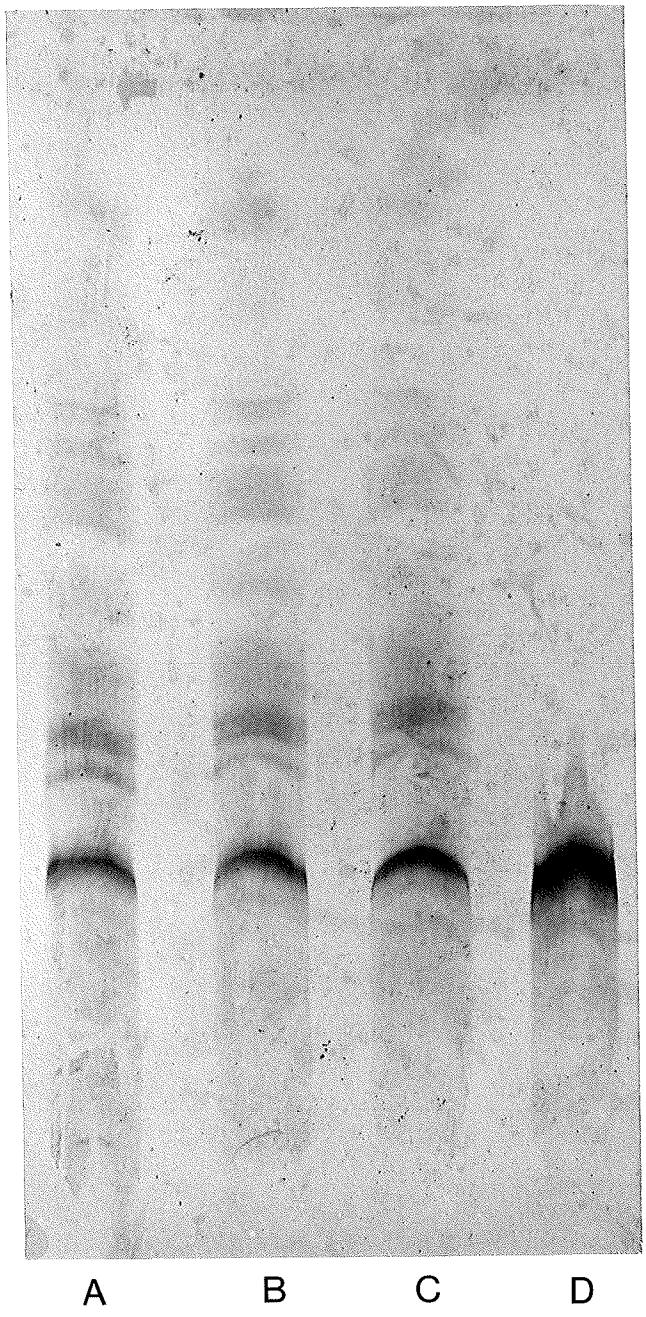
VEUILLEZ ECRIRE A L'AUTEUR POUR LES RENSEIGNEMENTS OU VEUILLEZ CONSULTER L'EXEMPLAIRE DONT POSSEDE LE DEPARTEMENT DES ARCHIVES ET DES COLLECTIONS SPECIALES, BIBLIOTHEQUE ELIZABETH DAFOE, UNIVERSITE DU MANITOBA, WINNIPEG, MANITOBA, CANADA, R3T 2N2.


5.7
5.4
5.2
5.0
A
B
C
D

72% for 0.01 M sodium citrate, pH 8.0; 96% for 0.025 M sodium citrate, pH 8.0; 98% for 0.025 M sodium phosphate, pH 8.0; 98% for 0.025 M sodium phosphate, pH 6.6.

In all samples, there was no evidence of legumin contamination. As determined in preliminary studies, legumin was characterized by a pI of 4.5. If present, this protein was distinctly visible on the gel at both its isoelectric point and as a precipitant at the point of application.

Subunit Determination

The vicilin fraction was analyzed by SDS-PAGE under reducing and non-reducing conditions. A comparison of the resulting electrophoregrams showed that the pattern and number of bands was not altered by the reductive influence of mercaptoethanol. The characteristic banding patterns for vicilin using Coomassie Brilliant Blue R-250 as a stain are given in Figure 1.3. Four subunits were observed having the following molecular weights: 42,000, 28,300, 19,100 and 15,700 . Of the four subunits, the band corresponding to the 28,300 polypeptide was always the least distinct on the electrophoregram. Similar SDS-PAGE gels treated with a silver stain exhibited only three faint bands corresponding to polypeptides with molecular weights of 44,000, 22,000 and 14,000.

Molecular Weight Determination

The molecular weight of vicilin, as estimated by gel filtration, was 158,000±15,000. This compares favorably with a value of 154,000±15,000 determined by the sedimentation diffu-

Figure 1.3. SDS-PAGE electrophoregrams of protein standards (A) and vicilin (B).

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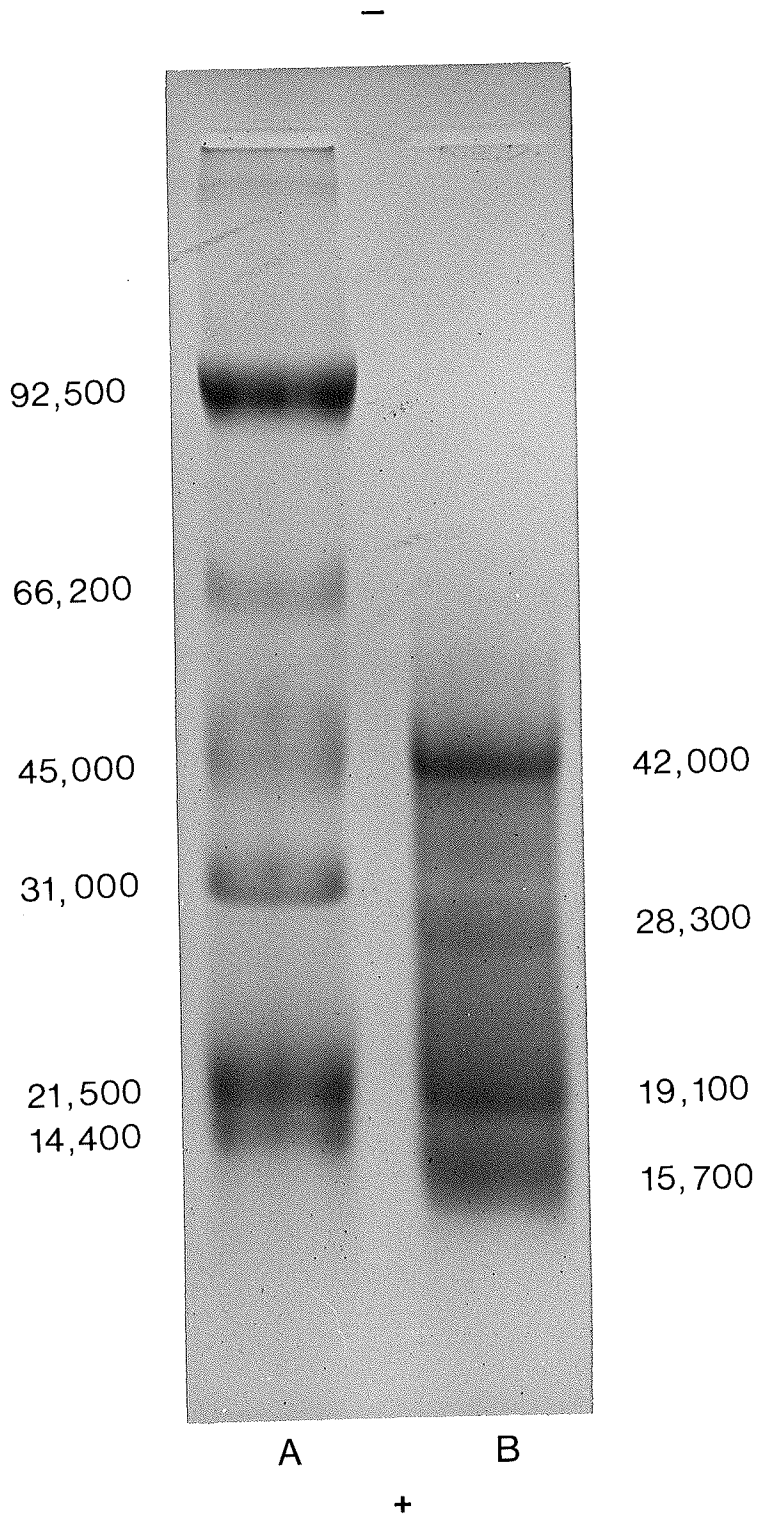
−
92,500
66,200
45,000
31,000
21,500
14,400
42,000
28,300
19,100
15,700
A
B
+

sion ultracentrifugal analysis. Sedimentation patterns for vicilin are given in Figure 1.4. The sequential photographs show symmetrical peaks characteristic of a homogeneous protein fraction. A sedimentation coefficient was calculated to be 6.5S.

For comparison, the molecular weight of vicilin was estimated by using a minimum residue approach based on the amino acid data. Cysteine(1/2 cystine) was selected as the amino acid present in the least amount. Although tryptophan was actually the minimum residue present, the value for this amino acid may be less reliable due to limitations of accurately assessing such reduced tryptophan levels. As given in Table 1.9, the ratio of the level of each amino acid to that of cysteine was determined. This ratio was subsequently converted to a total molecular weight for each amino acid. The molecular weight for a protein with this amino acid composition was calculated to be 56,543 after correction for water loss during peptide bond formation. From the molecular weight values estimated for vicilin by gel filtration and ultracentrifugal analysis, it appeared that each amino acid value should be increased by a factor of three. Such a manipulation resulted in a molecular weight approximation of 164,629 - a value not that dissimilar from the experimental values, considering the basic limitations of this method.

Figure 1.4. Ultracentrifugal patterns of vicilin. Photographs taken at intervals of eight minutes.

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TABLE 1.9. Estimation of vicilin molecular weight from amino acid data. Actual numbers of amino acids present approximated from experimental values for molecular weight.

Amino acid	Moles AA/100 Kg protein	Moles AA/moles minimum residue[1]	AA M.W.	Total M.W.[2]	Total AA present[3]
Glu	95.63	65	147	9555	195
Asp	64.14	44	133	5852	132
Gln[4]	33.23	23	146	3212	69
Asn[4]	33.23	23	132	3036	69
Ser	57.34	39	105	4095	117
Thr	21.83	15	119	1785	45
Arg	55.75	38	174	6612	114
Lys	50.14	34	146	4964	102
His	12.56	8	155	1240	24
Tyr	6.03	4	181	724	12
Trp[5]	0.40	1	204	204	3
Phe	35.24	24	165	3460	72
Pro	31.65	21	115	2415	63
Gly	41.97	28	75	2100	84
Ala	30.83	21	89	1869	63
Val	43.97	30	117	3510	90
Ile	40.95	28	131	3668	84
Leu	72.57	49	131	6419	147
Met	3.00	2	149	298	6
1/2Cys	1.48	1	121	121	3
Total		497		65639[6]	

[1] Cysteine selected as minimum amino acid.

[2] total M.W./AA = Ratio x AA M.W.

[3] Total AA present = ratio x 3. (Experimental vicilin M.W./56,729 $\simeq$ 3.)

[4] Gln/Asn residues are estimated to be present in equivalent amounts. Each value corresponds to one-half the value of the amide nitrogen.

[5] Tryptophan levels low; value estimated to be similar to minimum residue.

[6] Total M.W. = 65,639 - (495 x 18) = 56,729. Compensation for H_2O loss during peptide bond formation.

DISCUSSION

Previous studies indicated that legumin was more easily isolated from a legume globulin fraction than vicilin. However, in this study, using a rather different starting material, it was found that vicilin could be extracted from the PMM isolate by virtue of its differential solubility in a medium of 0.2 M sodium acetate, pH 7.5. Legumin was insoluble in this medium; many other protein contaminants were removed in the original PMM procedure(Murray *et al.*, 1981). Glycoside contaminants were removed chromatographically by gel filtration.

The isolated protein was identified as corresponding to the "classical" vicilin fraction as a result of its sedimentation coefficient(6.5S), molecular weight(154,000) and isoelectric point(5.0). Comparison of these characteristics with literature values for vicilin from *Vicia faba* is somewhat difficult due to isolation problems and lack of definition of the vicilin fraction. However, Wright and Boulter(1973) have reported a 7.1S value and a molecular weight of 150,000 for the main vicilin protein. Although there does not appear to be a specific pI value published for *Vicia faba* vicilin, Danielsson(1950) reported a pI of 5.5 for vicilin from *Pisum sativum* using a moving boundary electrophoretic technique. In contrast, legumin is an 11S protein with a molecular weight in excess of 300,000 and an approximate pI of 4.7(Derbyshire *et al.*, 1976). Therefore, in terms of the established physical characteristics, the protein isolated in this study definitely

appeared to be similar to a vicilin-type globulin.

Separation of the two main fractions, vicilin and legumin, was only one of the problems encountered in this protein procedure. The potential heterogeneity of the vicilin fraction from Vicia faba, (Wright and Boulter, 1972) represented an additional difficulty. In spite of these possible complications, the vicilin species isolated from PMM by solublization in sodium acetate appeared to be homogeneous. Chemical purity of any protein fraction is a difficult parameter to establish; often a definition of purity is a function of the protein under investigation. Basically, two physical attributes of protein molecules are exploited in purity studies - those of particle weight and charge. In terms of the first parameter, the vicilin isolated in this study exhibited symmetrical peaks during both gel filtration and ultracentrifugation. These are not, however, adequate techniques to substantiate homogeneity. For example, visual inspection of Schlerein profiles for Gaussian correctness will not discriminate between a truly monodisperse system and one in which up to 10% of a component differing in sedimentation coefficient by not more than 10% is present(Phelps, 1978). However, the presence of a single distinct band during analytical isoelectric focusing of vicilin originally suspended in a medium of 0.025 M sodium phosphate, pH 6.6, appeared to confirm the presence of a single protein species. Analytical isoelectric focusing is a highly powerful technique for the resolution of proteins due to the concentrating phenomenon that occurs. As such, it is frequently used

to detect the presence of protein species that may only differ by a few amino acid residues(Righetti et al., 1979). Interestingly, multiple banding patterns occurred if the vicilin focused was initially suspended in a low ionic strength medium at pH 8.0(Figure 1.2). Originally, pH 8.0 was chosen to increase solubilization of the protein in the low ionic strength environments required by traditional isoelectric focusing. With further experimentation, it was found that vicilin could be suspended in 0.025 M sodium phosphate, pH 6.6, without loss of protein solubilization. As multiple banding was only present with the pH 8.0 samples, it would appear that this was the result of a pH or a combined pH-ionic strength effect on the protein molecules.

Several authors have noted variations in electrophoretic patterns of what originally appeared to be single protein species. For example, Schlesier et al., (1978) have demonstrated that vicilin molecules isolated from Vicia faba will undergo various types of monomeric associations under changing environmental conditions. Samples of vicilin yielding single bands in polyacrylamide gel electrophoresis can be induced to produce a series of bands with decreasing mobilities correlated with associated protein forms after limited dialysis of the protein against distilled water or storage at room temperature. These authors caution that multiple electrophoretic bands with seed globulins may not necessarily be indicative of multiple protein species. This type of association phenomenon is probably not appropriate to explain the multiple banding

phenomena observed in this study. Although association of vicilin molecules might result in the shielding of negative residues and an increase in the isoelectric point, associated structures in excess of the dimeric form would probably have a tendency to precipitate during the focusing procedure.

In recent studies, it has been suggested that some of the multiple patterns exhibited by proteins during isoelectric focusing may also not represent true molecular heterogeneity (Gianazza and Righetti, 1978). For example, Basset et al., (1983) suggested that polymorphism of soluble rat brain guanylate cyclase observed during isoelectric focusing analysis is a result of various interactions between the protein and carrier ampholytes. Gianazza and Righetti(1978) have suggested that this phenomenon may be more prevalent in proteins containing polyanionic structures such as polyglutamic acid sequences. As such, vicilin could have responded similarly during isoelectric focusing; glutamic and aspartic acid residues comprised approximately 20.0% of the total number of amino acids in this molecule. Initial exposure of vicilin to a low ionic strength alkaline environment may have induced conformational changes such that more negative residues were exposed for potential interaction with carrier ampholytes. Such interaction could have resulted in the observed heterogeneity of the pH 8.0 isoelectric focusing patterns.

As the purpose of isolating vicilin in this study was to obtain a single protein system for further experimentation on noncovalent forces, it was important to establish additional

fundamental structural parameters characteristic of the vicilin molecules. These parameters included amino acid and subunit compositions. Examination of the amino acid data revealed that, similar to other vicilins of Vicia faba, this protein contained high levels of the dicarboxylic acids and reduced amounts of methionine, cysteine and tryptophan(Table 1.7). It is interesting to consider some of the parameters calculated from the amino acid data. The Bigelow hydrophobicity($H\Phi$) of the vicilin isolated in this study was 901.43 cal/AA residue. This is lower than the $H\phi$ values calculated from the two published amino acid compositions for Vicia faba vicilin - 930.13 cal/AA residue(Jackson et al., 1969) and 1054.07 cal/AA residue(Bailey and Boulter, 1972)(Table 1.8). The latter value is more directly comparable as the data of Jackson et al., (1969) did not include a value for proline. Decreased values for tyrosine and proline were the main contributing factors to the lower $H\Phi$ value for the vicilin isolated in this study as compared with that of Bailey and Boulter (1972). Repeated analyses with variations in hydrolysis times did not result in significant changes in these experimental amino acid values. Speculation as to the reality or significance of these differences is hampered by variations in the extraction procedures and the exact nature of the vicilin fractions. Both of the previous procedures used repeated isoelectric precipitation as a separation method. Jackson et al., (1969) observed some contamination with legumin; Bailey and Boulter(1972) described a heterogeneous but legumin-free vici-

lin preparation.

The frequency of charged groups calculated for the three sets of data(Table 1.8) were similar - 0.37(Bailey and Boulter, 1972); 0.38(this study); 0.39(Jackson et al., 1969). Once again these values are not directly comparable. In this situation, amide nitrogen values were not reported in the published data; an estimated value of 1.0% was used for the charge frequency determinations.

In comparison with other proteins, the vicilin isolated here had a lower H^{Φ} and a higher charge frequency than would be originally anticipated. Bigelow(1967), in a survey of more than 150 proteins, suggested that most globular proteins exhibit a narrow range of H^{Φ} from approximatey 1000 to 1200 cal/AA residue and a range of charge frequencies from 0.20 to 0.35. However, Bigelow also commented that some of the plant seed globulins, notably pumpkin globulin and hemp edestin were characterized by low H^{Φ} values of 980 and 950 cal/AA residue, respectively. In order to determine if this might be a trend in other seed globulins, H^{Φ} and charge frequency values were determined from complete amino acid data published for vicilin isolated from a variety of legumes other than Vicia faba. These values are presented in Table 1.10. Considerable variation exists in the average hydrophobicities - i.e. from 740.14 to 932.47 cal/AA residue; however, in all cases the values are much lower than those originally predicted by Bigelow(1967) for globular proteins. Values for charge frequencies also fluctuate widely; the range extends from a low of 0.25 for

TABLE 1.10. Calculated values for HΦ and charge frequencies for vicilins from a variety of legume species. Amide nitrogen levels and initial dicarboxylic amino acid data are included for comparison.

Source of vicilin	HΦ (cal/AA residue)	Charge frequency	Amide N_2 (%)	Combined % of dicarboxylic acids[1]
Arachis hypogaea[2]	740.14	0.27	2.30	33.0
Glycine max[3]	932.47	0.32	1.70	32.5
Lupinus angustifolius[4]	781.94	0.38	2.30	39.2
Lupinus luteus[4]	846.45	0.31	2.50	36.0
Phaseolus vulgaris[5]	916.79	0.25	1.80	30.2

[1] Represent initial values without correction for amide forms.

[2] Dawson(1971)

[3] Koshiyama(1968)

[4] Gerritsen(1956)

[5] Pusztai and Watt(1970)

vicilin from Phaseolus vulgaris to a high of 0.38 for that from Lupinus angustifolius. These differences can be attributed partially to variations in the levels of amide nitrogen, although fluctuations do exist in the percentages of dicarboxylic acids without correction for the amide forms(Table 1.10). Once again, such comparisons must be viewed with some degree of caution due to the variations in extraction techniques and the lack of definition of most vicilin fractions. From analysis of amino acid data, however, it appears that these globulins in general are less hydrophobic, more highly charged structures than was initially speculated.

Subunit analysis of vicilin with SDS-PAGE revealed four subunits having molecular weights of approximately 42,000, 28,000, 19,000 and 16,000. From this it would appear that vicilin is a multimeric protein with more than four subunits; the total weight of the four individual polypeptides, 105,000, is significantly less than the molecular weight estimated for the intact protein. Other studies have also shown the existence of four subunits for vicilin; however, the molecular weight characterization is variable. Bailey and Boulter(1972) identified four subunits for vicilin with molecular weights of 66,000, 60,000, 56,000 and 37,000. The total molecular weight exhibited by these polypeptides, 219,000, was not stoichiometrically compatible with the overall weight of the protein. As all four subunits could not be present in a single protein, it was speculated that vicilin represented a heterogeneous protein fraction resulting from complex polymorphism.

Wright(1973) also reported four subunits for the same protein; however, in this study, molecular weights were given as 55,500, 46,000, 33,300 and 31,500. The total of these values more closely approximated the estimated molecular weight for intact vicilin. Since then, Gatehouse et al., (1981) have speculated that the subunit profile for Vicia faba vicilin may parallel that proposed for Pisum sativum. In this situation, it was suggested that vicilin proteins contained mainly 50,000 molecular weight polypeptides, in addition to a number of smaller polypeptides. As a complicating factor, the subunit composition appeared to vary quantitatively and qualitatively during seed development(Gatehouse et al., 1981).

As previously discussed, it is difficult to compare published results with those of this study due to variations in extraction procedures and protein composition of the analysed fractions. Despite the variability in the characterization of the subunits, all results agree that vicilin is a multimeric protein. In addition, all studies report noncovalent associations among subunit polypeptides as electrophoretic patterns are not influenced by the presence of a reducing agent.

In this study, four subunits were always observed with the Coomassie Brilliant Blue R-250 as a stain; however, only three faint bands corresponding to molecular weights of 44,000, 22,000 and 14,000 were observed with the silver staining procedure. Apparently, only three of the subunits reacted with the silver stain as these molecular weights were similar to three of the polypeptides detected with Coomassie Blue. The

silver procedure was originally attempted as the advent of silver technology for protein detection seems to be a major advance due to its sensitivity; some silver staining techniques are described as 100 times more sensitive than procedures involving Coomassie Blue(Oakley et al., 1980, Switzer et al., 1979). However, the ability of silver nitrate to react stoichiometrically with proteins is a point of controversy and the sensitivity of the method can be drastically different for individual proteins(Merril et al., 1982, Guevara et al., 1982). As standard proteins were highly stained during this electrophoretic analysis, it would appear that the difficulty results from the characteristics of the vicilin subunits rather than from the actual staining procedure. Many suggestions have been put forward to explain the variability of protein response in silver staining; however,the exact mechanism for the basic staining procedure is not understood(Schleicher and Watterson, 1983). Proposed target sites for silver cations have included the amino acids phosphoserine and phosphothreonine(Satoh and Busch, 1981), or carboxyl and sulfhydryl groups(Olert et al., 1979). Although the vicilin subunits contain few sulfhydryl groups and their degree of phosphorylation is unknown but likely minimal, the presence of a large number of free carboxyl groups seemed to make them potential candidates for the technique. However, Schleicher and Watterson(1983), in their studies with the proteins calmodulin and troponin C, have recently demonstrated that the state of phosphorylation, the acidic nature of the protein and

the presence of sulfhydryl groups are not the only criteria for positive staining. As with other proteins, slight variations in the actual silver staining technique might make it more suitable for the vicilin subunits. At this time, these polypeptides can be categorized with those proteins for which a silver staining procedure is not an appropriate detection method.

In summary, a relatively gentle method has been developed to isolate an apparently homogeneous vicilin fraction from Vicia faba. As the procedure involved minimal protein manipulation, subsequent studies on specific protein conformational properties should not reflect characteristics that have been developed as a function of the extraction technique. The vicilin isolated was a multimer formed by the noncovalent combination of four possible subunits. In terms of physical parameters, the protein was characterized by an S value of 6.5, a molecular weight of 154,000 and a pI of 5.0.

CHAPTER TWO

THE SIGNIFICANCE OF ELECTROSTATIC PARAMETERS TO THE STABILITY OF CYTOCHROME C AND VICILIN

INTRODUCTION

The unique three-dimensional configuration of globular proteins is generally attributed to a complex set of noncovalent intramolecular interactions between specific amino acid residues(von Hippel and Wong, 1965). The structural forces implicated in the stabilization of globular proteins include hydrogen bonds, hydrophobic associations, short and long range van der Waals interactions and electrostatic attractions and repulsions. Although the contributions of all types of interactions are important to the structure of a protein, it is informative to attempt to isolate the relative influence of a single stabilizing force. Electrostatic interactions are an attractive "target" as these occur primarily between amino acid side-chains distributed over the protein surface. Therefore, in principle, it is possible to selectively manipulate the electrostatic contribution to protein conformation by suitable modification of charged residues, without directly affecting the forces that operate in the interior of the molecule(Schejter et al., 1979). Some appreciation for the contribution of electrostatic interactions to protein stability can be derived if protein conformational properties are followed through various degrees of surface amino acid modification.

Several basic considerations exist in this approach to the investigation of the significance of electrostatic interactions to protein structural stability. These include:

1. choice of a protein study system;

2. definition of a suitable technique to assess conformational changes;

3. identification of the amino acid residues to be modified;

4. determination of the specific method of modification.

With respect to the first consideration, the study system selected for this investigation was the storage protein vicilin, isolated from the legume Vicia faba. In general, little is known about the contribution of noncovalent forces to the structural stability of the seed globulins. Electrostatic interactions are especially interesting due to the capacity of vicilin to be a highly charged protein under appropriate conditions.

Fundamental to this study was the selection of an adequate technique to monitor conformational changes in vicilin with controlled modification of specific amino acid residues. Numerous methods have been used to follow protein structural changes after chemical modification, these have included the assessment of variations in spectra determined by circular dichroism(CD) or optical rotatary dispersion(ORD), patterns of intrinsic fluorescence, sedimentation coefficients, electrophoretic profiles, elution patterns during gel filtration and enzyme activity. The choice of the method is partially determined by the characteristics of the protein under consideration; however, some methods, such as CD, appear to be superior due to their overall sensitivity. In this study, an alternate approach was considered - that of assessing the thermal stability of a protein by differential scanning calorimetry(DSC). The use of the thermal characteristics of a protein, as deter-

mined by DSC analysis, to detect induced alterations in protein structure has been well documented in a number of studies(Biltonen and Freire, 1978; Privalov and Pfeil, 1979; Biliaderis, 1983). However, these alterations have been a function of environmental manipulation, ligand-binding or various types of intermolecular associations rather than of gradual selective modification of specific amino acid residues.

To assess the suitability of DSC as this type of conformational probe, an initial study was performed using an established method for chemical modification of the protein cytochrome c. Aviram et al. (1981) developed a procedure for the stepwise modification of lysine residues in horse heart cytochrome c with maleic anhydride as the modifying reagent. Circular dichroism was used to monitor protein conformational changes. In this study, the basic modification technique of Aviram et al. (1981) was repeated; however, DSC was substituted for CD in the analysis of protein conformational changes. Preliminary results demonstrated that DSC could be an informative technique in the assessment of structural changes.

With the identification of DSC as a viable technique for monitoring conformational variations, it seemed feasible to extend a similar chemical modification approach to vicilin, the main protein under investigation. This was attractive for several reasons. First, comparative observations could be made in terms of the response of two very different proteins to electrostatic manipulations. Structural considerations in vicilin, a large multimeric globular protein, could be com-

pared with those in cytochrome c, a small single domain cooperative unit. Secondly, the selection of lysine residues as a modification target is an appropriate approach. To substantiate this, it must be recognized initially that charged residues are usually found protruding from the surface of a protein. This general observation, originally initiated by Kendrew(1963) in the examination of the crystal structure of myoglobin, has been further supported by crystallographic studies on a number of proteins(Glazer, 1976). Lysine, in particular, seems to be mainly a surface residue as X-ray diffraction data and NMR studies in solution show considerable freedom of motion in lysine side-chains(Kendrew, 1963; Wyckoff et al., 1970; Glushko et al., 1972). This observation is not characteristic of restricted internal residues. As a result, the surface exposure of the lysine residues should theoretically enable the entire lysine complement of a protein to be manipulated by reaction with a modifying agent. The attractiveness of the location of lysine is enhanced by the existence of a specific modification technique for this residue involving maleic anhydride. The ε-amino group of lysine will react with maleic anhydride resulting in the substitution of a negative moiety for the positive component within the protein structure. The advantages of maleic anhydride as a modifying agent have been reviewed by Butler et al. (1969); one especially noteworthy property is the complete stability of the maleylated residues at neutral or alkaline pH. Although maleic anhydride is a highly specific reagent for amino groups, it

does not exhibit absolute specificity as O-maleic derivatives of serine, threonine and tyrosine residues may be formed(King and Perham, 1971). In addition, maleic anhydride may react with exposed thiol groups to form a stable complex. Although these side-chain reactions are detrimental attributes, these effects may be minimized with manipulation of experimental conditions. For example, O-maleyl tyrosine spontaneously hydrolyzes at an alkaline pH and O-maleyl derivatives of serine and threonine appear to be formed in reduced amounts only in the presence of large excesses of maleic anhydride(King and Perham, 1971). Therefore, if a protein is characterized by reduced or absent thiol groups and if modification is performed in an alkaline medium with excesses of reagent kept to a minimum, maleic anhydride becomes "operationally" specific for the amino side-chains of lysine(Glazer, 1976).

As a result of all these considerations, vicilin from Vicia faba and horse heart cytochrome c were modified in a step-wise procedure using the interaction of lysine residues with maleic anhydride. The significance of electrostatic parameters to protein conformation were examined by monitoring structural changes with DSC. Additional conformational information was derived from the measurement of surface hydrophobicity(So) values for vicilin with progressive modification. Most hydrophobic amino acids, in contrast to charged groups, are buried in the interior of native proteins; however, some of these residues are exposed at the molecular surface. This

group of exposed hydrophobic residues represents the "effective" hydrophobicity of a protein as these are the amino acids potentially capable of participating in intermolecular hydrophobic associations(Keshavarz and Nakai, 1979). As a protein destabilizes in response to a conformational stress such as increased electrostatic repulsion, the value of the surface or effective hydrophobicity should change. Measurement of So originally involved hydrophobic chromatography or hydrophobic partition(Keshavarz and Nakai, 1979); this approach has been considerably simplified by the development of a fluorescence procedure by Kato and Nakai(1980). Cis-parinaric acid, a natural polyene fatty acid, readily fluoresces on association with hydrophobic amino acids. Relative fluorescence intensities can be used to determine an So value; an index which is useful in the assessment of changes in the exposure of hydrophobic residues with conformational variations. Unfortunately this procedure is inappropriate for proteins such as cytochrome c due to the quenching nature of the iron moiety.

MATERIALS AND METHODS

Chemical Modification of Cytochrome c

Stepwise maleylation of lysine residues in horse heart cytochrome c(Sigma Chem. Co.,type III) was achieved according to the methods of Pettigrew et al. (1976) and Aviram et al. (1981). Three mL aliquots of 0.5 mM cytochrome c in 0.1 M sodium bicarbonate, pH 8.0 at 0°C were exposed to various levels of 1.0 M maleic anhydride(Sigma Chem. Co.) in dioxane (Table 2.1). In those labelling steps requiring multiple additions, 60 μL aliquots of maleic anhydride were added to single cytochrome c samples at 5 min intervals. The pH was maintained at 8.0 by the addition of 1 M sodium hydroxide. Excess maleic anhydride was removed by gel filtration using a Pharmacia K16-40 column containing Sephadex-G25 equilibrated with 0.025 M phosphate buffer, pH 7.5. Each desalted preparation was used immediately or else lyophilized.

The mean number of modified lysines in each preparation containing a mixture of derivatives was estimated by reaction with trinitrobenzene sulfonate(TNBS, Eastman Kodak Chem. Co.), according to the method of Habeeb(1966). One mL of 4% sodium bicarbonate, pH 8.5 and 1 mL of 0.1% TNBS were added to 1 mL of each protein solution(0.6 - 1.0 mg mL^{-1}). The solutions were allowed to react at 40°C; then 1 mL of 10% sodium dodecyl sulfate(SDS) was added to each to solubilize the protein and to prevent its precipitation with the subsequent addition of 0.5 mL of 1 N hydrochloric acid. The absorbances of the solutions were read at 400 nm against a blank in which

TABLE 2.1. Stepwise maleylation of lysine residues in horse heart cytochrome c.

Sample number	Volume of 1.0 M maleic anhydride (μL) per addition	Number of additions
1	6	1
2	15	1
3	30	1
4	45	1
5	60	1
6	60	2
7	60	4
8	60	12

the protein solution was replaced by 1 mL of water.

Calculation of the Number of Labelled Lysines

Two standard curves were used for estimating the number of labelled lysines in each cytochrome c preparation. One curve was established by plotting absorbance at 550 nm as a function of concentration for unlabelled cytochrome c samples ranging from 0.03 to 0.65 mg mL^{-1}. The second curve involved a similar relationship of absorbance as a function of concentration; however, the absorbance was determined at 400 nm using a series of unlabelled cytochrome c samples that had been assayed for lysine. Using these curves, two protein concentrations were determined for each labelled cytochrome c sample. The "actual" cytochrome c concentration was determined from the 550 nm curve; an "apparent" cytochrome c concentration was determined from the lysine plot. The observed "reduction" in cytochrome c concentration from each labelled sample at 550 nm to the corresponding lysine-assayed sample was proportional to the number of lysine residues labelled. As a result, the following relationship was used to determine the number of labelled lysines in each treated sample:

$$\% \text{ of labelled lysines} = ((A-B)/A)x100$$

A = "Actual" cytochrome c conc.(mg mL^{-1}) in labelled sample(550 nm)

B = "Apparent" cytochrome c conc.(mg mL^{-1})in labelled sample(lysine assay)

DSC Analyses of Labelled Cytochrome c

Conformational changes in cytochrome c, as a result of different degrees of lysine modification, were followed by monitoring the thermal properties of the labelled molecules. Thermal behavior was assessed by means of a DuPont 990 Thermal Analyzer with a 910 Differental Scanning Calorimeter cell base. In preparation for analysis by DSC, each lyophilized cytochrome c sample was dissolved in 0.025 M phosphate buffer, pH 7.5 to an approximate concentration of 10 mg mL^{-1}. Ten μL samples of labelled cytochrome c were then sealed hermetically in DuPont aluminum pans coated on the interior with an inert polymer. Sample weight was determined with a Cahn Gram Electrobalance by first weighing the empty pan and then the filled pan after it had been sealed. The reference material was a sealed pan containing an appropriate weight of sand. Silicon heat-sink compound(Dow Corning Corp.) was used to improve contact between pans and thermocouple detectors.

Thermograms, plots of heat flow as a function of temperature, were determined at a heating rate of 10°C min^{-1} over a temperature range of 25 to 150°C, with sensitivities of 0.01 or 0.02 mcal s^{-1} in^{-1}. During each run, the DSC cell was continuously flushed with nitrogen at a flow rate of 40 - 45 mL min^{-1}. On completion of the DSC scan, the sample pan was punctured, dried for 16 h and reweighed in order to determine the sample cytochrome c concentration. Due to the positioning of the sample and reference pans, an endothermic heat flow was recorded as a peak in the downward direction. To

obtain the area of this endotherm, a baseline was constructed as a straight line from the beginning to the end of the endotherm. The area of the enclosed peak was then measured with a planimeter. The following equation was used to calculate the enthalpy of denaturation(ΔH) from the peak area:

$$\Delta H = (A/W)(60xTxExS)$$

ΔH = Enthalpy(mcal mg^{-1})

A = Area(in^2)

W = Weight of protein sample(mg)

T = Time base(min in^{-1})

E = Calibration coefficient(dimensionless, adjusted to 1.00)

S = Sensitivity(mcal s^{-1} in^{-1})

For this study, the denaturation temperature(Td) was defined as the temperature corresponding to maximum heat flow into the sample - i.e. the "peak temperature". All Td values were obtained using a constant heating rate of 10°C min^{-1}. The half-band width(1/2bw), reflecting variations in the width of the endotherm, was calculated as a ratio of the endotherm area to the height of the enclosed triangle. All values(ΔH, Td, 1/2bw) were determined for at least quadruplicate samples. The mean values and standard deviations of the mean are reported for each.

Chemical Modification of Vicilin

The stepwise modification of lysine residues in vicilin was achieved using a method similar to that for cytochrome c,

with some exceptions. Vicilin in 0.2 M sodium acetate, pH 7.5, was dialysed initially against 0.2 M sodium bicarbonate, pH 8.0. The dialysed protein was then concentrated to approximately 6 mg mL^{-1} using a 200 mL Amicon stirred ultrafiltration cell fitted with a PM10 Diaflo ultrafiltration membrane. Six mL aliquots of concentrated vicilin were exposed to various levels of 0.1 M maleic anhydride in dioxane(Table 2.2). After each addition of maleic anhydride the pH was adjusted to 8.0 with 1 M sodium hydroxide. Following the removal of excess maleic anhydride, each sample was assayed for the number of labelled lysines using the method described for cytochrome c. Protein concentrations of the original samples were determined by the method of Lowry et al. (1951) using bovine serum albumin(Sigma Chem. Co.) as a standard.

DSC Analyses of Labelled Vicilin

Conformational changes in vicilin with different degrees of lysine modification were followed by monitoring the thermal properties of the labelled molecules using DSC as previously described. In preparation for DSC analysis, each protein sample was concentrated to approximately 10 mg mL^{-1} using a Minicon-B15 Macrosolute concentrator with a 15,000 M.W. cut-off. Protein levels in the concentrated samples were determined by the method of Lowry et al. (1951). The ΔH, Td and 1/2bw values were determined for at least quadruplicate samples; mean values and standard deviation of the mean are reported for each.

TABLE 2.2. Stepwise maleylation of lysine residues in vicilin.

Sample number	Volume of 0.1 M maleic anhydride (μL) per addition	Number of additions
1	36	1
2	72	1
3	72	2
4	72	3
5	72	4
6	72	5
7	72	8
8	72	9
9	72	12

Surface Hydrophobicity Determinations for Labelled Vicilin

Surface hydrophobicity(So) of the modified vicilin samples was assessed using the method of Kato and Nakai(1980) with some modifications. Cis-parinaric acid(Calbiochem-Behring Corp.) was used as a fluorescent probe. An ethanolic solution of cis-parinaric acid and butylated hydroxytoluene, equimolar at 3.6 mM, was purged with nitrogen and stored tightly capped and protected from light at -40°C. Aliquots of each original vicilin sample were diluted with 0.025 M phosphate buffer, pH 7.5, to give five samples ranging in concentration from approximately 0.04 to 0.4 mg mL^{-1}. Samples of each protein dilution(2 mL) were excited at 325 nm and the relative fluorescence intensity was measured at 420 nm in an Aminco-Bowman fluorescence spectrophotometer model No. 4-8202 using a slit width of 0.5 mm. The fluorescence measurements were then repeated after the addition of 10 μL of cis-parinaric acid to each sample. The method was standardized by initially adjusting the relative fluorescence to 5.0/10 full scale when 10 μL of cis-parinaric acid were added to 2 mL of decane.

The net fluorescence intensity at each protein concentration was determined as the difference between the fluorescence intensity of the cis-parinaric acid-protein conjuates and the intrinsic fluorescence of the protein in the absence of the probe. The initial slope of a plot of fluorescence intensity as a function of protein concentration was considered to be an index of surface hydrophobicity. This initial slope was determined by linear regression analysis. All protein concen-

trations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. All values of So were determined for a minimum of quadruplicate samples; mean values and standard deviations of the mean are reported for each.

Statistical Analysis

Due to the difficulty in duplicating exact levels of lysine modification, the data were grouped into closely related percentage levels with a maximum standard deviation of four percentage points. Differences in thermal parameters(ΔH, Td, 1/2bw) and So values for the various percentage levels of lysine modification were evaluated by a multiple T-test. Correlations between pairs of variables(ΔH and Td; Td and So), were assessed by standard linear regression analysis using individual data points.

RESULTS

DSC Analyses

Differential scanning calorimetry was used in this study to thermally induce unfolding of the two proteins, vicilin and cytochrome c, which had been subjected to various degrees of lysine modification. In general, as a protein undergoes transition from a native to a denatured state, the change in heat capacity plotted as a function of temperature results in an endothermic peak reflecting the interconversion of the two forms. Both the temperature corresponding to the largest change in heat capacity, or denaturation temperature(Td), and the heat required to effect this conversion reflect the stability and conformation of the initial state of the protein. Thermal parameters such as Td and the enthalpy of denaturation(ΔH) are characteristic of a specific unfolding process. As a result, any changes in these thermal parameters should reflect differences in the structure of the original or native state of the protein(Chlebowski and Williams, 1983) The effect of step-wise maleylation on the thermal properties of cytochrome c and vicilin is given in Tables 2.3 and 2.4. For both proteins, an increase in electrostatic perturbation resulted in changes in molecular thermal properties.

A. Cytochrome c

There was a progressive decrease in the thermal stability of cytochrome c, as indicated by a decrease in the Td value, with a corresponding increase in the degree of lysine modifi-

TABLE 2.3. Thermal properties (ΔH, Td, ½bw) of cytochrome c with varying degrees of lysine modification.

Labelled lysines (%)	Td (°C)	ΔH (cal g^{-1})	½bw (°C)
0(Control)	82.0±0.0	2.53±0.26[a]	5.3±0.2
9.0±0.5	78.8±1.3	2.18±0.25[a,b]	7.9±0.7[a,b,c]
17.2±0.0	75.6±1.6	2.17±0.33[a,b]	10.0±1.3[d,e]
21.5±0.9	71.7±0.4	2.20±0.20[a,b]	9.0±0.9[a,b,c,d]
27.7±1.7	70.7±0.4	2.30±0.37[a,b]	8.3±0.9[a,b,c,d]
51.7±1.1	67.9±1.1[a]	2.05±0.08[b]	7.6±0.3[b]
77.5±3.1	66.4±1.5[a,b]	1.09±0.58[c]	7.7±0.4[b]
96.4±2.5	66.7±0.6[b]	1.18±0.42[c]	8.7±1.2[c,e]

[1] Column values followed by the same letter do not differ significantly ($P \leq 0.05$).

TABLE 2.4. Thermal properties (ΔH, Td, ½bw) and surface hydrophobicities (So) of vicilin with varying degrees of lysine modification.

Labelled lysines (%)	Td (°C)	ΔH (cal g^{-1})	½bw (°C)	So
0(control)	83.4±1.7	4.30±1.9^{a,b,c}	11.3±0.7	212±24^{a,b,c,d,e}
11.0±4.0	80.8±1.6^{a,b}	4.93±1.13^{a}	13.7±1.4^{a,b}	212±26^{a,b,c,d,e}
31.2±4.0	81.0±1.4^{a}	4.23±0.74^{a}	13.1±0.9^{a}	184±9^{a}
43.9±1.6	80.4±1.6^{a,b}	4.67±0.88^{a}	13.3±0.8^{a}	167±9^{b}
51.9±1.0	80.9±0.9^{a}	3.79±0.82^{a,b}	13.1±1.2^{a}	194±16^{a,f}
63.0±1.6	78.6±3.2^{a,b,c,d}	3.90±1.22^{a,b,c,d}	14.8±0.8^{b}	213±17^{b,f,g,h}
68.1±0.5	78.7±3.2^{b,e}	2.78±0.94^{b,c,d}	14.3±1.2^{a,b}	221±21^{c,g,i}
76.4±1.5	76.9±2.4^{c,e}	2.84±0.63^{c}	14.4±1.8^{a,b}	244±22^{d,i,j}
82.2±2.5	75.1±0.9^{d}	2.16±0.36^{d}	14.3±1.2^{a,b}	238±39^{e,h,i,j}
93.8±1.9	74.4±1.3	1.79±0.42^{d}	15.4±0.8^{b}	294±76^{j}

[1] Column values followed by the same letter do not differ significantly ($P \leq 0.05$).

cation(Table 2.3, Figure 2.1). Initially, with only 27.7% of the lysine residues maleylated, the Td values decreased significantly from 82.0 to 72.7°C, a total change of 9.3°C. Although this seems to indicate a definite conformational response to modification, the enthalpy of denaturation(ΔH) over the same range of maleylation was unchanged(Table 2.3, Figure 2.2). In contrast, as the degree of lysine modification increased from 27.7 to 96.4% there was only a 4.0°C decrease in Td(from 70.7 to 66.7°C). The corresponding ΔH values remained constant to 51.7% of modification; however, there was a significant decrease to 1.09 cal g^{-1} at 77.5% maleylation reflecting considerable protein destabilization. This negative influence on ΔH and Td values was further evidenced by their significant correlation ($r = 0.5397$, $P \leq 0.0007$), described by the linear relationship: $\Delta H = 0.0713\ Td - 3.18$.

In addition to ΔH and Td parameters, the width of the denaturation thermogram may also reflect conformational changes in the original protein molecules. A relative increase in half-band width(1/2bw) may result from a decrease in "intramolecular cooperativity" or cooperative hydrogen bonding and hydrophobic interactions within a protein molecule(Privalov et al., 1971). For cytochrome c, there was a significant increase in 1/2bw with 9.0% lysine modification suggesting a decrease in intramolecular cooperativity. With further maleylation, the 1/2bw was relatively constant with no apparent trend towards an overall increase or decrease.

Figure 2.1. The effect of lysine modification on the denaturation temperature (Td) of vicilin and cytochrome c.

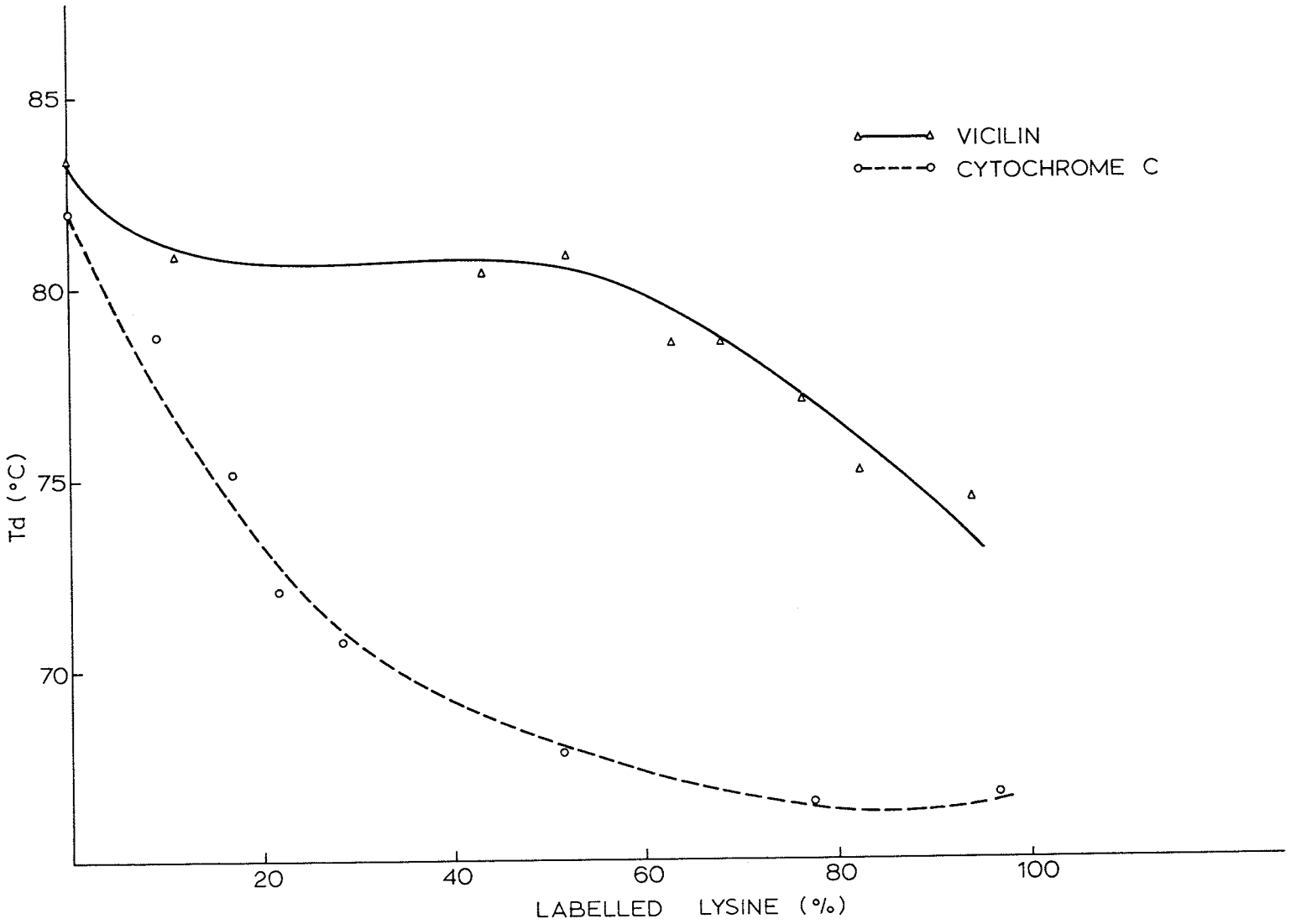
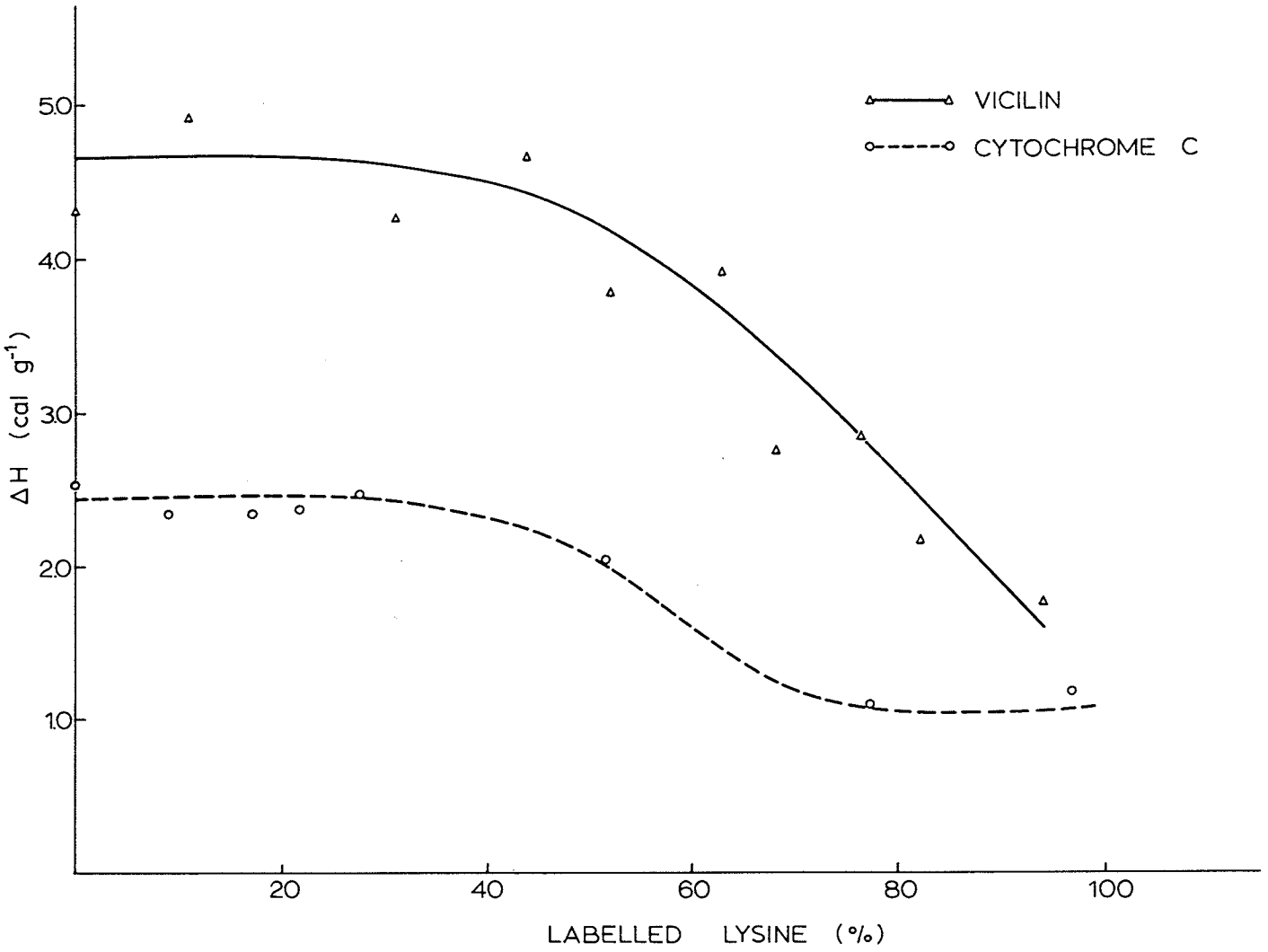

85
80
75
70
Td (°C)
20
40
60
80
100
LABELLED LYSINE (%)
VICILIN
CYTOCHROME C

Figure 2.2. The effect of lysine modification on the denaturation enthalpy (ΔH) of vicilin and cytochrome c.

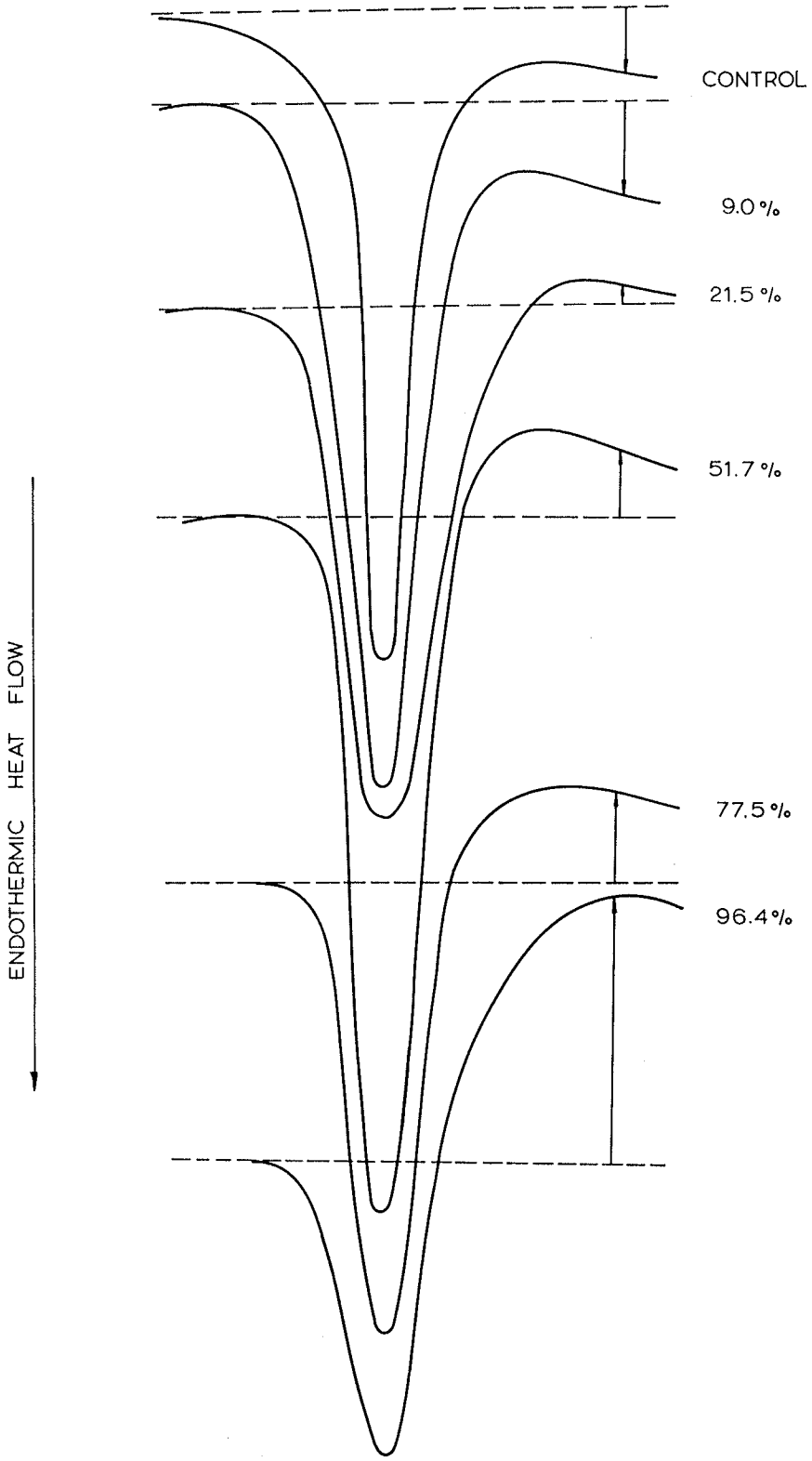

VICILIN
CYTOCHROME C
5.0
4.0
3.0
2.0
1.0
ΔH (cal g⁻¹)
20
40
60
80
100
LABELLED LYSINE (%)

Another consideration in the shape of a thermogram, in addition to the 1/2bw , is the relative position of the pre- and post-denaturation baselines. For a number of globular proteins, including cytochrome c, Privalov and Pfeil(1979) indicated that the level of the post-denaturation baseline reflected an increase in the heat capacity(Cp) of the native protein over the denatured state. An examination of the representative thermograms given in Figure 2.3 for cytochrome c with various levels of lysine modification revealed that this situation existed only for the control and the 9.0% modified cytochromes(i.e. a lower post-denaturation baseline is indicative of an increase in Cp or a positive ΔCp). As the degree of maleylation increased above 9.0%, the expected increase in Cp for the denatured state was replaced by an exothermic deviation from the original pre-denaturation baseline. The magnitude of the observed exotherm progressively increased with the degree of modification(Figure 2.3). This atypical exothermic phenomenon may have resulted from intermolecular aggregation of denatured cytochrome c molecules.

B. Vicilin

When vicilin was subjected to similar electrostatic manipulation, there was no significant change in Td up to 63.0% lysine modification(Table 2.4; Figure 2.1). Above this point, there was a gradual decrease in Td to 93.4% maleylation, resulting in a total decrease of 9°C in Td with nearly complete lysine modification. As shown in Figure 2.1, this trend differed from the biphasic cytochrome response in which

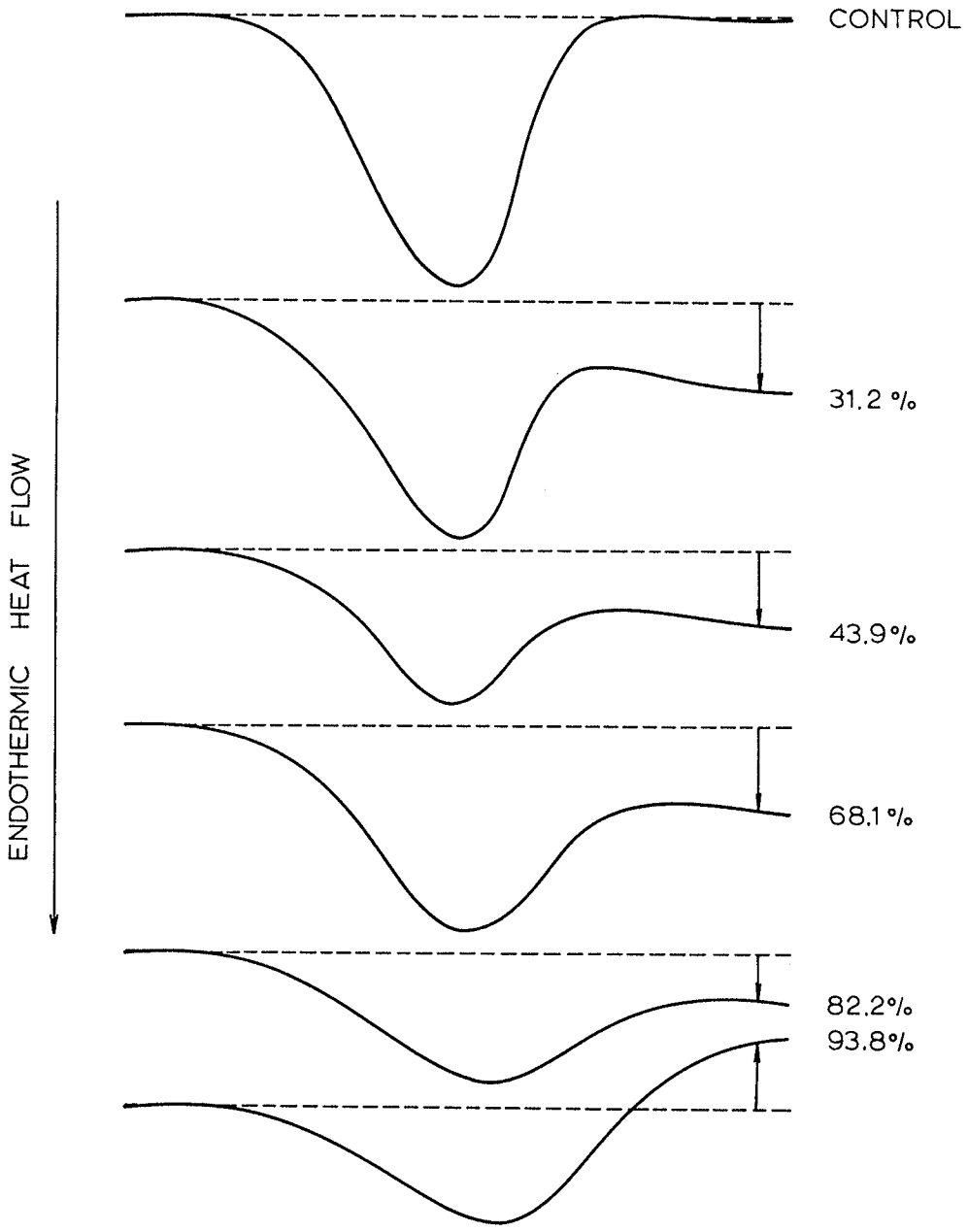
Figure 2.3. Representative endotherms for cytochrome c with various degrees of lysine modification. Percentages represent number of lysines labelled with respect to total number of lysines present. Arrows indicate changes in heat capacity from pre-denaturation to post-denaturation states. Thermogram scale was 0.02 mcal sec^{-1} in $^{-1}$.


CONTROL
9.0 %
21.5 %
51.7 %
77.5 %
96.4 %
ENDOTHERMIC HEAT FLOW

initial maleylation appeared to precipitate a definite molecular response, followed by a relative stabilization in Td values. However, the ΔH values for vicilin seemed to follow a similar pattern to those for cytochrome c. There was no significant change in ΔH to 63.0% modification(Table 2.4). As the degree of maleylation increased to 93.4%, the ΔH value decreased significantly to 1.79 cal g^{-1} reflecting apparent protein destabilization. As with cytochrome c, the ΔH and Td values for vicilin are positively correlated($r = 0.8293$; $P \leq 0.0001$). Their linear relationship is described by: $\Delta H = 0.345\ Td - 24.4$.

Representative thermograms for vicilin(Figure 2.4) exhibited several differences in comparison with those shown for cytochrome c(Figure 2.3). In contrast to cytochrome c, there was a progressive change in the width of the thermograms as reflected by the increase in 1/2bw values(Table 2.4). This observation may be indicative of differences in the cooperativity within a multi-subunit protein with decreased stabilization as compared with a simpler monomeric protein. In addition, there was only a slight exothermic shoulder following the denaturation endotherm for vicilin that had 93.8% lysine modification. At all other maleylation levels, the thermograms were characterized by a post-endotherm baseline that reflected an increase in the Cp of the denatured protein. This trend differed from that observed for cytochrome c, where post-denaturation exotherms were observed at modification levels of 21.5% and higher. The magnitude of the exotherms progressively

Figure 2.4. Representative endotherms for vicilin with various degrees of lysine modification. Percentages represent number of lysines labelled with respect to total number of lysines present. Arrows indicate changes in heat capacity from pre-denaturation to post-denaturation states. Thermogram scale was 0.02 mcal sec^{-1} in $^{-1}$.


CONTROL
31.2 %
43.9 %
68.1 %
82.2%
93.8%
ENDOTHERMIC HEAT FLOW

increased with the level of maleylation.

All data for both vicilin and cytochrome c have been expressed in terms of total lysine residues modified. Another perspective is to consider these characteristics with respect to the entire molecule, as reflected by the number of amino acids in each protein. As a result, Figures 2.5 and 2.6 were constructed to show Td and ΔH values as a function of the percentage of modified lysines in relation to the total number of amino acids present. Horse heart cytochrome c contains 104 residues with 19 being represented by lysine(Margoliash et al., 1961). As such, a maximum of 18.3% of the total amino acids could be labeled with maleic anhydride. Comparable values for vicilin were estimated from the amino acid data presented in Chapter 1. Using the minimum residue approach, the total number of amino acid residues in vicilin was approximated to be 1494(Table 1.9). Of this total, 102 residues were estimated to be lysine. As such, a maximum of 6.8% of the total amino acids in vicilin could be modified in the maleic anhydride labelling procedure.

Using this approach, the curves presented in Figures 2.1 and 2.2 for vicilin were compressed in relation to those for cytochrome c. The overall trend for a decrease in Td with a progressive increase in lysine modification was similar in the two molecules(Figure 2.5). The stable Td values in the initial stages of labelling were still apparent for vicilin; however, a comparable plateau region did not exist in the curve for cytochrome c. With this method of comparison, the significant

Figure 2.5. The effect of lysine modification on the denaturation temperature (Td) of vicilin and cytochrome c. The number of labelled lysines is expressed as a percentage of the total number of amino acids present in each protein.

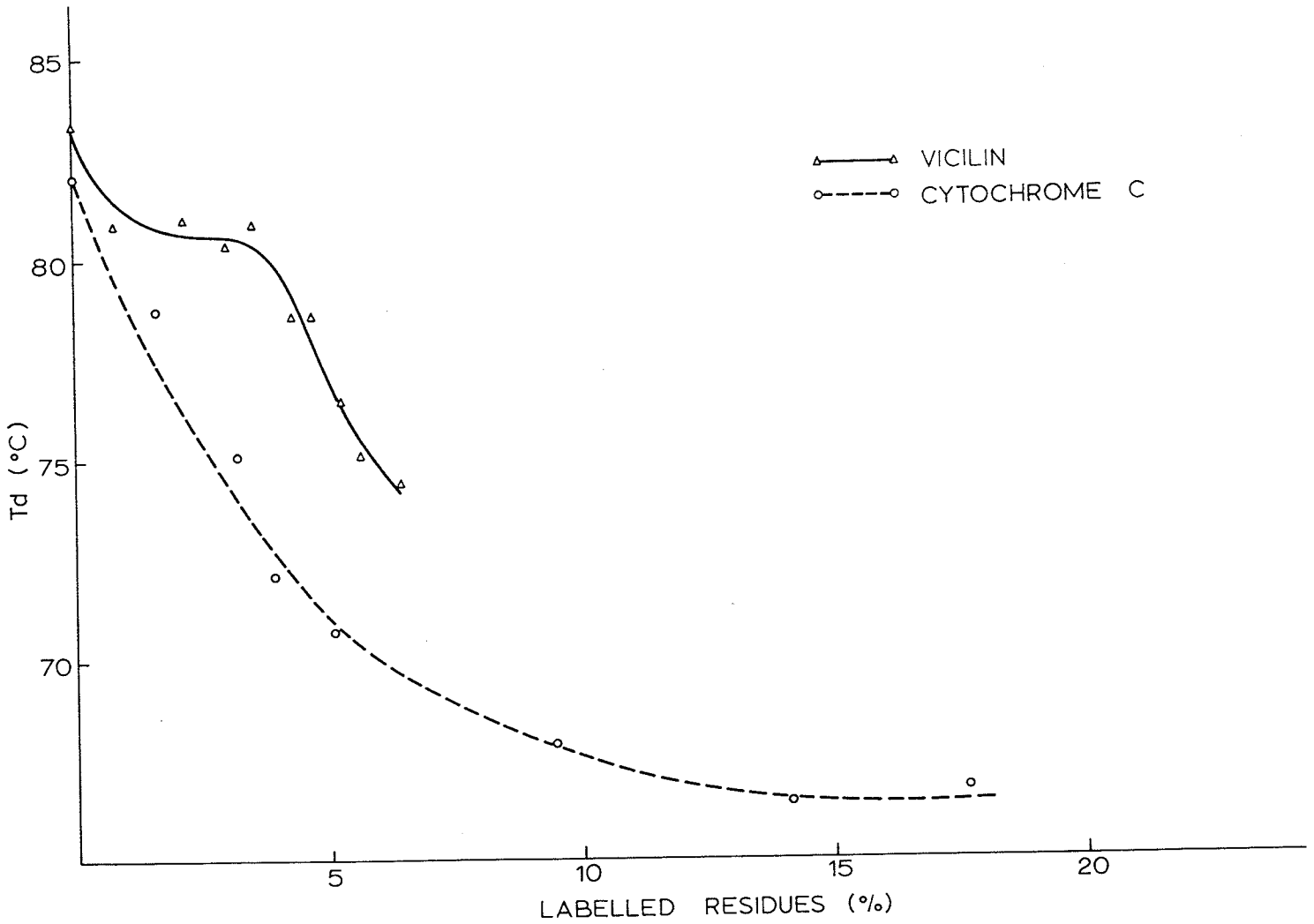
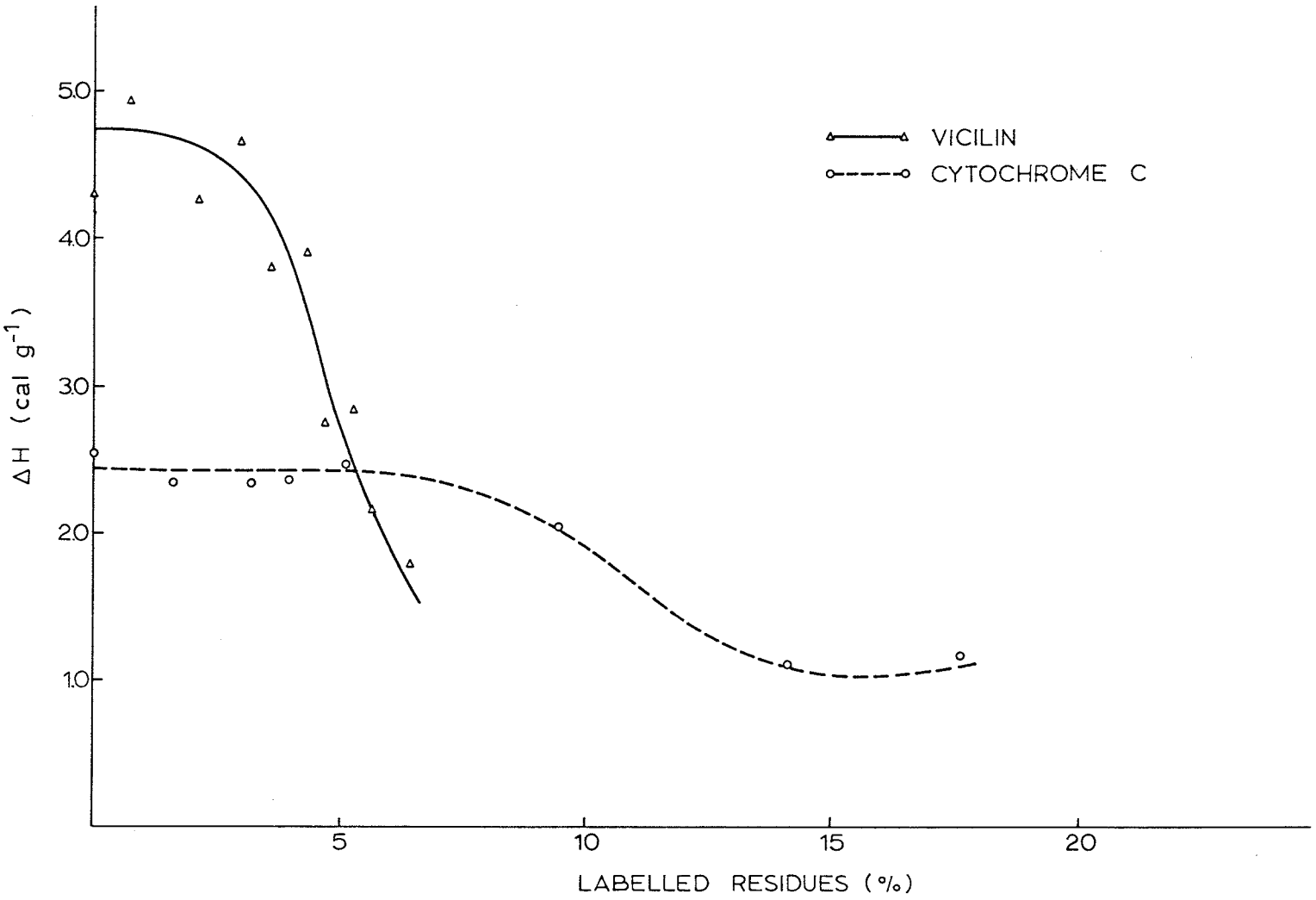

85
80
75
70
Td (°C)
5
10
15
20
LABELLED RESIDUES (%)
VICILIN
CYTOCHROME C

Figure 2.6. The effect of lysine modification on the denaturation enthalpy (ΔH) of vicilin and cytochrome c. The number of labelled lysines is expressed as a percentage of the total number of amino acids present in each protein.


5.0
4.0
3.0
2.0
1.0
ΔH (cal g⁻¹)
5
10
15
20
LABELLED RESIDUES (%)
VICILIN
CYTOCHROME C

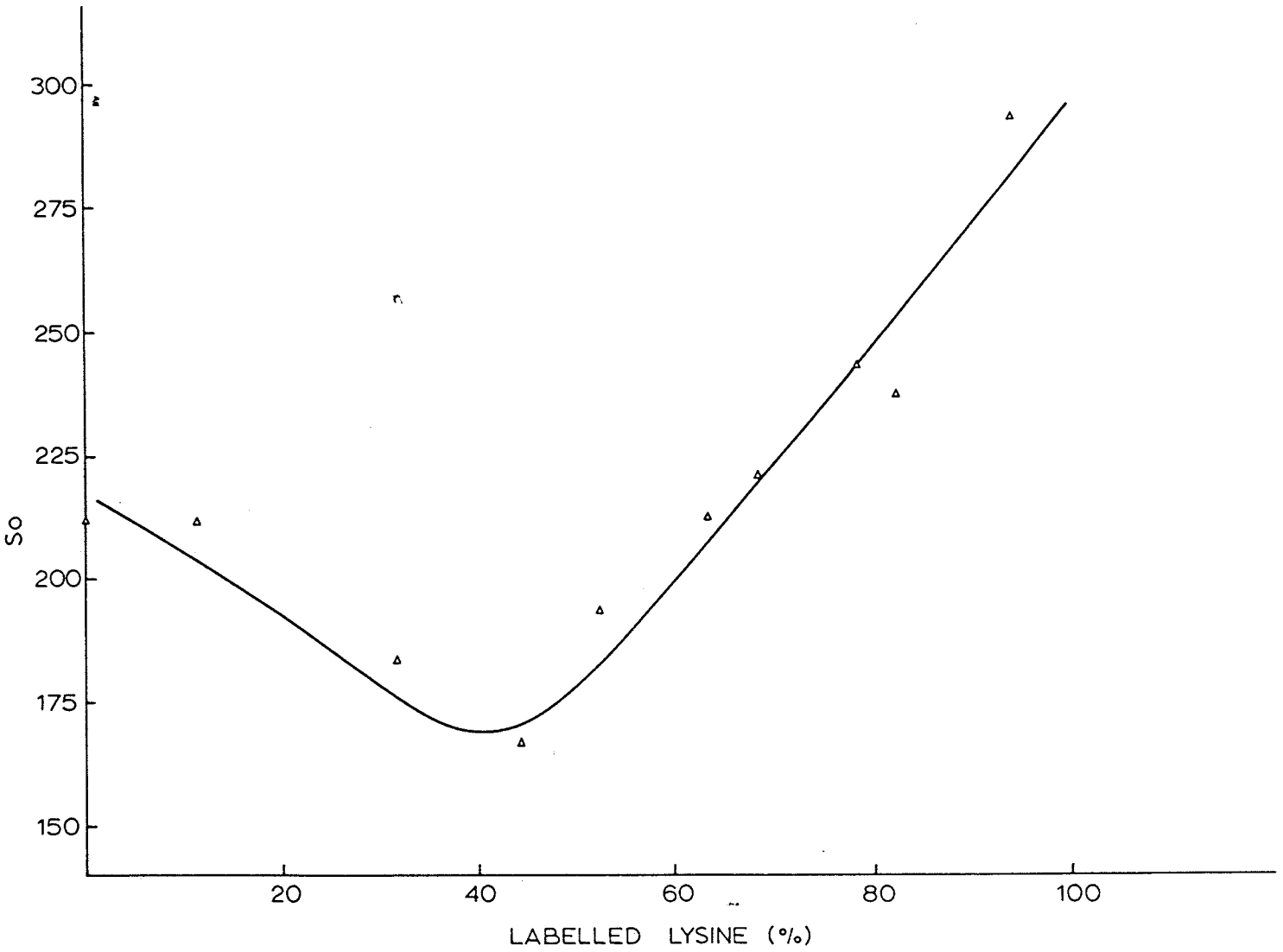
reduction in ΔH values for vicilin from the control proteins to those that have the maximum modification is emphasized relative to the nearly constant values for cytochrome c(Figure 2.6). Both proteins exhibited comparable reduced ΔH values at levels of maximum lysine labelling.

So of Vicilin with Various Levels of Lysine Modification

Conformational changes in response to step-wise molecular charge manipulation should be reflected in the surface properties of the molecule. As molecular destabilization occurs with abnormal charge interactions, it is expected that buried hydrophobic residues would gradually become exposed to the exterior. In order to investigate this possibility, the surface hydrophobicity(So) of vicilin subjected to various degrees of lysine modification was assessed. The results, given in Table 2.3 and Figure 2.7, were indicative of surface variations with progressive electrostatic manipulation. With initial lysine modification to 43.9%, the So of vicilin decreased significantly from 212 to 167(Table 2.4). At this point, further charge manipulation resulted in an increase of So to 294 at 93.4% maleylation. This change reflects an increase in the exposure of hydrophobic residues with destabilization. In fact, a significant negative correlation exists between So and the degree of molecular destabilization as assessed by Td measurements. The So-Td relationship is defined as follows:

$$So = 1140 - 11.7\ Td\ (r = 0.6901,\ P \leq 0.0001).$$

Figure 2.7. The effect of lysine modification on the surface hydrophobicity (So) of vicilin.


300
275
250
225
200
175
150
SO
20
40
60
80
100
LABELLED LYSINE (%)

DISCUSSION

The aim of this study was to partially understand the extent to which the electrostatic charge of a protein molecule contributes to its overall stability. Charged groups normally occur on the surface of a protein; consequently, modification of positively charged lysine residues by a negative moiety such as maleic anhydride should ensure continued orientation of the side-chains toward the exterior. As such, any change in the relationships among interior residues should arise from electrostatic repulsions between the negative surface side-chains. In addition, if the residues are modified in a stepwise sequence, the pattern of destabilization may be characterized. That is, conformational changes may occur gradually and progressively with increased electrostatic perturbance; or, alternately, a critical point of repulsion may be identified beyond which a three dimensional configuration is not possible.

Both proteins involved in this study are charged molecules under the pH conditions used. From the amino acid data, the net charge of the native cytochrome c at neutral pH is approximately +9(Margoliash and Schejter, 1966); this value represents the differential between 22 positive and 13 negative charges. A comparative net charge for vicilin using estimated amino acid data(Table 1.1) is -111, a value calculated from the difference between 328 negative charges and 217 positive charges. As such, it can be assumed that electrostatic attractions and repulsions are involved in the stabili-

zation of the native conformations of both proteins. Chemical modification of the lysine residues is expected to exert a destabilizing influence; the magnitude of this destabilization is considered to be a function of the complexity of the protein involved, and the significance of the specific electrostatic interactions to general conformational integrity. It should be noted that nearly complete maleylation was possible in both proteins i.e. approximately 96% of the total lysines were ultimately modified in cytochrome c, 94% in vicilin. Not all native proteins will allow such extensive modification. For example, Swenke and Rauschal(1983) could only modify 85% of the lysine residues in a sunflower globulin with succinic anhydride. Factors such as steric hindrance and resistance to nucleophilic attack created by proximal residues may interfere with the degree of experimental modification.

Although maleic anhydride is a relatively specific modifying reagent, it should be emphasized that the labelling approach used in this study was a "random" type of procedure. That is, the modification of any lysine residue is assumed to contribute equally and nonspecifically to molecular destabilization. The validity of such an assumption is questionable. For example, the results of X-ray crystallographic and model studies on a number of proteins including ferredoxin and hemoglobin(Perutz and Raidt, 1973),D-glyceraldehyde-3-phosphate dehydrogenase(Pettigrew et al., 1978) indicate that the activities and stabilities of these proteins are related to the electrostatic contributions of a small number of specific

interactions. Perutz(1978) emphasized that the general distribution of charges on a protein surface is of little importance compared with the interactions between charged groups responsible for subunit assembly, molecular cooperativity, thermostability or substrate binding. Even with horse heart cytochrome c, lysines 13 and 79 have been identified as key residues in maintaining a closed heme crevice(Osheroff et al., 1980). Therefore, with a random labelling approach, it would appear feasible to assume that modification of certain critical residues would have a greater destabilization effect. The influence of this possibility on overall experimental results must be considered. At any level of maleylation, a population of presumably randomly labelled molecules is assessed for conformational changes. If certain critical residues exist, it would be expected that considerable variations in conformational responses would be observed, based on random labelling of these more fundamental side-chains. As this type of variation was not apparent in the experimental results, it might be considered that such critical residues involved in maintaining particular molecular configurations do not exist. Alternatively, such residues may exist, but by virtue of specific locations and interactions, are more shielded from the effects of a modifying reagent. As such, they would not be maleylated until larger excesses of reagent are present. The validity of the latter supposition is enhanced by other protein studies. In general, it has been shown that the relative reactivity of lysine residues with a modifying agent is a

measure of the accessibility of the ε-amino groups to the solvent; those residues exhibiting reduced reactivities are known to be localized in the interior or involved in salt bridges with carboxyl groups(Kaplan et al., 1971; Kaplan, 1972; Bresciani, 1977).

As cytochrome c was initially included in this investigation in order to establish the feasibility of using DSC to monitor conformational changes with electrostatic modification, its response to lysine maleylation will be considered first. If all the lysine residues in cytochrome c are modified, the overall charge is shifted from +9 to -32(Schejter et al., 1979). As negative moieties are gradually introduced into the protein, the stabilizing positive forces will be gradually reduced, until their influence is virtually eliminated. This type of electrostatic perturbation should be expected, at some point, to have a significant effect on the overall stability of the molecule. Aviram et al. (1981) observed a definite conformational response of cytochrome c to lysine modification using circular dichroism(CD) as a monitoring technique. They found that the native conformation was preserved despite a considerable degree of maleylation; beyond a specific critical point, rapid destabilization occurred. In fact, only minor conformational changes were evidenced from the CD spectra with 42% of the lysines labelled; after 74% labelling, the spectra were similar to those for native cytochrome c denatured with 9 M urea. In this study, molecular thermal properties determined by DSC were used as a conforma-

tional probe. The trends exhibited by these parameters were somewhat different from those reported by Aviram et al. (1981). Significant decreases were observed in Td values even with minimal(9.0%) labelling. The Td decreased a total of 14.1°C to 51.7% labelling, then stabilized until maximum maleylation of 96.4% was achieved(Table 2.3, Figure 2.1). A decrease in Td value or thermal stability is usually correlated with molecular destabilization. Although a progressive conformational change is implicated with increased electrostatic manipulation, the relatively constant ΔH value over this temperature range precludes major structural alterations. It should be noted that interpretation of the ΔH values must be approached with caution as the ΔH parameter reflects a composite mixture of contributing factors. Privalov and Khechinashvili(1974) stated that the denaturation enthalpy(ΔH) for small globular proteins is the result of two opposite thermodynamic effects: an exothermic contribution resulting from the disruption of hydrophobic associations with denaturation and an endothermic contribution resulting from the dissolution of hydrogen bonds. The endothermic contribution resulting from the dissolution of van der Waals forces is considered to be negligible. Consequently, a decrease in ΔH may reflect an increase in intramolecular hydrophobic associations and/or a decrease in hydrogen bonding in the original protein prior to denaturation. As an additional complicating factor, the denaturation process may be immediately followed by an aggregation of the unfolded proteins. This type of aggregation, mediated

by hydrogen bond formation, is considered to be exothermic. Although this should result in reduction of observed ΔH values, the energies associated with such aggregations are considered to be minimal(Donovan and Ross, 1973). As a result of the complexity of factors contributing to a ΔH value, it would seem more appropriate to consider this thermal parameter from a qualitative rather than a strictly quantitative viewpoint. However, under carefully controlled experimental conditions, numerical differences may be used with caution, especially when significant changes appear to reflect conformational destabilization (Murray et al., 1984).

With respect to cytochrome c, a significant decrease in ΔH occured as the degree of lysine modification increased to 77.5% and 96.4%. This may be correlated with a major conformational shift in which original intramolecular hydrogen bonds have been disrupted due to increased electrostatic repulsion. This is in partial agreement with the CD observation of Aviram et al. (1981) which identified major conformational changes after 74% of the residues were modified. In this study, even with 96.4% labelling, the protein was not described as completely denatured in terms of thermal parameters. For example, preliminary DSC studies showed that cytochrome c in 9 M urea did not exhibit an endotherm; a situation which can result if the original protein is denatured. As previously mentioned, Aviram et al. (1981) reported that the CD spectra for cytochrome c with 74% labelling was similar to that for the protein denatured with 9 M urea.

These two comparable studies appear to reflect somewhat different responses of cytochrome c to electrostatic manipulation. Aviram et al. (1981) suggest that the protein is relatively tolerant to the increasing negativity up to a critical point; beyond this, it rapidly assumes a denatured form. Results from this study seem to imply gradual conformational fluctuations with progressive electrostatic disturbance. A critical point may exist(i.e. 77.5% labelling), beyond which major structural rearrangement has occurred. However, up to maximal labelling the protein is not completely intolerant of the change in charge characteristics; that is, from a thermodynamic viewpoint, it is not considered to be in a fully denatured form.

An interesting phenomenon associated with the thermal analysis of cytochrome c is the presence of post-denaturation exotherms observed with increasing cytochrome modification(Figure 2.3). This is considered to be an atypical response, reflecting a decrease in the heat capacity of the denatured molecules. Privalov and Pfeil(1979) found that an increase in heat capacity was characteristic of denatured proteins - an observation consistant with the original proposal by Kauzmann(1959) which stated that the unfolding of a compact protein structure was accompanied by a heat capacity change as a result of the interaction of non-polar groups with water. The type of post-denaturation exotherm observed with modified cytochrome c may be attributed to aggregation of denatured protein molecules mediated by exothermic hydrogen

bond formation. This seems to be somewhat of a contradictory assessment as it was previously mentioned that this type of aggregation seems to have a minimum energetic input and little effect on the ΔH value calculated from the DSC endotherm. In this case, the exothermic reaction appeared to occur in a temperature range beyond the endothermic transition, rather than as an immediate post-denaturation event. Although aggregation by hydrogen bonding is a feasible thermodynamic explanation for the observed exotherms, it is not an easily interpreted viewpoint due to the increased electrostatic repulsion among the extensively modified molecules.

Prior to a comparison of the effects of modification on vicilin, a large multimeric protein, with those observed for cytochrome c, it is interesting to consider the thermal properties of the unmodified proteins. Both proteins were characterized by similar Td values; however, they exhibited differences in ΔH and 1/2bw values. The ΔH value for unmodified vicilin was 4.30 cal g^{-1}, a value in the 4-6 cal g^{-1} range cited as typical for most globular proteins(Donovan and Ross, 1973; Privalov and Khechinashvili, 1974) whereas the ΔH for the unmodified cytochrome was 2.53 cal g^{-1}. In order to speculate on the possible reasons for differences in the two values, several fundamental premises regarding denaturation enthalpies as outlined by Privalov and Khechinashvili (1974), must be considered. Firstly, it is assumed that the maximum ΔH value possible for any globular protein is 13 cal g^{-1}. Secondly, the native structures of all compact globular proteins

appear to be equally saturated by hydrogen bonds. Finally, the saturation of proteins by nonpolar associations differs; however, these interactions attain a maximum stability from 60 to 80°C. Therefore, if the exothermic contributions to ΔH by van der Waals forces and the post-denaturation aggregation are considered to be minimal, any depression of ΔH values below the ideal 13 cal g^{-1} is the result of the exothermic disruption of hydrophobic associations. As both vicilin and cytochrome c have similar Td values, close to 80°C, the overall stability of hydrophobic interactions in the two proteins is assumed to be similar. Consequently, the remaining factor to cause a differential in the original ΔH values, is the actual number of hydrophobic contacts per unit mass in the two proteins. The average hydrophobicity for cytochrome c, calculated from the amino acid data of Margoliash et. al. (1961), is 1059.65 cal/AA residue as compared with 901.43 cal/AA residue(Table 1.8) for vicilin. This differential supports the concept of a higher number of hydrophobic contacts per unit mass in cytochrome c leading to an overall reduced ΔH value.

As mentioned previously, the second thermal parameter to differ between the two proteins is the 1/2bw. The value for unmodified cytochrome c(5.3°C) is less than one-half that for unmodified vicilin(11.3°C). This situation possibly reflects the multimeric-monomeric differential. A small monomeric globular protein may denature as a unit by a two-state transition from a native to a single denatured form(Privalov and Khechinashvili, 1974; Privalov, 1979). The denaturation process for

a multimeric protein is much more complex; it may be a multi-state procedure, with different conformational stages exhibiting different Td values(Privalov, 1982). Alternatively, individual subunits may express different Td values. In fact, these subunit Td values may vary as a function of the degree of subunit cooperativity within the native molecule. Both of these factors can contribute to a broadening of the endotherm, reflected by the numerical value of the 1/2bw.

In terms of step-wise chemical modification, the response of vicilin, as compared with cytochrome c, reflects the multimeric nature of the protein. With the maximum level of maleylation achieved(i.e. 93.8%), the overall net charge at pH 7.5 can be estimated to shift from approximately -111 to -206, a change reflecting the elimination of nearly one-half of the positive charges. The conformational responses of the protein up to this level of modification showed a somewhat different trend than those for cytochrome c. For vicilin, thermal parameters remained relatively constant to 63.0% modification; this was followed by a progressive decrease in both Td and ΔH with increased modification to 93.8%. In addition, the 1/2bw showed a definite increasing pattern, characterized by significant changes with 11.0% and 63.0% maleylation(Table 2.4, Figure 2.4). The question becomes how to assimilate these thermal data, which are a direct reflection of the intrinsic structure of vicilin and the nature of the stabilizing forces present therein, into a description of the actual molecular response. In terms of the constant thermal parameters to 63.0%

modification, it would appear that the conformation of the molecule is not initially influenced by the increasing negativity. Schwenke and Rauschal(1983), found a similar response of purified 11S globulin from sunflower to successive modification with succinic anhydride. Changes in electrophoretic patterns, as correlated with changes in tertiary and quaternary structures, could only be detected after 65% succinylation. As discussed with cytochrome c, it is assumed that some lysines will be more critical than others to the general assembly of the molecule. Subunit association in vicilin is a result of noncovalent forces(Chapter 1); consequently, lysine residues should be involved directly or indirectly in stabilizing electrostatic interactions. With greater excesses of the modifying reagent, maleylation of key residues in conjunction with the general increase in overall surface negativity had a destabilizing influence on the molecule. The subunit dissociation phenomenon may be evidenced in the changes in the two thermal parameters not observed in the monomeric cytochrome c. The significant decrease in ΔH from 4.30 cal g^{-1} for the unmodified protein to 1.79 cal g^{-1} at 93.8% maleylation may reflect hydrogen bond disruption in subunit disassembly; the corresponding increase in half-band width may be a consequence of exposed subunits having slightly different Td values in comparison with the Td values for the subunits associated as a cooperative unit in the native form. However, it should be noted, that similar to cytochrome c, maximal modification did not result in complete denaturation of the protein. A dis-

tinct endotherm was still apparent at 93.8% maleylation(Figure 2.4).

This conformational assessment for vicilin appears to be supported by the observed fluctuations in surface hydrophobicity. In response to increasing negativity, the So value initially decreased from a level of 212 in the unmodified protein to a minimal value of 167 with 43.9% maleylation(Table 2.4, Figure 2.7). This may reflect minor surface rearrangements as a function of increased electrostatic repulsion. With further modification, the progressive increase in So was indicative of an exposure of hydrophobic residues associated with a general unfolding phenomenon and/or partial subunit dissociation. Hydrophobic interactions are presumed to be important noncovalent forces in subunit assembly; exposure of those previously buried residues would cause an increase in apparent So for the overall molecule. It should be noted that a single So value may represent different conformational states. For example, the unmodified vicilin exhibited an So value of 212; vicilin with 63.0% maleylation was also characterized by a similar So. Having followed the dynamic So pattern from 0.0 to 63.0% modification, it is unlikely that the unmodified and modified proteins, despite apparent similarity in surface hydrophobicity characteristics, are in the same conformational arrangements.

It is interesting to compare the two molecules, vicilin and cytochrome c, in terms of the effect of lysine labelling with respect to the proportion of labelled residues as a func-

tion of the total number of amino acids in the entire protein(Figures 2.5 and 2.6). This approach perhaps illustrates that although electrostatic influences can be observed in the two very different proteins, an absolute comparison of the responses is invalid. In the first instance the type of electrostatic manipulation differs for cytochrome c and vicilin. Cytochrome c is a cationic protein that is stabilized by both positive and negative residues at neutral pH; due to the low level of arginine, maleylation results in almost complete elimination of positive residues. That is, approximately 91% of the potentially charged residues are negative. In comparison, vicilin is an anionic protein at neutral pH, also stabilized by a composite of negative and positive residues. Due to the higher level of arginine in vicilin, complete maleylation only results in 80% of the potentially charged residues becoming negative; some stabilizing positive residues remain. A second differential is the relative percentage of lysine in the two proteins; for cytochrome c lysine makes up approximately 18.5% of the total residues; in vicilin, only 6.5%.

Despite these obvious differences, some generalized comments can be made. Both proteins are relatively tolerant of major electrostatic modification; conformational shifts appear minor to approximately 60% maleylation. Conformational fluctuations upto this point were evidenced by decreases in Td for cytochrome c and decreases in So for vicilin. Although both proteins appeared relatively stable over this modification region, the enhanced stability of vicilin may reflect the

greater stabilizing influence of arginine residues or the lower amount of electrostatic modification that has occurred relative to the size of the entire molecule. At levels of modification in excess of 70%, more significant conformational changes were apparent in both proteins. These can be speculated to reflect unfolding in cytochrome c and a combination of subunit dissociation and unfolding in vicilin. In both vicilin and cytochrome c, maximal maleylation did not result in total denaturation as assessed by DSC. The critical labelling point for major conformational changes can be attributed to either the cumulative effect of electrostatic repulsion or the modification of key residues, previously inaccessible to the modifying reagent.

CHAPTER THREE

THE INFLUENCE OF NEUTRAL SALTS ON SELECTED CONFORMATIONAL PROPERTIES OF VICILIN

INTRODUCTION

The conformation adopted by a biological macromolecule, such as a protein, is a sensitive function of the residue composition, the sequence of residues and the solvent environment. As a specific example of environmental influences, it is well established that neutral salts have a profound effect on the conformational stability of proteins (von Hippel and Schleich, 1969). Recognition of these structural responses, however, has not necessarily been coupled with an understanding of the mechanism of action of a salt in the elucidation of specific protein changes. To facilitate the explanation of salt action, Eagland (1975) suggested that the effects of neutral salts may be arbitrarily divided into two major classes. The first involved the direct interaction of ions with specific charged groups on the macromolecule. At low salt concentrations, an electrical double layer is formed in association with protein molecules; this results in a salting-in or solubilizing effect based on the repression of interprotein electrostatic interactions. In addition, the charge-shielding influence of electrostatic effects may induce protein conformational variations as a result of the weakening of attractive or repulsive intramolecular forces (von Hippel and Schleich, 1969).

In contrast, the second class of salt effects is characterized by an indirect influence of the constituent ions on a macromolecule by means of their modification of the solvent environment. This phenomenon, normally associated with higher

concentrations of salts, has been examined from a variety of perspectives beginning with a fundamental study by Hofmeister (1888). In an attempt to categorize the influence of specific neutral salts on proteins, Hofmeister arranged various electrolytes into an hierarchical series based on the ability of the individual salts to induce a salting-out or precipitation of euglobulins from aqueous solution (Appendix V A). Since this particular study, many investigations have ranked the efficiency of the cationic and anionic components of neutral salts in the induction of protein precipitation. Representative hierarchical arrangements of several common anions and cations are given in Appendix V B. Although a variety of different electrolytes have been examined in this capacity, the original Hofmeister arrangement of salts is still considered to be a valid series for reference.

In addition to indicating protein precipitation effectiveness, the position of an ion within the Hofmeister (or lyotropic) series, with minor exceptions, was also recognized to be related to the ability of the ion to stabilize or destabilize a protein and to activate or inhibit a number of enzymes (Robinson and Jencks, 1965). For example, increased concentrations of salts such as NaCl and Na_2SO_4 are effective in both salting-out or precipitating proteins and in stabilizing certain proteins against denaturation or depolymerization. In contrast, high concentrations of salts such as NaSCN which increase salting-in or solubility of proteins also promote diverse conformational disturbances (von Hippel and Wong,

1964). These contrasting effects of high concentrations of neutral salts on proteins in terms of solubilization or precipitation and destabilization or stabilization were attributed to either some interaction between the salt and the solvent or a combined interaction among the salt, solvent and protein components. In one approach to this consideration, neutral salts have been classified as either chaotropic or nonchaotropic. Nonchaotropic salts have been considered to have a stabilizing influence on protein structure by promoting the ordering of water in such a way that exposure of nonpolar groups to the solvent becomes less thermodynamically favorable (Hatefi and Hanstein, 1969; Hanstein et al., 1971). An increase in the concentration of nonchaotropic salts results in intermolecular hydrophobic associations and eventual protein precipitation. In contrast, chaotropic salts have little influence on the order of water. In fact, Hatefi and Hanstein (1969) defined chaotropic ions as charged groups which promote the transfer of nonpolar groups to water. Therefore, in a chaotropic medium, the transfer of hydrophobic residues to an aqueous environment becomes more favorable; the protein destabilizes and exhibits a higher degree of solubility.

More recently, Melander and Horvath (1977) have suggested that the ability of a salt to increase protein stabilization and to eventually promote precipitation through hydrophobic associations is directly related to the capacity of the salt to raise the surface tension of water. A good correlation exists between the anionic lyotropic series and the molal sur-

face tension increment (σ) of a salt, a numerical index reflecting the ability of a salt to alter the surface tension of water. As a further development of this concept, Arakawa and Timasheff (1982) implied that the stabilizing effect of certain salts is a result of their capacity to increase the water surface tension in conjunction with their ability to induce preferential hydration of the protein. Preferential hydration of a macromolecule is an extension of the nonchaotropic concept; that is, it results from a specific solvent-salt interaction. As a consequence of the association of some salts with water, the immediate area around the protein molecule is either devoid of salt or contains reduced amounts relative to the bulk solvent (Lee and Timasheff, 1981). If the premise of preferential hydration is accepted, the next consideration is the rationale for a positive correlation of this phenomenon with increased molecular stability. One possible explanation involves the concept of the existence of a specific cavity within a solvent to accommodate a macromolecule such as a protein. A certain level of free energy is required to create such a cavity; the actual amount of this free energy is directly related to the cohesiveness of the solvent molecules, assessed by a factor called the solvent cohesive force. With respect to stabilizing salts, it is known that the salt concentrations required to cause preferential hydration of protein molecules also cause an increase in this solvent cohesive force (Arakawa and Timasheff, 1982). Therefore, in these media, a high level of free energy would be

required to convert the protein into an unfolded structure necessitating a larger cavity or contact area within the solvent. Consequently, a folded stabilized protein conformation is favored.

In order to explain the effects of destabilizing or chaotropic salts, it has been suggested that these salts, at high concentrations, do not induce preferential hydration but bind directly to the protein (Bull and Breese, 1970: Arakawa and Timasheff, 1982). One ion of a neutral salt may become strongly bound to a specific protein site such as a charged residue or a polarized peptide bond; the other ion interacts nonspecifically with the protein as a component of the Debye-Huckel double layer (Aune et al., 1971). An increase in electrostatic repulsive forces as a consequence of such protein-ion interactions will cause a decrease in the stability of the protein. The established electrostatic repulsive force should also prevent intermolecular aggregation; in other words, it should have a salting-in effect whereby the solubility of the protein is increased.

Several authors (Melander and Horvath, 1977; Pahlman et al., 1977) have suggested that the effects of increasing concentrations of various salts from the Hofmeister series on protein properties could be indicative of the relative contributions of some noncovalent forces to protein structural stability. Significant changes in conformational properties with exposure of proteins to salt environments differing in concentration and identity should imply a dependence on hydrophobic

associations whereas a reduced conformational response would suggest the predominance of electrostatic factors or other forces such as hydrogen bonding. From this viewpoint, a study was designed to investigate the effects of different concentrations of various salts on the conformational stability of the globulin vicilin. As discussed in Chapter 1, vicilin is the product of the noncovalent association of several subunits. The relative importance of the type of noncovalent forces fundamental to this association is of interest. In an attempt to understand some of these forces, conformational responses of vicilin to various salt environments were monitored by assessment of protein thermal properties using differential scanning calorimetry (DSC). The surface hydrophobicity of the protein molecules was also monitored to evaluate possible changes in the exposure of hydrophobic residues with varying environmental conditions.

MATERIALS AND METHODS

Influence of pH on Conformational Parameters

If a number of unbuffered neutral salts are used as separate protein solubilizing media, some pH variation will occur among the different solutions. This variation is a function of the identity and concentration of the specific salt. Therefore, prior to investigating the influence of a number of diverse electrolytes on selected aspects of vicilin conformation, several experiments were performed to define a pH range in which pH as a single parameter, did not appear to exert an effect on protein structural characteristics. In all experiments, the initial protein solution contained vicilin, as isolated by gel filtration in 0.2 M sodium acetate, pH 7.5, at an approximate concentration of 1 mg mL^{-1}.

In the first preliminary study, vicilin was dialysed extensively against a series of 0.1 M phosphate buffers, ranging in pH from 6.0 to 8.0. The dialysis procedure involved exposure of 10 mL protein samples to a minimum of six 400 mL buffer changes over a 36 h period with continuous stirring. The buffers differed in pH by 0.1 units from 6.0 to 6.8 and by 0.5 units from 7.0 to 8.0. The pH of all electrolyte solutions and dialysed protein samples was measured with a Radiometer model 26 expanded scale pH meter using a combination microelectrode. The thermal properties of vicilin in all pH environments were assessed using DSC as described in Chapter 2. Prior to DSC analysis, each vicilin sample, was concentrated 40 to 50 times using a Minicon-B15 Macrosolute concentrator

with a 15,000 M.W. cut-off. All values for Td, ΔH and 1/2bw were determined for quadruplicate samples; mean values and standard deviations of the mean are reported for each.

In addition, the surface hydrophobicity(So) was determined for vicilin samples at pH intervals of 0.5 units from 6.0 to 8.0 using the modified method of Kato and Nakai(1980) described in Chapter 2. In preparation for fluorescence measurements, each dialysed protein sample was diluted with 0.1 M phosphate buffer of the corresponding pH to give five vicilin samples ranging in concentration from approximately 0.04 to 0.4 mg mL^{-1}. All values of So were determined using a minimum of four samples; mean values and standard deviations of the mean are given for each.

The composite results from this first experiment were indicative of relatively stable conformational properties for vicilin in the pH range 6.0 to 7.0; however, significant variations occurred at alkaline pH values. To pursue this further, a second preliminary experiment was designed to assess possible conformational fluctuations in vicilin exposed to two types of pH environments involving the salts sodium acetate and sodium citrate. If unbuffered, both of these salts impart an alkaline pH to the protein media. For each salt, vicilin was dialysed extensively against a series of electrolyte solutions with varying concentrations(Table 3.1). At each salt concentration, the pH was either uncontrolled, but monitored, or adjusted to 6.6 - a value representing the midpoint of the acidic pH range exhibited by other neutral salts used in sub-

TABLE 3.1. Salt concentrations used in the conformational studies of vicilin. The pH remained uncontrolled unless indicated.

Salt	Concentration(M)
sodium acetate	0.2, 0.5, 1.0, 2.0
sodium acetate, pH 6.6	0.2, 0.5, 1.0, 2.0
sodium bromide	0.1, 0.2, 0.5, 1.0
sodium chloride	0.2, 0.5, 1.0, 2.0
sodium citrate	0.01, 0.1, 0.2, 0.5, 1.0
sodium citrate, pH 6.6	0.1, 0.2, 0.5, 1.0
sodium iodide	0.1, 0.2, 0.5
sodium phosphate, pH 6.6	0.025, 0.1, 0.2, 0.5
sodium sulfate	0.1, 0.2, 0.5, 1.0
sodium thiocyanate	0.1, 0.2, 0.5, 1.0
potassium chloride	0.1, 0.2, 0.5, 1.0
potassium sulfate	0.1, 0.2, 0.5
lithium chloride	0.1, 0.2, 0.5, 1.0
lithium sulfate, pH 6.6	0.1, 0.2, 0.5

sequent studies. All pH levels were adjusted with an acid having the identical anion to that of the original electrolyte. The pH values for the individual protein samples are presented in Table 3.2. Thermal properties and So values were determined for quadruplicate samples as previously described; mean values and standard deviations of the mean are reported for each. On the basis of the results from these two studies, all subsequent electrolyte media exhibiting alkaline pH values were adjusted to pH 6.6 prior to dialysis; however, pH was allowed to remain an uncontrolled parameter in those salt solutions having a neutral or acidic pH. One consequence of the pH 6.6 adjustment was the production of a mixture of anions, specifically HPO_4^{-2} and $H_2PO_4^-$, in the phosphate environment. As the pKa for this dissociation is 6.8, it was assumed that the $H_2PO_4^-$ anion would be slightly dominant at pH 6.6.

Influence of Various Electrolyte Media on Conformational Parameters

The initial stage in the assessment of the influence of various electrolytes on selected aspects of vicilin conformation involved the exposure of vicilin to a series of different salt environments by means of extensive dialysis. The experimental salt environments are given in Table 3.1. Some variation existed in the ranges of the concentrations used among the various electrolyte media; this mainly reflected limitations associated with protein and/or salt solubility. All salts were Analytical Reagent Grade and were used without fur-

TABLE 3.2. The influence of pH on thermal properties (Td, ΔH, ½bw) and So values for vicilin in various concentrations of sodium acetate and sodium citrate. For all concentrations, pH values were either uncontrolled or adjusted to 6.6.

Conc. (M)	pH	Td (°C)	ΔH (cal g^{-1})	½bw (°C)	So
Sodium acetate					
0.2	7.5	86.9±0.5[a]	2.14±0.53[a]	9.6±1.0[a,b]	161±6[a]
0.5	7.9	88.7±0.2[b]	3.47±0.17[b]	10.4±0.7[a,b]	137±16[b]
1.0	8.3	94.8±0.8[c]	4.23±0.17[c]	10.6±0.4[a,b]	138±17[b]
2.0	8.6	103.6±0.4	4.56±0.27[c]	11.2±1.2[a,b]	187±16[c]
0.2	6.6	87.7±0.7[a,b]	2.56±0.80[a]	11.3±0.4[a]	207±24[c]
0.5	6.6	91.5±0.7	2.50±0.17[a]	10.6±0.4[a,b]	201±15[c]
1.0	6.6	97.8±1.9[c]	3.49±0.23[b]	10.8±0.3[a]	200±35[a,c]
2.0	6.6	105.4±0.7	3.54±0.19[b]	9.8±0.3[b]	185±20[a,c]
Sodium citrate					
0.01	7.6	78.0±1.6	1.89±0.18	11.4±1.3[a,b]	176±24[a,b]
0.1	7.9	86.8±1.3[a]	3.33±0.43[a]	12.6±1.2[a,b]	127±34[a,c,d]
0.2	8.3	90.3±1.0[b]	4.04±0.18[b]	11.7±0.2[a]	147±17[a,c,d]
0.5	8.6	100.2±0.7[c]	nd[2]	nd[2]	197±22[b]
1.0	8.7	111.8±2.5[d]	nd[2]	nd[2]	280±78[b]
0.1	6.6	86.8±0.8[a]	3.07±0.67[a,b]	13.3±1.6[a,b]	191±23[b,c]
0.2	6.6	91.3±1.6[b]	4.19±0.55[a,b]	12.5±0.2[b]	183±18[b,c]
0.5	6.6	101.5±2.3[c]	nd[2]	nd[2]	137±8[d]
1.0	6.6	114.6±1.4[d]	nd[2]	nd[2]	124±11[d]

[1] Column values followed by the same letter are not significantly different ($P \leq 0.05$). Statistical comparisons apply only within a single salt group.

[2] Value not determined due to large post-denaturation enotherms.

ther purification. Details concerning the initial protein solution and the dialysis procedure were as described in the preceeding section. The pH of all electrolyte solutions and dialysed protein samples was measured; values for the individual protein samples are presented in Table 3.3. As previously mentioned, pH was allowed to remain an uncontrolled parameter for solutions in the neutral and acidic pH range. With alkaline salt environments, the pH was adjusted to 6.6 by means of an acid having the anion identical to that of the original electrolyte.

The thermal stability of vicilin in various salt environments was assessed using DSC as described previously. All values for Td, ΔH and 1/2bw were determined for at least quadruplicate samples; mean values and standard deviations of the mean are reported for each. The DSC analyses were not performed on vicilin in 0.1 M potassium chloride due to difficulties in sample concentration and recovery as a result of reduced protein solubility.

Surface hydrophobicity of vicilin in the various electrolyte environments was determined using the method described previously. The So values were not established for vicilin in sodium iodide or sodium bromide due to the quenching properties of the two anions. All values of So were determined using a minimum of four samples; mean values and standard deviations of the mean are given for each.

Statistical Analysis

Differences in thermal parameters(Td, ΔH, 1/2bw) and So

TABLE 3.3. Thermal properties (Td, ΔH, ½bw) and So values for vicilin in various concentrations of sodium salts, pH remained uncontrolled unless indicated.

Conc. (M)	pH	Td (°C)	ΔH (cal g^{-1})	½bw (°C)	So
Sodium bromide					
0.2	6.4	89.8±1.2	2.28±0.27	10.6±1.2[a]	nd[2]
0.5	6.3	92.4±0.4	4.08±0.35[a]	11.5±0.3[a]	nd[2]
1.0	6.3	95.4±0.7	3.43±0.16[a]	11.9±1.2[a]	nd[2]
Sodium chloride					
0.2	6.3	88.0±0.8	3.63±0.13[a]	11.6±0.6[a]	248±18
0.5	6.5	92.5±0.6	3.49±0.62[a]	9.9±0.5[b]	202±16[a]
1.0	6.2	98.0±0.0	3.84±0.01[a]	10.4±0.4[a,b]	201±9[a]
2.0	6.4	105.8±0.8	4.99±0.45	10.5±0.1[a,b]	184±5[a]
Sodium iodide					
0.1	6.3	87.8±0.2	1.83±0.16[a]	11.6±0.4[a]	nd[2]
0.2	6.3	85.4±1.7[a]	2.88±0.24[b]	11.8±0.8[a]	nd[2]
0.5	6.8	86.1±0.7[a]	2.65±0.80[a,b]	12.5±1.0[a]	nd[2]
Sodium phosphate					
0.025	6.6[3]	85.5±0.5[a]	2.83±0.86[a]	11.2±1.1[a]	156±25[a]
0.1	6.6	86.7±0.9[a]	2.48±0.82[a]	11.7±0.7[a]	219±16[b]
0.2	6.6	91.5±1.1	2.28±0.15[a]	10.4±1.0[a]	269±60[b]
0.5	6.6	98.8±0.8	2.73±0.79[a]	11.1±0.6[a]	148±44[a]
Sodium sulfate					
0.1	6.6	88.5±0.8	2.74±0.25[a]	9.8±0.4[a]	220±4[a]
0.2	6.6	90.6±0.5	3.06±0.90[a,b]	10.4±0.2[a]	273±39[a]
0.5	7.0	99.1±0.5	3.37±0.30[a,b]	10.0±0.6[a]	164±11
1.0	6.0	109.3±0.4	4.39±0.22[b]	10.2±0.8[a]	129±15
Sodium thiocyanate					
0.1	6.6	79.0±3.0[a,b,c]	3.75±0.19	12.4±1.0[a,b,c]	179±37
0.2	6.4	82.3±0.8[a]	2.76±0.61[a]	11.0±0.9[c,d]	318±25[a]
0.5	6.3	80.8±1.2[b]	2.58±0.46[a]	13.4±1.3[b]	373±56[a]
1.0	6.4	75.9±0.7[c]	1.01±0.19	10.9±1.1[c,d]	384±52[a]

[1] Column values followed by the same letter are not significantly different ($P \leq 0.05$). Statistical comparisons only apply to a single salt group.

[2] Value not determined due to quenching effect of anion.

[3] pH adjusted to 6.6 for all concentration levels within this salt group.

values for different concentrations within a specific salt group and for values between different salt groups were evaluated by a multiple T-test. Correlations between pairs of variables were assesssed by standard linear regression using individual data points.

RESULTS

Influence of pH on Vicilin Conformational Parameters

The effects of varying pH from 6.0 to 8.0 on the thermal properties and So of vicilin exposed to 0.1 M sodium phosphate are given in Table 3.4. In terms of thermal parameters, Td values showed no significant change from pH 6.0 to 6.5. There appeared to be some trend towards an overall decrease in Td from pH 6.5 to 7.0; however the Td at pH 7.0 was not significantly different from that at pH 6.5. An important observation was the situation at pH 7.5 and 8.0. At these pH levels, the Td values were significantly lower than those values in the pH 6.0 to 7.0 range. In fact, there was a Td differential of 5.7°C from pH 6.0 to 8.0. With respect to the other thermal properties, ΔH values over the experimental range showed some fluctuations; however, there was no apparent trend involving significant changes. In contrast, the changes in 1/2bw values were more comparable to those of the Td values; their pattern of variation also implicated some degree of vicilin destabilization with increasing pH. Although the absolute values for 1/2bw increased from pH 6.0 to 7.0, there was no significant difference in these values from pH 6.3 through to 7.0. At pH 7.5 and 8.0, the 1/2bw values of 12.6 and 14.3°C, respectively, appeared distinctly higher than those from pH 6.0 to 7.0. However, due to the variation in experimental 1/2bw values and the reduced range of values normally observed, the 1/2bw at pH 7.5 and 8.0 only differed significantly from the 1/2bw at pH 6.0 to pH 6.4 and pH 6.8. As a result, although

TABLE 3.4. Thermal properties (ΔH, Td, ½bw) and So values for vicilin exposed to a series of 0.1 M phosphate buffers ranging in pH from 6.0 to 8.0.

pH	Td (°C)	ΔH (cal g^{-1})	½bw (°C)	So
6.0	86.9±1.1[a,b]	2.81±0.29[a,b]	9.9±2.3[a,b]	286±22[a]
6.1	86.8±1.0[a,b]	3.08±0.20[a,b]	8.9±0.2[a]	nd[2]
6.2	86.4±0.4[a]	3.27±0.38[a,b]	10.3±0.1[b,c]	nd[2]
6.3	86.3±0.5[a]	3.78±0.96[a,c,d,e]	10.5±0.9[b,d]	nd[2]
6.4	86.3±0.9[a,b]	3.09±0.51[a,e]	10.7±1.0[b,d]	nd[2]
6.5	85.6±0.4[b,c]	4.30±0.56[c]	11.4±0.8[b,d,e]	296±17[a]
6.6	84.6±0.5[d]	3.60±0.62[b,c,e]	11.2±1.0[b.d.e]	nd[2]
6.7	83.8±0.3[d,e]	3.31±0.21[b,d]	11.8±0.3[d,e]	nd[2]
6.8	83.8±0.3[d,e]	3.00±0.10[b,e]	11.3±0.1[d,f]	nd[2]
7.0	83.9±1.3[c,d,e]	3.21±0.54[b,e]	11.2±0.9[c,d,f,g]	246±14
7.5	82.0±0.6[f]	2.87±0.23[b,e]	12.6±0.8[e,g]	162±16[b]
8.0	81.2±1.2[f]	3.27±0.20[b,e]	14.3±1.6[e,f,g]	158±12[b]

[1] Column values followed by the same letterare not significantly different ($P \leq 0.05$).

[2] Value not determined.

the 1/2bw appeared to increase with increasing pH, only the values at the extreme ends of the pH range were different statistically.

A collective assessment of all the thermal parameters seemed to indicate that conformational characteristics for vicilin were relatively constant from pH 6.0 to 7.0. Some structural perturbations may have occurred at pH 7.5 and 8.0. Analyses of the So data appeared to confirm the implications derived from the thermal properties. The So values decreased significantly with an increase in pH from 7.0 to 7.5; values for pH 7.5 and 8.0 were similar. As a result of the combined data for thermal properties and So(Table 3.4) it was decided that pH 7.0 would be the upper maximum pH limit for the electrolyte solutions used in subsequent conformational studies. As previously indicated, conformational variations in vicilin as a function of pH appeared to be minimal from pH 6.0 to 7.0.

To further assess if pH effects might be a factor in considering the influence of some electrolytes on protein conformation, vicilin was exposed to varying concentrations of two salt environments with two different pH regimes. Sodium acetate and sodium citrate were selected as experimental media. These salts contain anions of interest in terms of their possible effects on protein structure; however, if the pH is uncontrolled, they result in alkaline environments with the degree of alkalinity increasing as a function of salt concentration. Therefore, the thermal properties and So values of vicilin in these two salt environments with uncontrolled pH

were compared with those of vicilin in similar salt media(of the same concentration) with the pH adjusted to 6.6(Table 3.2). In terms of thermal stability, the Td values for vicilin in sodium acetate were similar for the two pH conditions at 0.2 and 1.0 M but were significantly higher for the pH 6.6 environment at 0.5 and 2.0 M. For sodium citrate, the pH had no effect on Td values at comparable concentration levels. The second thermal parameter, 1/2bw, did not exhibit any significant trends for either of the salt environments in terms of differences in pH conditions at comparable concentration levels. With respect to ΔH values, those for vicilin in sodium acetate(pH uncontrolled) were significantly higher than those for pH 6.6 at 0.5, 1.0 and 2.0 M; ΔH values at 0.2 M were similar. For vicilin in sodium citrate, the results for ΔH values were comparable to those for the Td values. That is, there was no significant difference in ΔH values for the two pH situations.

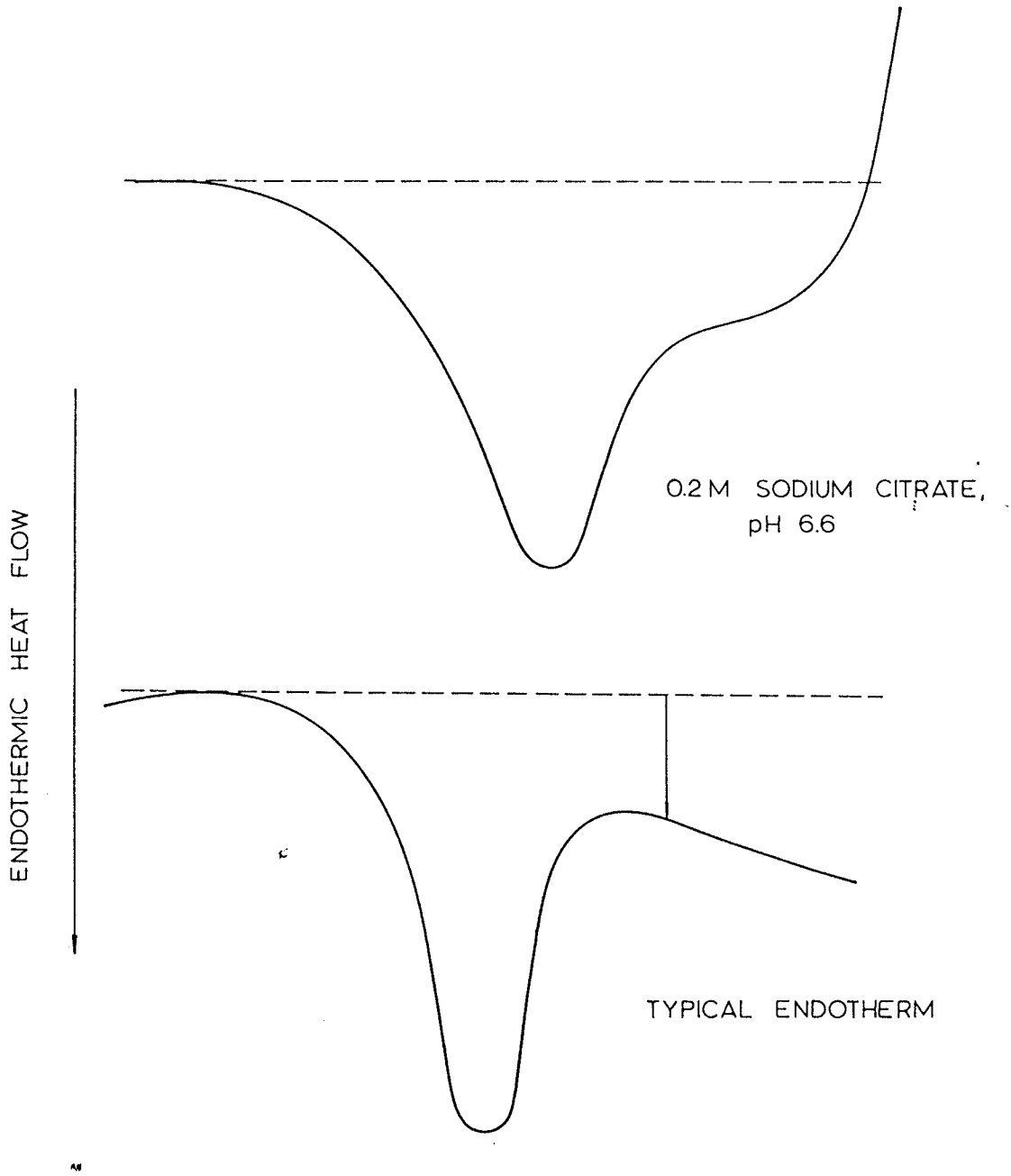
It should be mentioned that vicilin exposed to sodium citrate exhibited some atypical thermal characteristics. For example, ΔH and 1/2bw values could not be determined for vicilin in 0.5 M and 1.0 M sodium citrate due to the large post-denaturation exotherms in the temperature range immediately following the denaturation temperature. Post-denaturation exotherms were also observed to a minimal extent at the lower concentrations of sodium citrate. At 0.5 M and 1.0 M, the exotherms were so exaggerated that it was not possible to maintain the post-denaturation baseline on scale during DSC

analyses. As a result, only Td values could be determined at these concentration levels. Post-denaturation exotherms are considered to be an atypical phenomenon due to the heat capacity implications. A typical denaturation endotherm for vicilin exposed to sodium citrate was characterized by a decrease in the heat capacity of the denatured molecules, as reflected by the elevated position of the post-denaturation baseline relative to the pre-denaturation state(Figure 3.1). With sodium acetate and all other subsequent salt environments, the denatured vicilin molecules exhibited the expected increased heat capacity as described by Privalov and Pfeil(1979). An example of this type of denaturation endotherm, with the position of the pre- and post-denaturation baselines indicative of a positive heat capacity change is given in Figure 3.1.

Significant variations, as a result of the pH effect, occurred in the So values for vicilin in both salt media at most concentration levels. In general, the So values were higher for vicilin in sodium acetate, pH 6.6. A similar trend existed for vicilin in sodium citrate at 0.1 and 0.2 M; however, the situation was reversed at 0.5 and 1.0 M with So values being lower at pH 6.6.

In summary, the differential in thermal and So parameters between the two types of pH environments for both salts was not consistent or easily interpreted. From these results, however, it was evident that even within a relatively narrow range, pH can be an influential factor in the types of protein conformational responses elicited by various electrolytes.

Figure 3.1. Representative endotherms comparing the post-denaturation state of vicilin in 0.2 M sodium citrate, pH 6.6 with that of vicilin in all other electrolyte media examined. Thermogram scale was 0.02 mcal sec^{-1} in $^{-1}$.


ENDOTHERMIC HEAT FLOW
0.2 M SODIUM CITRATE, pH 6.6
TYPICAL ENDOTHERM

Therefore it would seem appropriate to restrict pH variations in studying the effects of different electrolytes.

Influence of Various Electrolytes on Vicilin Conformational Parameters

A. Effects of Specific Anions

Vicilin was exposed to a series of different sodium salts to assess the influence of various anions on protein thermal stability and So values. If necessary, pH levels were adjusted to 6.6. These results are summarized in Tables 3.2 and 3.3 and Figures 3.2 and 3.3. It should be mentioned that sodium salts were chosen to examine anionic effects as the sodium cation itself does not appear to influence protein conformational parameters. In fact, sodium does not seem to be able to penetrate the primary hydration shell of the protein and, as such, does not bind to the molecule(Ikegami, 1968).

For each salt, the concentrations investigated ranged from a minimum level of 0.01 M to a maximum of 2.0 M. As mentioned previously, the concentration ranges were not constant among the various salts mainly due to limitations imposed by salt and/or protein solubility characteristics. However, for comparative purposes, the influences of most salts on vicilin were assessed at concentrations representing two levels of salt action. Specifically, concentrations at which salt effects on the protein were primarily electrostatic were contrasted with concentrations at which salt effects were more lyotropic, or related to interactions with the solvent. This

Figure 3.2. The effect of increasing the concentration of various sodium salts on the denaturation temperature (Td) of vicilin.

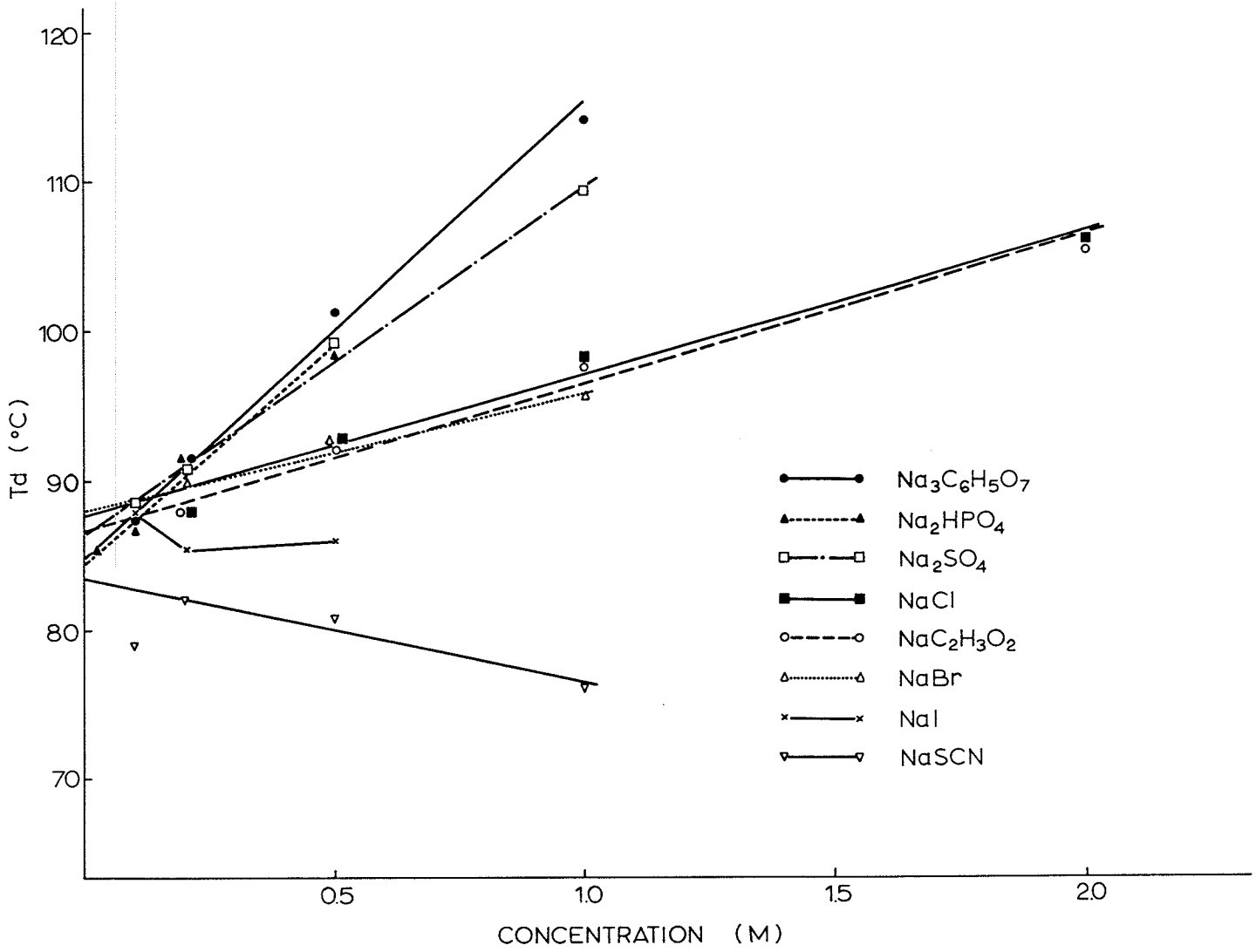
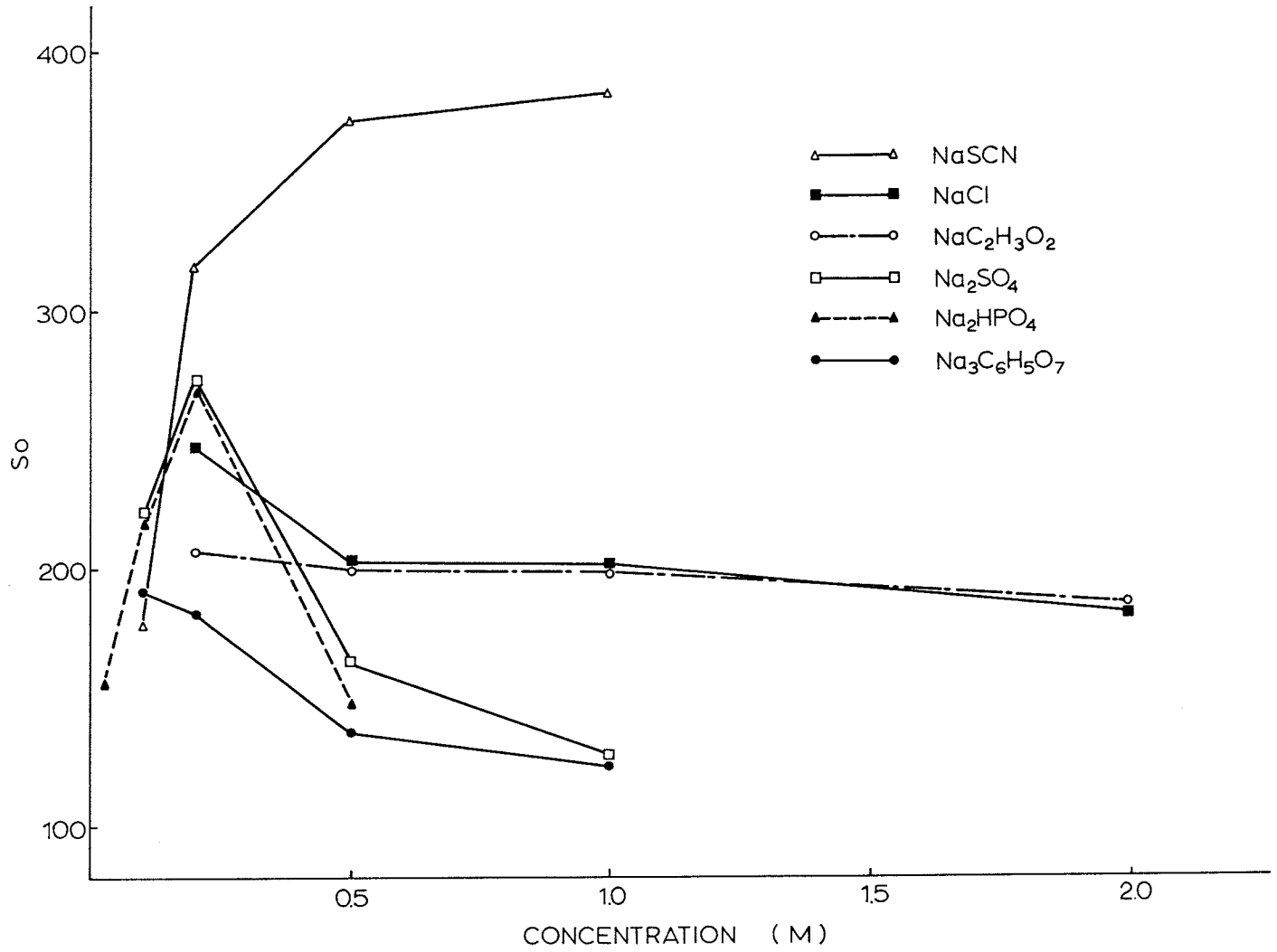

120
110
100
90
80
70
Td (°C)
0.5
1.0
1.5
2.0
CONCENTRATION (M)
$Na_3C_6H_5O_7$
Na_2HPO_4
Na_2SO_4
NaCl
$NaC_2H_3O_2$
NaBr
NaI
NaSCN

Figure 3.3. The effect of increasing the concentration of various sodium salts on the surface hydrophobicity (So) of vicilin.


400
300
200
100
So
0.5
1.0
1.5
2.0
CONCENTRATION (M)
NaSCN
NaCl
$NaC_2H_3O_2$
Na_2SO_4
Na_2HPO_4
$Na_3C_6H_5O_7$

is not an easily defined boundary as these represent two extreme types of salt behavior with various intermediate situations possible(Eagland, 1975). Although identification of an absolute transition point is difficult, it is often possible to attribute a particular salt effect to either a dominant electrostatic or lyotropic influence. The actual mode of influence of a particular salt on a protein is dependent upon ionic strength levels rather than molar concentrations. In this respect, electrostatic salt effects predominate at low ionic strengths; these, in turn, usually become negligible from an ionic strength(μ) of 0.5 to 1.0(von Hippel and Schleich, 1969).

Using this guideline, the effects of all univalent anionic salts in this study were considered to be electrostatic at 0.1 and 0.2 M(μ = 0.1 and 0.2) and predominantly lyotropic at 0.5 and 1.0 M(μ = 0.5 and 1.0). For the divalent sulfate salts, only effects at 0.1 M(μ = 0.3) were mainly electrostatic; all other concentration levels($\mu \geq 0.6$) were considered to be predominantly lyotropic. Although the phosphate medium should be similar to the sulfate, the pH 6.6 conditions were expected to result in approximately equal populations of the univalent($H_2PO_4^-$) and the divalent(HPO_4^{-2}) phosphate anions. As a result, it is expected that electrostatic salt effects prevail at 0.1 and 0.2 M($\mu \simeq 0.2$ and 0.4) whereas lyotropic effects are dominant at 0.5 and 1.0 M($\mu \simeq$ 1.0 and 2.0) For sodium citrate, lyotropic influences appear to be dominant at all concentration levels(0.1 to

1.0 M; μ 0.45); although electrostatic effects may still be important at 0.1 M.

The variations that occurred in vicilin conformational parameters can be considered from two viewpoints - first, the changes in structural characteristics with increasing salt concentration within an individual salt; secondly, the variations in effects among the different salts. The thermal properties of vicilin were considered initially from the former perspective, specifically, structural changes that occurred at different concentrations of a single anion. With most anions, there was an increase in Td with an increase in salt concentration; an observation that implies an increased stabilization of the vicilin molecule(Tables 3.2 and 3.3; Figure 3.2). The only exceptions to this trend were exhibited by vicilin exposed to NaI and NaSCN. In fact, for all other anions, there was a significant positive correlation between salt concentration and Td, as defined by the relationships given in Table 3.5. The destabilizing influences of NaI and NaSCN on vicilin, as evidenced by the depression of Td values, is illustrated in Figure 3.2. With NaI, there was a significant decrease in Td from 0.1 to 0.2 M; the Td then remained constant to 0.5 M(Table 3.3, Figure 3.2). With vicilin exposed to NaSCN, there was a significant negative correlation between Td and salt concentration as defined by the relationship given in Table 3.5. As a result of the effect observed with NaI and NaSCN, the anionic components of these salts will be referred to as "destabilizing anions". In contrast, the anionic compo-

TABLE 3.5. Relationships between pairs of variables as defined by standard linear regression analysis. All equations are for vicilin exposed to various sodium salts.

A. Td(°C) as a function of salt concentration(M).

i. acetate	:	Td = 9.62 M + 86.6	(r = 0.9784, P < 0.001)
ii. bromide	:	Td = 7.65 M + 88.0	(r = 0.9440, P < 0.001)
iii. chloride	:	Td = 9.47 M + 87.4	(r = 0.9856, P < 0.001)
iv. citrate	:	Td = 30.5 M + 84.8	(r = 0.9847, P < 0.001)
v. phosphate	:	Td = 29.5 M + 84.4	(r = 0.9697, P < 0.001)
vi. sulfate	:	Td = 23.4 M + 86.4	(r = 0.9942, P < 0.001)
vii. thiocyanate	:	Td = -6.72 M + 83.2	(r = -0.7555, P< 0.001)

B. Td(°C) as function of σ(dyn g cm^{-1} mol^{-1}).
All concentrations refer to groups of sodium salts.

i. 0.2 M	:	Td = 2.91 σ + 83.3	(r = 0.8158, P < 0.001)
ii. 0.5 M	:	Td = 7.21 σ + 80.3	(r = 0.9241, P < 0.001)
iii. 1.0 M	:	Td = 12.6 σ + 75.9	(r = 0.9371, P < 0.001)

C. So as a function of σ(dyn g cm^{-1} mol^{-1}).

i. 0.5 M	:	So = -74.0 σ + 346	(r = -0.7699, P < 0.001)
ii. 1.0 M	:	So = -84.6 σ + 369	(r = -0.8369, P < 0.001)

D. Td(°C) as a function of So.

i. 0.5 M	:	Td = -0.085 So + 111	(r = -0.9685, P < 0.001)
ii. 1.0 M	:	Td = -0.139 So + 128	(r = -0.9847, P < 0.001)

nents of all other sodium salts examined will be designated as "stabilizing anions". With the exception of the inclusion of bromide as a stabilizing anion, this description is similar to that used by von Hippel and Wong(1964) in relation to the anionic stabilization of ribonuclease.

In terms of other thermal parameters, ΔH values for vicilin in the stabilizing anionic environments followed one of two patterns. The ΔH values either paralleled the changes in Td values by exhibiting an overall increase with increasing salt concentration, especially at the higher molarities, or they showed no significant change over the salt concentration range used(Tables 3.2 and 3.3). For example, vicilin exposed to the sulfate, acetate, chloride and bromide anions showed a significant increase in ΔH at some concentration level. However, vicilin in citrate and phosphate environments was characterized by a constant ΔH value over the entire range of salt concentrations.

The differential in the effect of the destabilizing anions on the thermal properties of vicilin was not as distinct for ΔH considerations as for Td values. For example, with vicilin exposed to NaI, there was no significant change in ΔH values as the salt concentration increased. With NaSCN, however, the destabilizing influence was apparent. The ΔH values decreased significantly from 3.75 cal g^{-1} at 0.1 M NaSCN to 1.01 cal g^{-1} at 1.0 M NaSCN.

Changes in 1/2bw were not particularily informative with respect to the stabilization or destabilization phenomena.

Some significant changes in 1/2bw did occur within the different salt groups; however, no specific trends were apparent In fact, for most salts, there was no significant change in 1/2bw with changes in concentration.

Results from So determinations for vicilin were similar to those from the thermal analyses in that conformational changes were observed with increases in the concentration of specific anions(Tables 3.2 and 3.3, Figure 3.3). In general, the stabilizing anions caused a decrease in vicilin So with an increase in salt concentration. However, some exceptions to this generalization were observed. With vicilin exposed to sodium phosphate, the eventual decrease in So with increasing salt concentration was preceeded by a significant So increase from 0.025 to 0.2 M. A nearly identical trend seemed to exist for Na_2SO_4; however, the apparent So increase from 0.1 to 0.2 M was not statistically significant. Increases in concentration of the chloride and acetate anions had virtually no effect on the So properties of vicilin; all So values were similar with the exception of a significantly higher So for 0.2 M NaCl as compared with other NaCl concentrations. With respect to the destabilizing anions, only the effects of thiocyanate on So could be assessed due to the quenching nature of the iodide moiety. In this case, there was a significant increase in So with increasing NaSCN concentration, especially from 0.1 to 0.2 M. As a result of these data the stabilizing and destabilizing anions appeared to have opposing effects on both So characteristics and thermal properties of

vicilin.

To this point, the results have been presented with a view to examining the effects of increasing the concentration of a specific anion on the conformational properties of vicilin. In order to assess the relative significance of various noncovalent forces with respect to vicilin structural stability, it was essential to compare the effects of different anions at different concentration levels on vicilin conformational properties. Accordingly, most anionic comparisons were performed among results obtained at constant molar concentrations with consideration given to ionic strength values for the multivalent salts. Using this approach, Tables 3.6, 3.7 and 3.8 were constructed.

These tables relate So values as well as specific thermal properties(Td and ΔH) for vicilin to the molal surface tension increment(σ) of each salt at most concentrations examined. This value(σ) represents a numerical assessment for the position of an anion within the Hofmeister series; as a result, it has been correlated directly with the ability of an anion to stabilize or destabilize protein structure(Melander and Horvath, 1977). However, in this correlation, salt concentrations are critical. At low ionic strength values($\mu < 0.5$), the electrostatic influence of the anions is not related to σ values; at higher salt levels($\mu > 0.5$), the predominant effect of anions is usually a lyotropic interaction with the solvent and σ considerations are fundamental(Melander and Horvath, 1977). In this study, the influence of all salts at 0.5 and

TABLE 3.6. Denaturation temperature (Td) values for vicilin in various concentrations of different sodium salts. Salts are listed in sequence according to their molal surface tension increments (σ).

Salt	σ[2]	Td (°C) 0.1 M	0.2 M	0.5 M	1.0 M
NaSCN	0.60[3]	79.0±3.0	82.3±0.8[a]	80.8±1.2	75.9±0.7
NaI	1.02[4]	87.8±0.2[a]	85.4±1.7[a]	86.1±0.7	nd[5]
Na-acetate	1.27[4]	nd[5]	87.7±0.7[a]	91.5±0.7	94.8±0.8[a,b]
NaBr	1.32[4]	nd[5]	89.8±1.2[b,c]	92.4±0.4[a]	95.4±0.7[a]
NaCl	1.64[4]	nd[5]	88.0±0.8[a,b]	92.5±0.6[a]	98.0±0.0[b]
Na_2HPO_4	2.02[3]	86.7±0.9[a]	91.5±1.1[c]	98.8±0.8[b]	nd[5]
Na_2SO_4	2.73[3]	88.5±0.8[a]	90.6±0.5[c]	99.1±0.5[b]	109.3±0.4
Na_3-citrate	3.27[3]	86.8±0.8[a]	91.3±1.6[c]	101.5±2.3[b]	114.6±1.4

[1] Column values followed by the same letter are not significantly different ($P \leq 0.05$). Statistical comparisons apply to a single salt concentration.

[2] Units are 10^{-3} dyn g cm^{-1} mol^{-1}

[3] Melander and Horvath (1977).

[4] International Critical Tables (1929).

[5] Value not determined due to solubility limitations.

TABLE 3.7. Denaturation enthalpy (ΔH) values for vicilin in various concentrations of different sodium salts. Salts are listed in sequence according to their molal surface tension increments (σ).

Salt	σ[2]	ΔH (cal g^{-1}) 0.1 M	0.2 M	0.5 M	1.0 M
NaSCN	0.60[3]	3.75±0.19[a]	2.76±0.61[a,b]	2.58±0.46[a,b]	1.01±0.19
NaI	1.02[4]	1.83±0.16[b]	2.88±0.24[a]	2.65±0.80[a,b]	nd[5]
Na-acetate	1.27[4]	nd[5]	2.56±0.80[a,b]	2.50±0.17[a]	3.49±0.23[a]
NaBr	1.32[4]	nd[5]	2.28±0.80[b]	4.08±0.35[c]	3.43±0.16[a]
NaCl	1.64[4]	nd[5]	3.63±0.13[c]	3.49±0.62[b,c]	3.84±0.01
Na_2HPO_4	2.02[3]	2.48±0.82[b,c]	2.28±0.15[b]	2.73±0.79[a,b]	nd[5]
Na_2SO_4	2.73[4]	2.74±0.25[c]	3.06±0.90[c]	3.37±0.30[b.c]	4.39±0.22
Na_3-citrate	3.27[3]	3.07±0.67[c]	4.19±0.55[c]	nd[6]	nd[6]

[1] Column values followed by the same letter are not significantly different ($P \leq 0.05$). Statistical comparisons apply to a single salt concentration.

[2] Units are 10^{-3} dyn g cm^{-1} mol^{-1}.

[3] Melander and Horvath (1977).

[4] International Critical Tables (1929).

[5] Value not determined due to solubility limitations.

[6] Value not determined due to large post-denaturation exotherms.

TABLE 3.8. Surface hydrophobicity (So) values for vicilin in various concentrations of different sodium salts. Salts are listed in sequence according to their molal surface tension increments (σ).

Salt	σ[2]	So 0.1 M	0.2 M	0.5 M	1.0 M
NaSCN	0.60[3]	179±37[a]	318±25	373±56	384±52
NaI	1.02[4]	nd[5]	nd[5]	nd[5]	nd[5]
Na-acetate	1.27[4]	nd[6]	207±24[a]	201±15[a]	200±35[a]
NaBr	1.32[4]	nd[5]	nd[5]	nd[5]	nd[5]
NaCl	1.64[4]	nd[6]	248±18[b]	202±16[a]	201±9[a]
Na_2HPO_4	2.02[3]	219±16[a]	269±60[a,b]	148±44[b,c]	nd[6]
Na_2SO_4	2.73[4]	220±4[a]	273±39[b]	164±11[b]	129±15[b]
Na_3-citrate	3.27[3]	191±23[a]	183±18[a]	137±8[c]	124±11[b]

[1] Column values followed by the same letter are not significantly different ($P<0.05$). Statistical comparisons apply to a single salt concentration.

[2] Units are 10^{-3} dyn g cm^{-1} mol^{-1}

[3] Melander and Horvath (1977)

[4] International Critical Tables (1929).

[5] Value not determined due to quenching effect of anion.

[6] Value not determined due to solubility limitations.

1.0 M was considered to be lyotropic; for 0.1 and 0.2 M, most salts had an electrostatic effect. To elaborate on the latter generalization, the ionic strength values for sodium citrate at 0.1 and 0.2 M(μ = 0.45 and 1.2) and sodium sulfate at 0.2 M(μ = 0.6) were such that these specific environments were considered initially to have a predominantly lyotropic influence on vicilin properties.

In terms of relationships between thermal properties of vicilin and σ, there was no correlation between Td and σ at 0.1 M salt concentrations(Table 3.6). At all other concentrations(0.2 M, 0.5 M, 1.0 M), there was a significant positive correlation between Td and σ defined by the relationships given in Table 3.5. A comparison of the correlation coefficients for these three relationships indicated an increasing degree of correlation between the two variables with increasing salt concentrations.

The relationship between ΔH and σ was not as clearly defined as that for Td and σ(Table 3.7). There was no correlation between the two variables at 0.1, 0.2 and 0.5 M salt concentrations; however, a significant positive relationship existed at 1.0 M($\Delta H = 1.51\ \sigma + 0.89$; $r = 0.8491$, $P < 0.001$).

From previous considerations of the influence of individual electrolytes on vicilin properties, it would appear that the stabilizing phenomenon observed with increasing concentrations of some anions is also frequently characterized by a progressive decrease in the So of vicilin. This was especially apparent at the higher salt concentrations. In this respect,

Td and So showed a significant negative correlation for the anions of sodium salts at concentrations of 0.5 and 1.0 M. Equations describing these relationships are given in Table 3.5. As a result, it would be expected that So and σ might also show an inverse relationship, particularily at 0.5 and 1.0 M salt concentrations where lyotropic effects for all experimental salts are important. Therefore, So-σ correlations were examined for all salt concentrations. At 0.1 M, there was no significant difference among the So values(Table 3.8). At 0.2 M, a trend became more apparent in that the extreme values were correlated. The highest So for vicilin(318) was associated with a thiocyanate environment having the lowest σ(0.6 dyn g cm^{-1} mol^{-1}), whereas the lowest So(183) was associated with a citrate medium having the greatest σ(3.27 dyn g cm^{-1} mol^{-1}). The intermediate So values did not exhibit a hierarchical pattern. As anticipated, at 0.5 and 1.0 M salt concentrations, there was a significant negative correlation between So and σ defined by the relationship given in Table 3.5. In summary, the tendency for the thermal properties and surface hydrophobicity of vicilin to be correlated with σ increased with increasing salt concentrations.

B. Effects of Specific Cations

As the main effects of electrolytes on protein conformational characteristics have been attributed to the anionic components(Robinson and Jencks, 1965), only a limited examination of the influence of cations on the thermal properties and So of vicilin was undertaken in this study. Three cations were

selected - sodium, potassium and lithium in association with each of the two stabilizing anions, chloride and sulfate. The results are presented in Tables 3.9 and 3.10 and Figures 3.4 to 3.7. In an assessment of the results, the first consideration was the general effects of increasing salt concentration on vicilin conformational parameters. In all instances, similar to the results associated with the stabilizing anions, there was a significant positive correlation between Td and salt concentration as described by the equations given in Table 3.11. However, ΔH and 1/2bw values remained relatively constant within the individual salts. The only notable exception was a significant increase in ΔH to 4.99 cal g^{-1} at 2.0 M NaCl(Table 3.9).

The influence of increasing salt concentration on the So characteristics of vicilin varied among the different cations. For both lithium and potassium, in association with either the chloride or sulfate anions, the values of So were relatively constant with increasing salt concentration(Tables 3.9 and 3.10; Figures 3.6 and 3.7). In contrast, there was a significant decrease in vicilin So with increasing concentrations of both NaCl and Na_2SO_4. The only deviation from the progressive decrease in So for the two sodium salts was a significant initial increase in the So value for vicilin in 0.2 M Na_2SO_4 as compared with 0.1 M Na_2SO_4(Figure 3.6).

Attempts to correlate stabilizing or non-stabilizing effects of the different cations at constant concentration levels(most importantly at $\mu > 0.5$) with σ, the molal surface

TABLE 3.9. Thermal properties (Td, ΔH, ½bw) and So values for vicilin in various concentrations of lithium potassium and sodium chloride. The pH was an uncontrolled parameter.

Conc. (M)	pH	Td (°C)	ΔH (cal g^{-1})	½bw (°C)	So
Lithium chloride					
0.1	6.5	86.0±0.0	2.48±0.92[a]	8.5±1.2[a,b]	279±31[a]
0.2	6.2	88.5±0.4	2.68±0.63[a]	12.9±0.9[a]	278±38[a]
0.5	6.4	90.5±0.8	3.26±0.26[a]	10.4±0.5[b]	246±21[a]
1.0	6.3	95.5±0.9	3.96±0.85[a]	9.6±1.2[b]	229±42[a]
Potassium chloride					
0.1	6.5	nd[2]	nd[2]	nd[2]	212±8[a]
0.2	6.2	86.5±0.0	2.87±0.41[a]	10.3±0.8[a]	236±24
0.5	6.3	93.5±0.5	3.36±0.07[a]	10.6±0.7[a]	197±20[a]
1.0	6.2	95.9±0.5	3.59±0.50[a]	10.3±0.6[a]	199±28[a]
Sodium chloride					
0.2	6.3	88.0±0.8	3.36±0.13[a]	11.6±0.69[a]	248±18
0.5	6.5	92.5±0.6	3.49±0.62[a]	9.9±0.5[b]	202±16[a]
1.0	6.2	98.0±0.0	3.84±0.01[a]	10.4±0.4[a,b]	201±9[a]
2.0	6.4	105.8±0.8	4.99±0.45	10.5±0.1[a,b]	184±5

[1] Column values followed by the same letter are not significantly different ($P \leq 0.05$). Statistical comparisons only apply to a single salt group.

[2] Value not determined due to solubility limitations.

TABLE 3.10. Thermal properties (Td, ΔH, ½bw) and So values for vicilin in various concentrations of lithium, potassium and sodium sulfate.

Conc. (M)	pH	Td (°C)	ΔH (cal g^{-1})	½bw (°C)	So
Lithium sulfate					
0.1	6.6[2]	91.5±1.1	3.48±0.44[a]	10.6±0.6[a]	179±20[a]
0.2	6.6	93.5±1.0	3.67±0.71[a]	11.0±0.3[a]	144±25[a]
0.5	6.6	98.8±0.8	4.03±0.24[a]	11.1±0.9[a]	162±8[a]
Potassium sulfate					
0.1	6.4	88.7±0.7[a]	2.63±0.72[a]	10.1±0.1[a]	296±30[a]
0.2	6.3	90.2±2.0[a]	3.42±0.45[a]	10.2±1.8[a]	301±27[a]
0.5	6.3	98.9±2.0	3.27±0.45[a]	11.4±1.4[a]	293±36[a]
Sodium sulfate					
0.1	6.6	88.5±0.8	2.74±0.25[a]	9.8±0.4[a]	220±4
0.2	6.6	90.6±0.5	3.06±0.90[a,b]	10.4±0.2[a]	273±39
0.5	6.5	99.1±0.5	3.37±0.30[a,b]	10.0±0.6[a]	164±11
1.0	6.6	109.3±0.4	4.39±0.22[a]	10.2±0.8[a]	129±15

[1] Column values followed by the same letter are not significantly different ($P \leq 0.05$). Statistical comparisons only apply to a single salt group.

[2] pH adjusted to 6.6 for all concentration levels within this salt group.

Figure 3.4. Denaturation temperature (Td) as a function of salt concentration for vicilin exposed to three chloride salts.

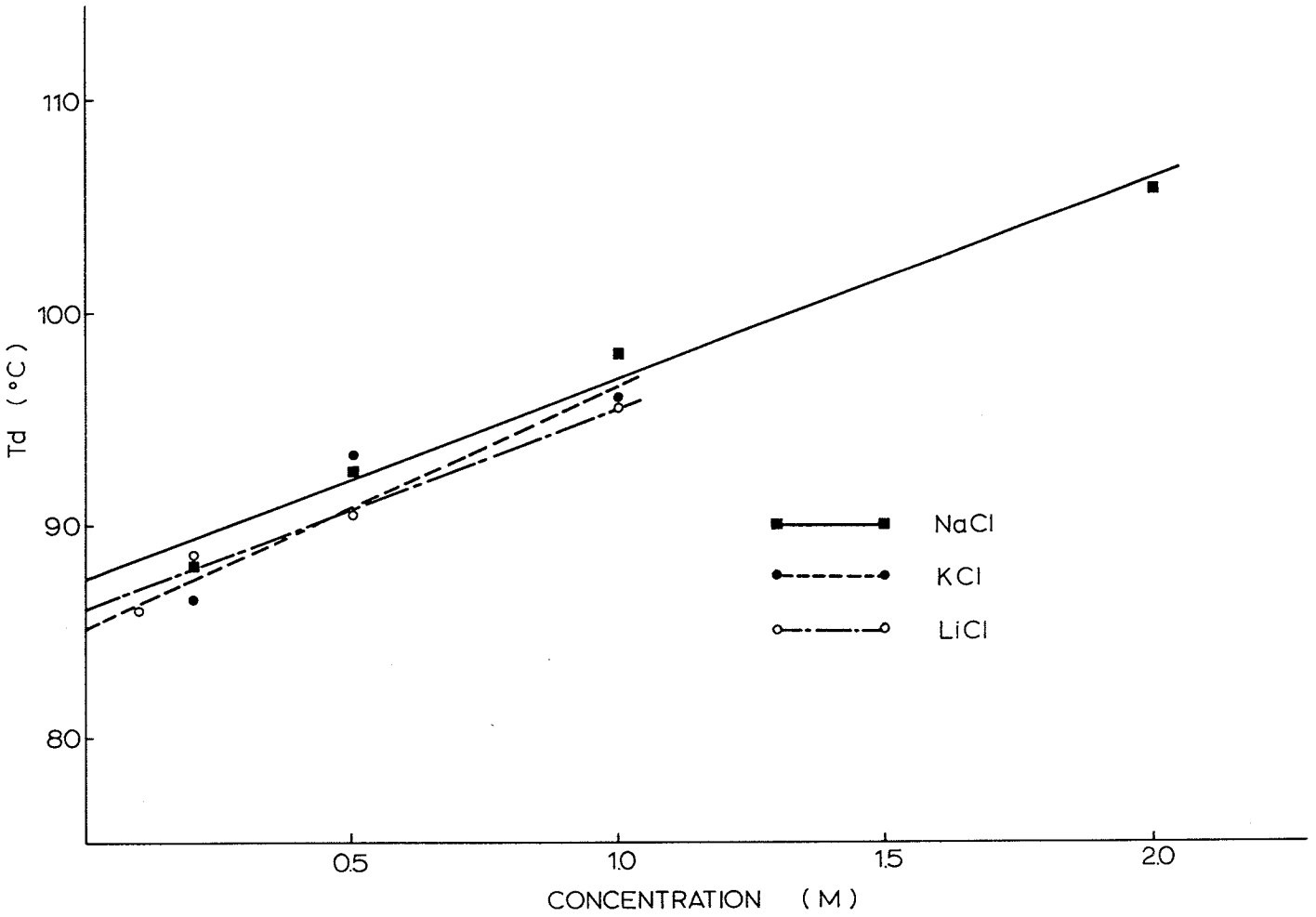

110
100
90
80
Td (°C)
0.5
1.0
1.5
2.0
CONCENTRATION (M)
NaCl
KCl
LiCl

Figure 3.5. Denaturation temperature (Td) as a function of salt concentration for vicilin exposed to three sulfate salts.

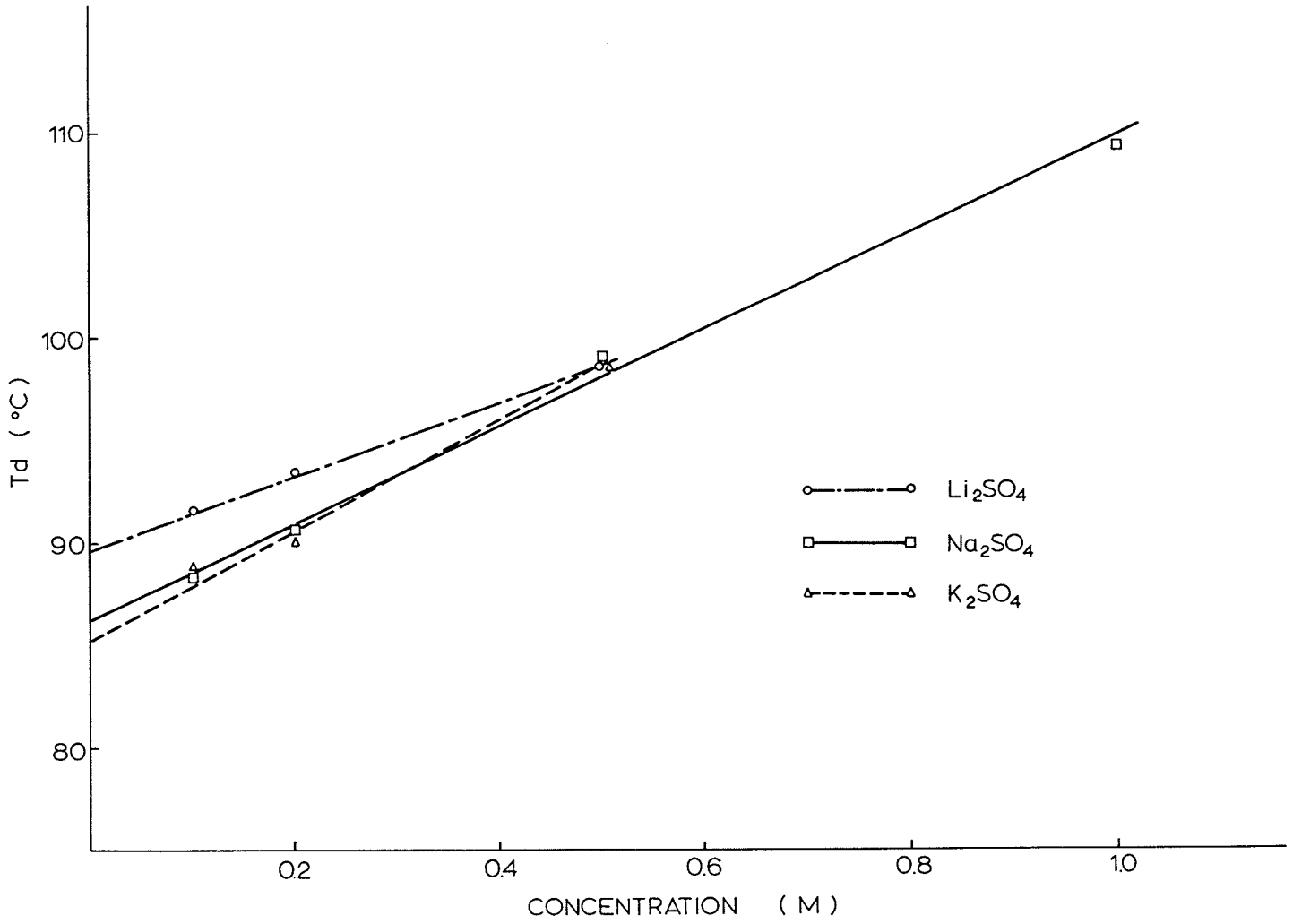

110
100
90
80
Td (°C)
0.2
0.4
0.6
0.8
1.0
CONCENTRATION (M)
Li2SO4
Na2SO4
K2SO4

Figure 3.6. Surface hydrophobicity (So) as a function of salt concentration for vicilin exposed to three chloride salts.

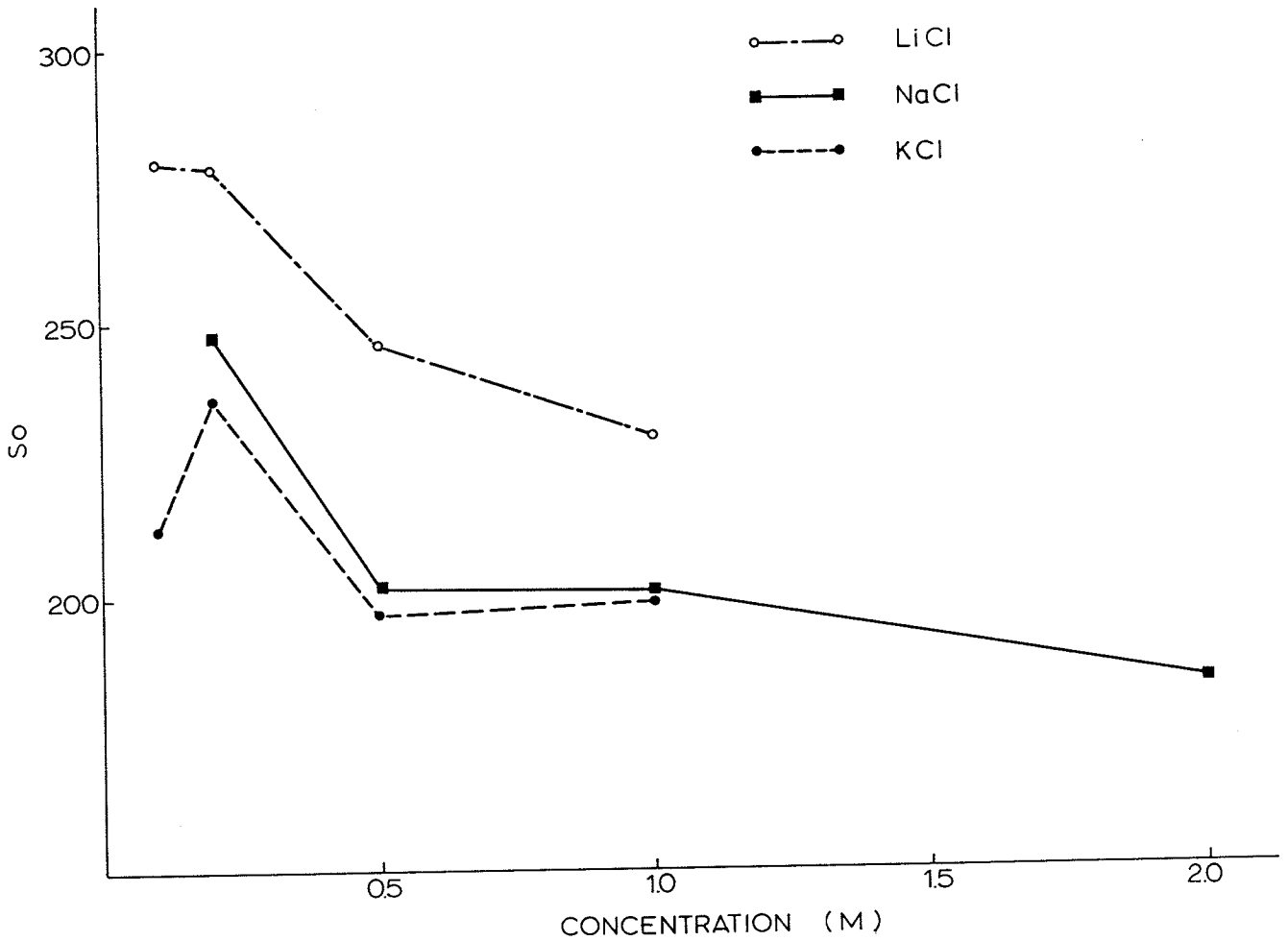

LiCl
NaCl
KCl
300
250
200
So
0.5
1.0
1.5
2.0
CONCENTRATION (M)

Figure 3.7. Surface hydrophobicity (So) as a function of salt concentration for vicilin exposed to three sulfate salts.

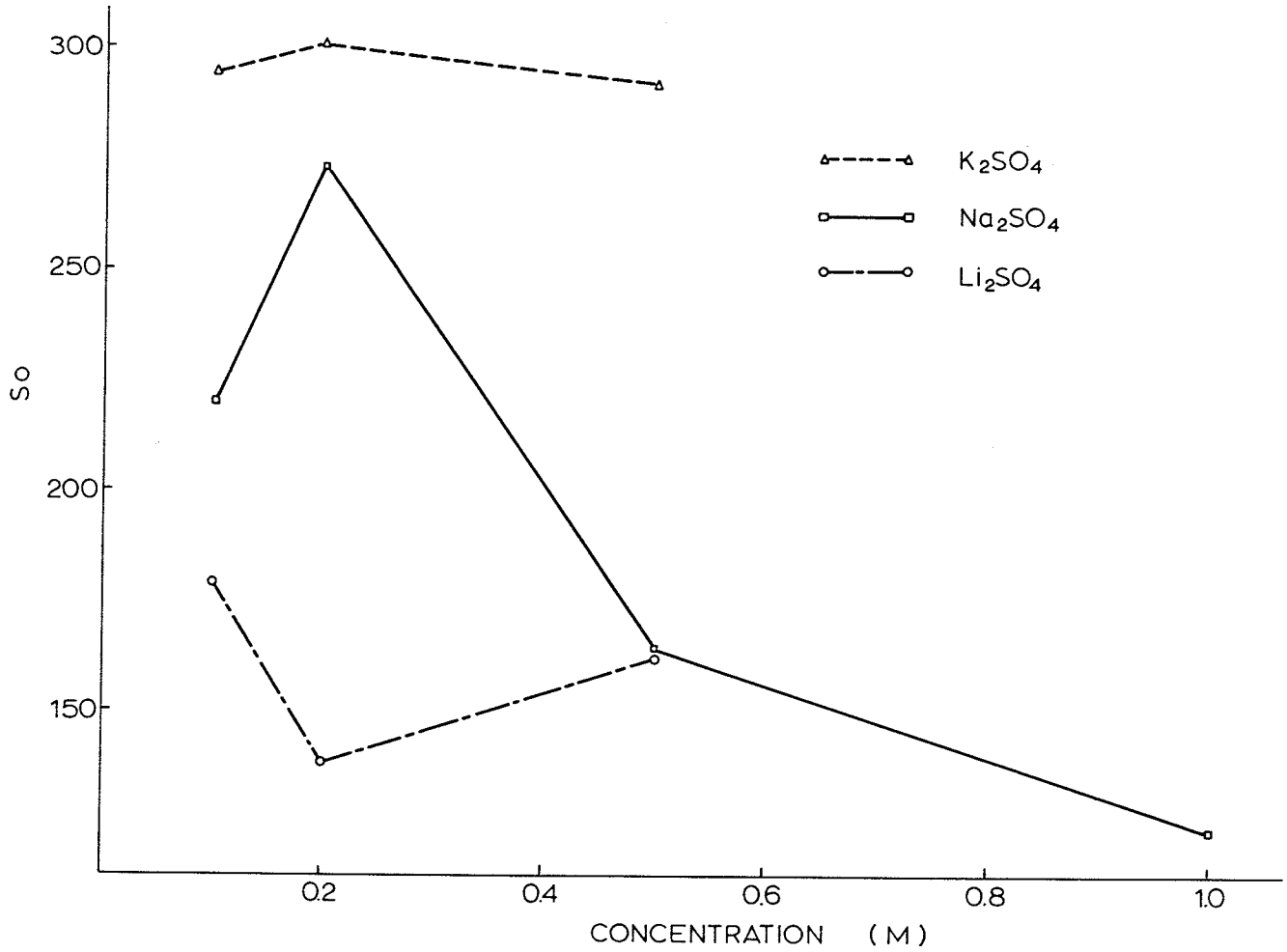

300
250
200
150
So
0.2
0.4
0.6
0.8
1.0
CONCENTRATION (M)
K2SO4
Na2SO4
Li2SO4

TABLE 3.11. Relationships between Td(°C) and salt concentrations(M) as defined by standard linear regression analysis. All equations are for vicilin exposed to various electrolytes.

i. LiCl	:	Td = 9.36 M + 86.1	(r = 0.9694, P < 0.001)
ii. KCl	:	Td = 11.6 M + 85.1	(r = 0.9071, P < 0.001)
iii. NaCl	:	Td = 9.47 M + 87.4	(r = 0.9856, P < 0.001)
iv. Li_2SO_4	:	Td = 18.0 M + 89.8	(r = 0.9530, P < 0.001)
v. K_2SO_4	:	Td = 27.1 M + 85.2	(r = 0.9345, P < 0.001)
vi. Na_2SO_4	:	Td = 23.4 M + 86.4	(r = 0.9942, P < 0.001)

tension increment of a salt, were not successful as with the various anions(Tables 3.12 and 3.13). One of the major drawbacks appeared to be the similarity of σ values among the three cations. Although no statistical correlation existed between σ and the parameters Td, ΔH or So, some of the trends observed in the cationic studies were similar to the relationships established with the anions. For example, the highest Td values for vicilin exposed to sulfate salts were observed with Li_2SO_4, the salt having the highest σ within this group. In contrast to the Td-σ relationship for the anions, significantly different Td values were only observed at 0.1 and 0.2 M; at 0.5 M, there was no difference in the Td values among the three sulfate salts(Table 3.13). The thermal stability-σ relationship differed with the chloride salts. At 0.2 and 0.5 M there was no difference in Td values; however, at 1.0 M, NaCl, with the highest σ in this salt group, had the most stabilizing effect(Table 3.12).

With respect to the So of vicilin, the results varied between the sulfate and chloride salts. A similar relationship to that of the anions appeared to exist with respect to the sulfate salts. The highest So values were associated with K_2SO_4, having the lowest σ; the lowest So values were associated with Li_2SO_4 having the greatest σ(Table 3.13). However, unlike the anions, this trend occurred at all concentration levels. With the chloride salts, the different cations had little apparent effect on So values. The So values for vicilin were relatively constant at all concentration levels.

TABLE 3.12. Thermal properties (Td, ΔH) and So values for vicilin in various concentrations of three chloride salts. Salts are listed in sequence according to their molal surface tension increments (σ).

Salt	σ^2	Concentration 0.2 M	0.5 M	1.0 M
		Td (°C)		
KCl	1.49	86.5±0.0[a]	93.5±2.2[a]	95.9±0.5[a]
LiCl	1.63	88.5±0.4[b]	90.5±0.8[a]	95.5±0.9[a]
NaCl	1.64	88.0±0.8[b]	92.6±0.6[a]	98.0±0.0
		ΔH (cal g^{-1})		
KCl	1.49	2.87±0.41[a]	3.36±0.07[a]	3.59±0.50[a]
LiCl	1.63	2.68±0.92[a]	3.26±0.26[a]	3.96±0.85[a]
NaCl	1.64	3.63±0.13[a]	3.49±0.62[a]	3.84±0.01[a]
		So		
KCl	1.49	236±24[a]	197±20[a]	199±28[a]
LiCl	1.63	278±38[a]	246±21[a]	229±42[a]
NaCl	1.64	248±18[a]	202±16[a]	201±9[a]

[1] Column values followed by the same letter are not significantly different ($P \leq 0.05$). Statistical comparisons apply to a single parameter at one concentration level.

[2] International Critical Tables (1929). Units are 10^{-3} dyn cm^{-1} mol^{-1}.

TABLE 3.13. Thermal properties (Td, ΔH) and So values for vicilin in various concentrations of three sulfate salts. Salts are listed in sequence according to their molal surface tension increments (σ).

Salt	σ^2	Concentration 0.1 M	0.2 M	0.5 M
			Td (^oC)	
K_2SO_4	2.58	88.7±0.7[a]	90.2±2.0[a]	98.9±2.0[a]
Li_2SO_4	2.73	88.5±0.8[a]	90.6±0.5[a]	99.1±0.5[a]
Na_2SO_4	2.78	91.5±1.1	93.5±1.0	98.8±0.8[a]
			H (cal g^{-1})	
K_2SO_4	2.58	2.63±0.72[a]	3.42±0.45[a]	3.27±0.45[a]
Li_2SO_4	2.73	2.74±0.25[a]	3.06±0.90[a]	3.37±0.30[a]
Na_2SO_4	2.78	3.48±0.44[a]	3.67±0.71[a]	4.03±0.24[a]
			So	
K_2SO_4	2.58	296±30	301±27[a]	293±36
Li_2SO_4	2.73	220±4	273±39[a]	164±11[a]
Na_2SO_4	2.78	179±20	144±25	162±8[a]

[1] Column values followed by the same letter are not significantly different ($P \leq 0.05$). Statistical comparisons apply to a single parameter at one concentration level.

[2] International Critical Tables (1929).
Units are 10^{-3} dyn cm^{-1} mol^{-1}.

In summary, the variations in the conformational responses of vicilin to different cationic environments were not dramatic; however, cationic stabilizing influences differed between the two types of associated anions. With the chloride anion, sodium appeared to have the most stabilizing influence whereas with the sulfate, a differential effect among the cations was not established.

DISCUSSION

Influence of pH on Vicilin Conformational Parameters

Dependence of protein conformational parameters on environmental properties is a consequence of the basic structural design of a protein molecule. Maximum stability of the protein macromolecule in an aqueous environment may be achieved if all hydrophobic residues are oriented toward the molecular interior and all hydrophilic residues are exposed to complete solvation. However, as a protein is a product of the specific sequence of hydrophilic and hydrophobic residues, it cannot completely attain this thermodynamically favorable orientation of the two groups of amino acids(Fisher, 1965). In fact, even a series of residues with nonpolar side-chains cannot provide an area of complete hydrophobicity, as these amino acids are covalently linked by hydrophilic peptide bonds(von Hippel and Schleich, 1969). The result of this overall structural arrangement is the marginal stability of a protein molecule and the susceptibility of its conformation to environmental parameters(Cooper, 1980).

Exposure of proteins to media containing various neutral salts is known to be one method of provoking protein conformational changes as a result of solvent modification(von Hippel and Schleich, 1969). By definition, neutral salts are considered to be strong electrolytes which are significantly soluble in water without bringing about a major change in solution pH(von Hippel and Schleich, 1969). Despite this definition, the pH of different neutral salt media may show considerable

variation. In this study, therefore, the possible impact of pH on the conformational properties of vicilin represented an additional variable in the assessment of the influence of various electrolytes on the structural integrity of the protein. To pursue this potential complication of combined pH-electrolyte effects, the influence of limited pH variations on some structural properties of vicilin maintained in a sodium phosphate environment at a constant molar concentration(0.1 M) was investigated. The observed changes in some of the examined conformational properties over the pH range 6.0 to 8.0 appeared to reflect subtle structural changes in vicilin with increasing pH, especially from pH 7.0 to 8.0. The relatively stable ΔH values over the entire pH range were responsible for the designation of these conformational changes as "subtle" rather than "major".

In terms of specific thermal parameters, there was an overall decrease in Td and an overall increase in 1/2bw with increasing pH(Table 3.4). Similar changes in these thermal properties were reported by Privalov and Khechinashvili(1974) in an analysis of five globular proteins as pH conditions were shifted away from the isoelectric points of the individual proteins. An overall decrease in Td for vicilin from pH 6.0 to 8.0 was indicative of a destabilization of the molecule; an overall increase in 1/2bw, especially from pH 7.0 to 8.0, may have reflected a gradual distortion in subunit association accompanied by a reduction in cooperativity within the multimeric molecule. Due to the low ionic strength environ-

ment($\mu \simeq 0.2$), both thermal responses may be related to changes in the charge profile of the vicilin molecules. As the pH increased above the isoelectric point(for vicilin, pH 5.0), the overall negativity of the molecule increased. This increase in repulsive negative surface charge may have stressed the structure and induced conformational changes of varying magnitudes within the molecule. In addition, Perutz(1978) suggested that progressive ionization of internal residues results in the attraction and incorporation of numerous hydration shells. This leads to a shift in the equilibrium from a native to an altered conformation. In addition, the contribution of all internal residues to overall molecular stability is not equal; the ionization of some residues may exert more deleterious effects than others. Consequently, molecular destabilization is a complex phenomenon not necessarily occurring in a sequential relationship with maniplulation of an environmental parameter such as pH.

Although the thermal parameters, 1/2bw and Td, appeared to reflect a gradual destabilization of vicilin, the corresponding decrease in So over the same pH range was not characteristic of a progressive unfolding of the molecule(Table 3.4). With preliminary studies, exposure of vicilin to a pH extreme of 10.0 resulted in an elevated So value of 500; an expected response if the conformation of the molecule was deteriorating. In contrast to this exposure of hydrophobic side-chains with more extreme alkaline pH values, the conformational fluctuations observed from pH 6.0 to 8.0 seemed to be

characterized by an increased burial of hydrophobic residues. This observation would appear to reinforce the concept of a gradual distortion in molecular structure with minimal pH changes being related to disturbed subunit associations at the quaternary level rather than a deterioration of more fundamental secondary structural arrangements.

It should be mentioned that although the influence of pH on vicilin structural characteristics was assessed at one electrolyte concentration(0.1 M), the relative proportion of the phosphate anions would be expected to shift with increasing pH from a dominant univalent anion($H_2PO_4^-$) at pH 6.0 to a dominant divalent anion(HPO_4^{-2}) at pH 8.0. A possible consequence of this effect is an increase in electrostatic shielding of charged surface residues by the divalent anion at higher pH values. Although this phenomenon might have an increased molecular stabilizing effect, this stabilization was not sufficient to negate the destabilizing influences of pH adjustment on vicilin as evidenced by the Td and 1/2bw thermal parameters.

The observation that vicilin conformational properties varied with changes in pH prompted a further investigation into the possibility of combined pH-electrolyte effects on specific protein structural parameters. As most variations in the previous study occurred from pH 7.0 to 8.0, emphasis was placed on the properties of vicilin in alkaline media versus media at pH 6.6. A comparison of the structural changes in vicilin exposed to these two different pH regimes(pH 6.6,

adjusted; alkaline, unadjusted) for each of the sodium salts showed some interesting trends. With vicilin exposed to sodium acetate, the thermal stability results differed from those expected on the basis of theoretical electrostatic-lyotropic effects. At low salt concentrations($\mu < 0.5$), electrostatic interactions of the protein and salt are a function of the net charge of the protein molecule and thus, are strongly pH dependent(Melander and Horvath, 1977). As a result, conformational responses to different pH levels were anticipated at salt concentrations of 0.2 M. However, there was no significant difference in the Td values for vicilin exposed to the two pH conditions at this concentration level(Table 3.2). At higher salt concentrations($\mu > 0.5$), pH influences should be reduced as the resulting ionic shielding makes the charged macromolecule effectively behave as a neutral dipole(Kirkwood, 1943). Despite this theoretical assessment, the Td values for vicilin at pH 6.6 were significantly higher than those for the alkaline media at 0.5 and 2.0 M concentration levels(Table 3.2). This anomolous behavior may be partially explained by the fact that acetate exhibits some binding to proteins at low and high salt concentrations(Arakawa and Timasheff, 1982). Although vicilin carries an overall negative charge in an alkaline environment, many positive residues are available for potential interaction with negatively charged ions such as acetate. The relative amount of acetate binding may be greater under alkaline conditions as a result of vicilin conformational changes. As a result, this salt-protein interaction

may induce slight molecular destabilization as evidenced by the lower Td values at 0.5 and 2.0 M.

Some interesting variations also occurred in the So values for vicilin exposed to sodium acetate. The most notable results were the lower So values for vicilin in the alkaline media as compared with those at pH 6.6 for salt concentrations of 0.2, 0.5 and 1.0 M(Table 3.2). These lower values may reflect a conformational response to an increased surface negativity resulting from either the pH differential at 0.2 M or the preferential binding of acetate at 0.5 and 1.0 M. At this point it should be mentioned that interpretations of the conformational implications of So values must be approached with some caution. For example, on a thermodynamic basis, it might be expected that a low So value reflects a highly stabilized conformation with a large number of the hydrophobic surface residues being removed from the aqueous environment. However, vicilin in 0.5 M sodium acetate, pH 7.9 had an So of 137 and a Td of 88.7°C; vicilin in a similar medium at pH 6.6 had an So of 201 and a significantly higher Td of 91.5°C. As a result of the Td values, the low So at pH 7.9 actually appears to be associated with a more destabilized conformation. This observation confirms the necessity of examining more than one molecular parameter in the assessment of the conformational response of a protein to its environment.

A similar pH investigation was repeated using sodium citrate as the electrolyte variable. Due to the trivalent status of the citrate anion and the range of sodium citrate con-

centrations used in this study, the response of vicilin to pH differences was associated with the lyotropic rather than the electrostatic influence of the salt. Even at the lowest citrate concentration(0.1 M), the ionic strength(μ = 0.45) was sufficient to elicit a predominantly lyotropic effect. With respect to thermal properties of vicilin exposed to the two types of citrate media, there was no effect of pH despite relatively high pH values of 8.6 and 8.7 at salt concentrations of 0.5 and 1.0 M, respectively(Table 3.3). There was, however, an opposite relationship between So and salt concentration for vicilin in the two pH environments. With vicilin in the alkaline sodium citrate, the So increased as a function of salt concentration from 127 at 0.1 M to 280 at 1.0 M; with the pH 6.6 environment, the So decreased from 191 at 0.1 M to 124 at 1.0 M(Table 3.2). The latter relationship is a more consistant response with the concept of preferential hydration, a lyotropic effect in which the solvent adjacent to the molecular surface becomes nearly devoid of salt with increasing salt concentrations(Arakawa and Timasheff, 1982). An expected molecular response to this hydration phenomenon would be a gradual decrease in So with increasing hydration, as observed with citrate at pH 6.6. In contrast, the overall increase in So values observed for vicilin in the alkaline environment appeared to represent a more unusual protein reaction. Molecular destabilization in terms of hydrophobic residue exposure seems to have occurred; this may be a consequence of citrate-protein binding, especially considering the increased ioniza-

tion of citrate at these pH levels. In fact, ionic binding at high salt concentrations is frequently accompanied by molecular destabilization(Bull and Breese, 1970; Arakawa and Timasheff, 1982). However, if citrate-protein binding did occur at alkaline pH values to create a destabilization effect, this phenomenon was not evidenced in the thermal properties of vicilin. As a result, the possibility of citrate preferential binding to vicilin at various pH levels can only be assessed with further experimentation.

Even from this restricted study, it is apparent that protein-electrolyte relationships are complex phenomena. Several factors are operative; these include the identity and concentration of the electrolyte and the pH of the protein-electrolyte environment. The effect of any one of these factors may or may not be influenced by the other two. As variations in salt concentration and identity were fundamental to further experimental studies, it appeared logical to eliminate pH as a potential variable. Since most pH complications appeared to be associated with an alkaline environment, it was decided to restrict pH variations to a range from 6.2 to 7.0. The pH value of 6.2 was a natural lower limit for further electrolyte studies as this represented the lowest pH value exhibited by any media used in subsequent experiments.

Effects of Specific Anions on Vicilin Conformational Parameters

A complete mechanistic explanation of the effects of neutral salts on protein conformational parameters is difficult

due to the complexity of multicomponent interactions. In general, however, the different structural influences of these electrolytes have been attributed to variations in the anionic constituents(Robinson and Jencks, 1965). The de-emphasis of cationic effects is a consequence of hydration requirements. As a result of extensive hydration shells, the cations are almost completely excluded from the layer of water at the protein interface; the anions, on the other hand, show a wide variation in their extent of exclusion due to their limited hydration spheres(Eagland, 1975). With the recognition of this cation-anion differential in neutral salt considerations, it must also be acknowledged that both the identity and the concentration of the anion are important in assessing protein conformational responses. At low electrolyte concentrations(μ < 0.5), the anions can be treated more as a collective group as their effects can be attributed to electrostatic interactions related to the polar polyionic nature of the protein(von Hippel and Schleich, 1969). In fact, the charged protein is assumed to be a simple ion and the Debye-Huckel theory applies(Kirkwood, 1943). Therefore, most anions at low concentrations have a solubilizing, salting-in effect with the magnitude of the electrostatic influence related primarily to the ionic strength of the salt and the density and distribution of charged residues on the protein surface(von Hippel and Schleich, 1969). In this study, the general electrostatic influences of various anions at low concentrations(μ < 0.5) on the thermal stability and So values of vicilin were evidenced

by the minimal differences observed among most of the experimental parameters(Tables 3.2 and 3.3). Despite the apparent non-specificity of most anionic effects, von Hippel and Schleich(1969) reported that anionic influences at low concentrations may not simply be a function of charge-shielding, identical concentrations of different anions having the same valence may result in different protein responses. In fact, for vicilin exposed to different anions at 0.1 M salt concentrations, all Td and So values were similar with the exception of a significantly lower Td for vicilin exposed to thiocyanate(Table 3.3). With 0.2 M NaSCN, vicilin was characterized by a further significant decrease in Td in conjunction with a significant increase in surface hydrophobicity. This destabilizing influence of thiocyanate, apparent at even low concentrations of the anion, may be related to its capacity to exhibit a high degree of nonspecific binding to a number of exposed protein polar sites(Arakawa and Timasheff, 1982).

In addition to the thiocyanate results, other differences among specific anions were observed at salt concentrations of 0.2 M, the most notable being elevated Td values for vicilin exposed to the divalent and trivalent anions(sulfate, phosphate, citrate) in comparison with those for the univalent anions(acetate, bromide, chloride, iodide). At this molar salt concentration, the ionic strength differential among the multivalent and univalent anions appeared to influence the degree of protein stabilization. Tatham et al. (1983) observed a similar effect with the ability of the divalent anions, phosphate

and sulfate, to promote α-helix formation in the protein melittin, as compared with that of the univalent chloride anion. This stabilization phenomenon observed from 0.1 to 1.0 M was attributed to the increased capacity of multivalent negative ions to suppress electrostatic repulsion between basic residues and also to the potential of these residues to form cross-linkage sites between arginine residues(Cotton et al., 1973; 1974).

At 0.2 M salt levels, however, the effective electrostatic shielding by the multivalent anions may be supplemented by a stabilizing lyotropic effect in terms of preferential hydration of the surface of the protein. In fact, the effects of both sodium citrate(μ = 1.2) and Na_2SO_4(μ = 0.6) on vicilin conformational parameters should be predominantly lyotropic at this concentration level; the situation for sodium phosphate($\mu \simeq 0.4$) may be related more to a combination electrostatic-lyotropic effect.

In the progression from considering the effects of salts at low concentrations on protein conformational properties to interpreting the effects of high salt concentrations($\mu > 0.5$), several basic differences must be acknowledged. One consideration is the charge status of the protein. At high salt concentrations, electrostatic interactions between charged residues are effectively neutralized by extensive ionic shielding(Kirkwood, 1943). As a result, the protein behaves as a neutral dipole and general anionic electrostatic influences become less important. The factor that does become more sig-

nificant at high salt levels is the specific identity of the anion. As a preliminary step in considering the effects of individual anions on protein conformational properties, the entire group of anions can be initially subdivided into two general categories. Those designated as destabilizing anions promote continual protein solubilization in addition to progressive molecular destabilization with increasing salt concentrations. In contrast, the stabilizing anions result in general conformational stabilization coupled with eventual protein precipitation as a consequence of increased intermolecular hydrophobic associations(von Hippel and Wong, 1964).

Several theories have been put forward to explain the differential action of the two groups of anions evidenced at high salt concentrations. At this time, it appears that satisfactory interpretations involve the integration of several factors. With respect to the stabilizing anions, the effect of a salt on protein structure seems to be related primarily to its ability to increase the surface tension of the protein aqueous environment(Melander and Horvath, 1977) in conjunction with its ability to cause preferential hydration of the protein molecule(Arakawa and Timasheff, 1982). Consequently, as the concentration of the stabilizing salt increases beyond the level associated with electrostatic effects, the resulting salt-water interaction results in a gradual exclusion of the salt from the protein surface. This lyotropic effect results in an aqueous surface environment that would appear to be thermodynamically unfavorable for the unfolding of the protein

molecule and exposure of hydrophobic residues. Results from this study correlate well with this explanation; in fact, as the lyotropic effect increased, vicilin gradually assumed a more highly stabilized conformation. This is evidenced by a significant positive correlation between the thermal stability of vicilin, as assessed by Td values, and the concentration of the various stabilizing salts(Figure 3.1).

It is also apparent from the thermal properties of vicilin, that different anions within the stabilizing group had different capacities to affect the thermal stability of the protein. Using the Td values as a guideline, the relative stabilizing effectiveness of the anions examined could be described by the following hierarchy:

citrate > sulfate, phosphate > chloride, acetate, bromide

With the exception of chloride and acetate, this order of anions is similar to that given by Hofmeister(1888) based on the ability of salts to induce a protein salting-out effect. In the original Hofmeister scheme, sodium acetate was considered to be a more effective protein precipitant than NaCl. This view was substantiated in a more recent review by Eagland(1975) in which acetate was descibed as a more stabilizing anion than chloride. The similar effect of the two anions with respect to vicilin, in contrast to other proteins, may reflect a composite of parameters including structural differences in the proteins examined, variations in the properties assessed and diversities in the assay techniques used.

With respect to the hierarchy of anions in relation to the stabilization of vicilin, the position and effects of the citrate anion warrant some consideration. Citrate has been recognized previously as a potent stabilizing factor both in the original series established by Hofmeister and in later studies such as that by Robinson and Jencks(1965). In this latter study, increasing concentrations of citrate were found to have the greatest effect of a number of anions on the activity coefficient of the model peptide acetyltetraglycine ethyl ester(ATGEE), as determined by solubility measurements. The magnitude of the stabilizing influence of citrate on vicilin was remarkable; an increase of approximately 30°C was observed for vicilin Td values with an increase in salt concentration from 0.1 to 1.0 M(μ = 0.45 to 6.0). From a conformational viewpoint, the driving force created by the preferential hydration phenomenon associated with increasing sodium citrate concentrations must induce a considerable stress on the molecule to assume a more stabilized form. As will be noted later, this concept is also supported by a significant decrease in the exposure of surface hydrophobic residues of vicilin with increasing preferential hydration. The capacity for such structural responses only further reinforces the general concept of a protein as a tremendously dynamic molecule.

At the opposite end of the hierarchy, the designation of bromide as a stabilizing anion in terms of its influence on vicilin is contradictory to a number of protein studies. For example, the structures of ribonuclease(von Hippel and Wong,

1964), sesame α-globulin(Prakash and Nandi, 1977) and a variety of biomembranes and multi-protein complexes(Hanstein et al., 1977) have been destabilized with exposure to even low concentrations of the bromide anion. In this study, although the overall increase in Td values with increasing NaBr concentrations was among the lower of the values for the stabilizing anions, the bromide anion did not result in vicilin destabilization as assessed by calorimetry. As a result, it would appear that NaBr may exert a mildly stabilizing or destabilizing influence depending on the conformational properties of the protein in question and on the actual method of analysis used.

The progressive increase in Td values for vicilin exposed to increasing concentration levels of the stabilizing salts was not, however, correlated with a similar increase in ΔH values. The ΔH values either remained constant over the salt concentration range or showed a significant increase(Tables 3.2 and 3.3). As discussed in Chapter 2, ΔH is a complex parameter that is determined by the composite interaction of a number of factors. Consequently, speculations concerning the elements contributing to changes in this thermal parameter must be made with caution. It is interesting to note, however, that the highest ΔH values observed(4.99 and 4.39 cal g^{-1}) were associated with salt environments in which the Td values were also high, specifically 105.8°C for vicilin in 2.0 M NaCl and 109.3°C for 1.0 M Na_2SO_4. At Td values in excess of 100°C, the contribution of the exothermic dissociation of hydrophobic

forces during denaturation to the ΔH parameter becomes negligible(Privalov and Pfeil, 1979). As this exothermic dissociation has a negative influence on the absolute value of ΔH, it would appear that the absence of this exotherm could contribute to the ΔH values that are observed at these temperatures. However, other elements may be involved. For example, although vicilin in 2.0 M sodium acetate denatured at 105.4°C, the corresponding ΔH value(3.54 cal g^{-1}) was significantly lower than those associated with other Td values in a similar temperature range.

In addition to defining degrees of stabilization, a critical issue in characterizing the response of a protein to high levels of neutral salts is an appreciation for the surface properties of the molecules in question. It seems logical to anticipate that the thermodynamic response of a protein to the lyotropic effect of preferential hydration might also involve a decrease in So values. For vicilin, this projected response was evident with increasing concentration of the highly stabilizing citrate, phosphate and sulfate anions. A similar trend was not observed for the more moderately-stabilizing anions. With chloride anions, the So values for vicilin decreased significantly from 0.2 to 0.5 M, then remained constant to 2.0 M; with acetate anions the So remained constant over the entire concentration range(Figure 3.3). The relatively constant and elevated So values for vicilin exposed to chloride and acetate may reflect the tendency of these anions to bind to the protein, especially at high salt concentrations. Arakawa and

Timasheff(1982) demonstrated that acetate, unlike the more stabilizing anions, is not completely excluded from the surface of the protein at high concentrations. Similarily, others(Scatchard et al., 1957) have shown that chloride anions may exhibit limited binding to proteins. Therefore, the attractive forces between these anions and vicilin may somewhat overcome the highly stabilizing environment imposed by preferential hydration when salt molecules are excluded from the surface of the protein.

Up to this point, the discussion of the effects of neutral salts at high concentrations has only involved those which exert a stabilizing influence. With vicilin, similar to other proteins(von Hippel and Schleich, 1969), the two anions, thiocyanate and iodide, were shown to have a destabilizing influence on molecular thermal properties. The thiocyanate anion had the most pronounced effect as evidenced by a significant negative correlation between Td and NaSCN concentration and a significant decrease in ΔH from 3.75 cal g^{-1} at 0.1 M NaSCN to 1.01 cal g^{-1} at 1.0 M NaSCN(Table 3.3 and 3.5). To explain the general mechanism of the destabilizing anions, in comparison with the stabilizing salts, two factors are usually considered. First, it has been suggested that destabilizing anions are characterized by a low capacity to increase the surface tension of water(Melander and Horvath, 1977) and consequently, by a reduced ability to induce preferential protein hydration(Arakawa and Timasheff, 1982). Secondly, these destabilizing anions actually appear to remain preferentially bound

to the protein structure creating conformational disturbances(Bull and Breese, 1970; Arakawa and Timasheff, 1982). In fact, with specific reference to the thiocyanate anion, Arakawa and Timasheff(1982) demonstrated that a significant amount of thiocyanate would preferentially bind to bovine serum albumin at high concentrations of the salt. These results were indicative of a correlation between this binding phenomenon and a destabilization of the protein, similar to that observed for vicilin in this study. Binding of the thiocyanate anion should increase the electrostatic free energy of the protein with the resulting repulsive forces causing a decrease in protein stability. This premise appears to be substantiated by the increase in So values for vicilin with increasing thiocyanate concentrations. If protein destabilization involves a progressive unfolding of the molecule, it is expected that So values would reflect the increased exposure of hydrophobic residues.

In the preceeding discussion, the response of vicilin to various neutral salt environments has been considered in terms of general molecular trends with increasing concentrations of either stabilizing or destabilizing anions. Once these trends have been recognized, the relative significance of hydrophobic forces to the overall structural stability of vicilin can be evaluated subjectively by a more detailed comparison of the responses of the protein to different concentrations of various anions. Theoretical protein studies, reviewed by Franks(1978) and Melander and Horvath(1977), have shown that,

at ionic strengths sufficient to minimize electrostatic interactions, changes in protein properties can be attributed to hydrophobic interactions, if the changes can be correlated with the position of these anions in the Hofmeister series. For example, Prakash and Nandi(1977) found that anions at extreme positions in the Hofmeister series had opposite effects on the stability of sesame α-globulin, a protein whose subunits are held together by hydrophobic associations. The sulfate and chloride anions had a stabilizing, associating effect whereas iodide and thiocyanate anions were destabilizing and dissociating. However, difficulty in establishing these types of correlations between protein conformational parameters and the position of anions in the Hofmeister series has previously resulted from the empirical nature of the lyotropic hierarchy. Bruins(1934) and Voet(1937) attempted to resolve this problem by quantifying the lyotropic properties of certain anions. Specific lyotropic numbers, referred to as H and N values respectively, were assigned to individual anions on a semi-quantitative basis using data derived from protein flocculation experiments. Despite these attempts, a completely quantitative approach for the anionic series was not established until Melander and Horvath(1977) demonstrated that the molal surface tension increment(σ) of a salt, an index of the influence of a salt on water surface tension, could be used to numerically arrange salts into a specific hierarchy which correlated with the position of the anions in the original Hofmeister series.

The Melander and Horvath(1977) approach represented a method of quantifying the relative lyotropic effectiveness of an anion, or the relative ability of an anion to increase the surface tension of water and increase protein preferential hydration(Arakawa and Timasheff, 1982). As previously discussed, these lyotropic effects are evident at high salt concentrations($\mu > 0.5$) whereas electrostatic interactions are predominant at low salt concentrations($\mu < 0.5$)(von Hippel and Schleich, 1969). As a result, if various anions had a lyotropic effect on the conformation of vicilin, it was assumed that correlations between parameters assessing protein stability, such as Td values, and σ, the index of lyotropy, would exist at high salt concentrations. If the original premise associating the relative importance of hydrophobicity to molecular structure with protein responses to the Hofmeister series is accepted, such correlations would be affirmative of the significance of hydrophobic associations to the molecular integrity of vicilin. With this viewpoint, it was found that a significant positive correlation between Td and σ existed for the anionic series at 0.2, 0.5 and 1.0 M salt concentrations(Tables 3.5 and 3.6). Consequently, the increase in thermal stability of vicilin, as assessed by Td values, exactly paralleled the original Hofmeister series both in an empirical and a numerical sense. Although the correlation between Td and σ was unexpected at low salt concentrations(0.2 M), the actual degree of correlation increased dramatically at high salt levels(0.5 and 1.0 M), reflecting the

lyotropic salt-protein relationship(Table 3.5). As further support for the hierarchical anionic effect on vicilin thermal stability, there was also a significant positive correlation between ΔH and σ at 1.0 M salt concentrations(Tables 3.5 and 3.7).

In addition to the thermal stability data, relationships observed for vicilin between So and σ were indicative of an anionic lyotropic effect. At salt concentrations where electrostatic interactions were important, there was no significant correlation between So and σ. However, as salt concentrations were increased to levels at which lyotropic effects predominated, there was a significant negative correlation between So and σ(Tables 3.5 and 3.8). In fact, this negative relationship appears to substantiate the premise that hydrophobic associative forces are important in the stabilization of vicilin. As the level of vicilin preferential hydration increases, a factor determined by the σ and concentration of the environmental salt, removal of hydrophobic residues from a surface orientation would appear to be entropically favorable. Increased internalization of hydrophobic residues implies increased intramolecular hydrophobic associations. As a result, if these intramolecular hydrophobic associations are important to vicilin stability, an increase in these interactions should be manifested by an increase in protein thermal stability. Evidence for this proposal exists in the observed significant negative correlation between So and Td for vicilin at salt levels of 0.5 and 1.0 M(Table 3.5), concentrations at

which preferential hydration is a major parameter.

Another phenomenon that may be partially related to σ, the molal surface tension increment, is the existence of extreme exotherms following the endothermic denaturation of vicilin exposed to the highly stabilizing citrate environment. These exotherms were evident at all concentrations of citrate(0.1 to 1.0 M), however, their relative magnitude increased with increasing salt concentration. As discussed in Chapter 2, such exotherms may result from extensive post-denaturation molecular aggregation. Consequently, the lyotropic effects of sodium citrate may result in a repulsive driving force between the solvent and protein molecules which thermodynamically favors massive aggregation of the denatured protein.

Effects of Specific Cations on Vicilin Conformational Parameters

A limited investigation of the effects of specific cations on the conformation of vicilin was undertaken to determine if some intramolecular structural relationships could be indentified using an approach similar to that with the anionic neutral salts. An hierarchical arrangement of cations has been previously reported; for example, Bull and Breese(1970) arranged a number of cations in a lyotropic series on the basis of their hydrated ionic radii (eg. lithium > sodium > potassium). In fact, the position of a cation in this series was related to its effectiveness as a protein denaturant. Those cations with the largest radii were consid-

ered to be the most potent denaturants as a result of their capacity to dehydrate the surface of the protein. Despite this rating or categorization approach, it is generally recognized that the differences in the influence of various cations on protein conformation are not of the magnitude of those seen with the anions(Warren and Cheatum, 1966).

With specific reference to the thermal properties of vicilin, all three cations examined(lithium, sodium and potassium) had a stabilizing effect on the protein as evidenced by the significant positive correlation between Td and salt concentration for each salt environment(Table 3.11). Despite this general stabilizing phenomenon, an hierarchy of the relative effectiveness of each cation was not readily apparent. At low salt concentrations(0.1 and 0.2 M), the thermal properties of vicilin in all salt environments were relatively stable; an expected result if general salt-protein electrostatic interactions were predominant. The electrostatic phenomenon was further substantiated by a lack of correlation between Td and σ and ΔH and σ. One interesting observation was the differential in Td values between comparable cations associated with the two different anions(chloride and sulfate). At 0.2 M salt levels, the higher Td values associated with the sulfate salts may in fact, reflect a difference in the electrostatic effectiveness of the divalent anion in conjunction with an increasing preferential hydration of the vicilin surface as a result of the sulfate ionic strength level(μ = 0.6) in comparison with that of the chloride salts(μ = 0.2).

At higher salt concentrations(0.5 and 1.0 M), it was anticipated that if the three cations had a differential lyotropic effect, this would be manifested by variations among the thermal properties of vicilin. However, there were no significant correlations between Td and σ or ΔH and σ. The absence of these relationships may partially reflect the reduced spectrum of cations examined in conjunction with the minimum differences in σ values within the chloride or sulfate salts. Despite the inability to establish a specific cation-lyotropic relationship with these data, some notable trends occurred. One aspect was the greater stabilizing influence of the sodium cation in association with the chloride anion at 1.0 M salt levels. This was evidenced by a significantly higher Td value for vicilin exposed to NaCl in comparison with KCl or LiCl(Table 3.12). As a comparison, a similar NaCl stabilizing influence was observed by Bull and Breese(1973) in an analysis of egg albumin melting temperatures. In this study, the experimental cations were arranged in a series according to their protein stabilizing effectiveness (i.e. NaCl > KCl > LiCl); however, the hierarchy was apparent at 3.0 M salt concentrations, not at 1.0 M levels.

The lithium cation, with its extensive hydration radius, has been identified as a protein denaturant on the basis of its capacity to dehydrate the surface of a protein(Bull and Breese, 1970). However, this perspective on the mechanism of action of lithium has been somewhat modified as a result of the observation that lithium in association with the chloride

anion exhibits some binding to proteins at high salt concentrations(Altekar, 1977). Accordingly, this binding may reduce some of the repulsive forces due to the molal surface tension increment resulting in decreased preferential hydration and either destabilization or decreased stabilization in comparison with other ions. As an example of the latter situation, LiCl has the lowest capacity for vicilin stabilization of the chloride salts; however, it does not have a destabilizing influence comparable to that described by von Hippel and Schleich(1964) and Warren and Cheatum(1966). This observation may be a function of the salt concentrations used. For example, Altekar(1977) reported a denaturing influence of lithium in a study which monitored the intrinsic fluorescence of a number of proteins exposed to various electrolyte regimes. This specific influence was only observed at very high salt concentrations, usually in excess of 2.0 M.

The cationic differential at 1.0 M(μ = 1.0) chloride salt concentrations was not apparent for the cations associated with the sulfate anion at 0.5 M(μ = 1.5) salt levels. Although these results are not directly comparable due to the difference in ionic strength values for the two groups of salts, it seems that the general effect of cations is influenced by the associated anions. At 0.5 M salt concentrations, the Td values for the sulfate salts were significantly higher than those for the chloride salts. This appears to be a function of the differential in σ values, a factor determined mainly by the identity of the anion(Table 3.12). As a result,

the preferential hydration effect caused by the sulfate anion may overcome any tendency for differential binding of the cations to the protein, as may have occurred with the chloride salts. This is not an unsubstantiated supposition. Arakawa and Timasheff(1982) described different actions for the magnesium cation associated with different anions. Destabilization of bovine serum albumen exposed to $MgCl_2$ was attributed to binding of the cation. In contrast, preferential hydration and stabilization of the protein occurred with a $MgSO_4$ environment as a consequence of the high σ imposed by the sulfate component. In general, it appears that the possible binding effects of a cation may be influenced by the identity of the anion. As the salt concentration increases, cation binding, if originally present may continue or preferential hydration of the protein surface may occur depending on the effects of the associated anionic component.

Up to this point only the thermal stability of vicilin with respect to cationic effects has been considered. In terms of So values, there were few significant changes for a specific electrolyte with increasing salt concentrations. Most changes involved a decrease in So with increasing salt levels as expected if preferential hydration of the protein surface were occurring. Several interesting trends were observed with respect to relative So values among the different types of salts. The elevated So values for vicilin in 0.5 and 1.0 M LiCl may be indicative of a somewhat destabilized molecule with increased exposure of hydrophobic surface residues(Table

3.9, Figure 3.6). This is feasible if lithium were binding specifically to vicilin preventing the increased hydrophobic associations as a consequence of preferential hydration. At this point, however, the destabilizing influence was not sufficient to be reflected in thermal parameters. The highly elevated So values for vicilin exposed to K_2SO_4 are more difficult to explain(Table 3.10, Figure 3.7). These extreme values have been previously associated with a destabilized vicilin molecule, as evidenced in the NaSCN environment. However, the thermal properties of vicilin in K_2SO_4 were not characteristic of a destabilized molecule. In fact, considering the high σ value for K_2SO_4, it would be expected to observe relatively low So values especially at 0.5 M levels similar to those obtained for Li_2SO_4 and Na_2SO_4. The So values for the latter two salts are more in accordance with the anticipated preferential hydration phenomenon. The elevated values for vicilin in K_2SO_4 may reflect a possible binding of potassium cations to the protein. Although this possibility exists(Arakawa and Timasheff, 1982), a similar trend was not evidenced for So values of vicilin in KCl(Table 3.9, Figure 3.6). In general, cationic influences on protein structure will be more easily interpreted as detailed information becomes available with respect to the specific ability of cations to bind to proteins. However, this binding ability must also be assessed with respect to the associated anion. As previously mentioned, the influential capacity of any cation on protein structure appears to be related to the identity of the associated anion.

Conclusion

The structural integrity of vicilin is influenced by environmental parameters as evidenced by conformational changes observed with defined variations in pH and solvent electrolyte components. Considering the specific structural responses of vicilin to different anions, this conformational sensitivity was extrapolated to imply a dependence of vicilin structure on hydrophobic associations. Recognizing both the subtle environmental changes involved and the multimeric nature of vicilin, it is assumed that this hydrophobic dependence is being assessed mainly at the quaternary level. Stabilization of subunit associations in vicilin by hydrophobic interactions is not only a logical interpretation from the experimental results but also from an accepted energetic viewpoint. In this respect, it is recognized that protein subunits or molecules contain patches of exposed hydrophobic residues as steric restraints imposed by protein primary structure do not allow complete internal burial of these residues(Fisher, 1965). With exposure of proteins to an aqueous environment, it is thermodynamically feasible for hydrophobic areas to associate. The subsequent disordering of water provides an entropically favorable driving force for the continuation of these hydrophobic interactions(Kauzmann, 1959).

Despite the apparent importance of hydrophobic associations, other noncovalent forces must also be considered as potential stabilizing or destabilizing factors in the intramolecular organization of subunit components. Hydrogen bonding,

for example, represents one possible type of associative interaction. In general, the contribution of hydrogen bonding to structural integrity has been recognized at the secondary and tertiary levels; however, its significance at the quaternary level has been questioned(Murray et al., 1981). Although the relative importance of this factor was not directly established with vicilin, it is felt that the observed correlation of protein conformational responses with the Hofmeister series of anions as observed in this study are more supportive of a major hydrophobic contribution(Melander and Horvath, 1977; Pahlman et al., 1977; Preston, 1981).

A second noncovalent parameter involved in subunit associations is the electrostatic interaction of charged residues. However, similar to hydrogen bonding, its relative importance in comparison with hydrophobic associations is poorly understood. With vicilin, it was shown by selective charge manipulation that electrostatic interactions were a factor in molecular stabilization(Chapter 2). This was further substantiated by the subtle, but existant, conformational changes that occurred with vicilin exposed to minimal pH variation. Despite these basic observations, extensive charge modifications were required before major structural aberrations, possibly corresponding to subunit dissociations, were evidenced(Chapter 2). Therefore, based on the collective experimental results, subunit assembly in vicilin appears to result from a specific balance of noncovalent forces. Of these forces, hydrophobic associations appear to play a major stabilizing role.

CHAPTER FOUR

IMPLICATIONS OF ENVIRONMENTAL MANIPULATION ON MICELLE FORMATION BY VICILIN

INTRODUCTION

Macromolecules may undergo various types of association reactions in response to different environmental conditions. An example of a specific type of aggregation product is the micelle; a spherical structure formed by amphiphilic molecules exposed to an aqueous environment in an attempt to achieve segregation of hydrophobic portions from the solvent medium (Tanford, 1972). As a consequence of the micelle arrangement, hydrophilic segments of the associated molecules remain in contact with water at the surface of the aggregated structure. On the basis of the interactions involved, this micelle phenomenon has been traditionally attributed to molecules such as lipids which are characterized by distinct, spatially-separate hydrophobic and hydrophilic regions (Tanford, 1972). Although lipids have an ideal configuration for the micelle arrangement, evidence now exists that proteins, as amphiphilic molecules, may also aggregate in a micelle-type structure (Murray et al., 1978; Simons et al., 1978; Evans and Philips, 1979). However, despite the potentiality for this type of arrangement, the protein micelle is a relatively uncommon occurrence; definite limitations are imposed by the general structure of the interacting molecules. One of the major conformational difficulties associated with proteins involves the hydrophilic and hydrophobic amino acids. These two types of residues not only occur in scattered patches on the surface of a molecule (Klotz, 1970; Lee and Richards, 1971), but also vary in relative amounts among dif-

ferent proteins (Bigelow, 1967). As a further complication, protein surface properties are not constant; the dynamic, flexible nature of a protein may allow considerable conformational variations with environmental changes (Cooper, 1980).

With the recognition of the structural restrictions inherent in a protein molecule, it appears that the actual formation of micelles by associating proteins must be a sensitive function of environmental parameters. As a result, the associative forces necessary to derive such a structure become a fundamental consideration in the comprehension and prediction of the protein micelle phenomenon. From a thermodynamic viewpoint, it is entropically favorable if the hydrophobic surface residues of a protein can be removed from the aqueous environment, possibly through intermolecular associations. Although complete burial of these nonpolar residues would be energetically ideal, protein structural restrictions necessitate that even after molecular association, a certain number of hydrophobic moieties remain exposed at the solvent interface (Fisher, 1965). Consequently, the aggregated protein superstructure formed in response to the driving force imparted by noncovalent hydrophobic interactions appears to be characterized by minimal exposure of hydrophobic residues and maximal exposure of hydrophilic side-chains (Murray et al., 1978) - a structure comparable to the classical detergent micelle described by Tanford (1972). Although it seems thermodynamically appropriate to attribute molecular orientation in the establishment of protein micelles to hydrophobic forces,

it should be recognized that surface hydrophobic residues do not have a strong attractive capacity. In fact, intermolecular distances must be less than approximately 3A° before hydrophobic areas will associate (Murray et al., 1981). As a result, other noncovalent parameters such as the electrostatic profile of the interacting proteins may influence the capacity of the molecules to aggregate. In this respect, protein molecules are not expected to assemble into a micelle arrangement if intermolecular electrostatic repulsions are great enough to discourage hydrophobic interactions.

As a delicate balance of noncovalent forces is apparently fundamental to protein micelle formation, it is not surprising that few studies have described this type of aggregation. A protein micelle system that has been investigated extensively is the casein micelle; however, this structure appears to represent a unique association phenomenon. As reviewed by Griffin and Anderson (1983), the casein micelle involves a heterogeneous mixture of polypeptides bound together through hydrophobic associations and the undefined action of calcium, phosphate and citrate ions. This dependence on ionic interactions makes the casein protein arrangement somewhat different from the traditional description of a micelle, as a structure formed and stabilized through a specific hydrophobic response to the environment (Tanford, 1972). Interestingly, the casein arrangement does not seem to typify the characteristic structure of a protein micelle. Several examples of protein micelles similar to the hydrophobically-established and

hydrophobically-maintained detergent micelle (Tanford, 1972) have been observed; these, in fact, appear to represent a more non-specific phenomenon with respect to proteins in general. In one study, β-casein, isolated as a single polypeptide, underwent self-association as a suggested consequence of hydrophobic interactions to form defined micelles (Evans and Philips, 1979). In another, Simons et al. (1978) observed the formation of micelles by several different bacterial and viral membrane proteins after detergent removal from a membrane preparation originally solubilized with Triton-X. Similar to β-casein, the establishment of these micelles was attributed to intermolecular hydrophobic associations.

According to Murray et al. (1978), a number of diverse proteins under appropriate experimental conditions can be induced to aggregate into a detergent-like micelle arrangement. The results from this study, in fact, represent the most extensive description of non-casein protein micelles to date. In the original micelle experimentation of Murray et al. (1978), an air-classified protein concentrate from Vicia faba, suspended in a specific ionic strength medium (0.3 to 0.5 M NaCl), was found to precipitate extensively on sudden dilution in cold tap water. Microscopic analysis of the precipitate revealed massive numbers of spherical micelles. With more detailed structural analyses, it became apparent that the micelle interior was composed of a random, non-crystalline arrangement of densely packed protein molecules. From a compositional viewpoint, there was no evidence of associated

lipid molecules; the micelles appeared to be entirely proteinaceous structures (Murray et al., 1981). In fact, the selective nature of this protein micelle phenomenon proved to be significant from a protein isolation point of view. The protein micellar mass (PMM) formed under these conditions contained approximately 96% protein(N x 5.85). In addition, with fababean concentrate as the starting material for micelle formation, only two main protein species were present in the isolated PMM This selectivity coupled with the mildness of the extraction technique was a positive attribute not frequently observed in protein isolation methods.

Once the existence and reproducibility of the protein micelle were established, the specific forces promoting this intermolecular association became of fundamental concern. In order to predict and manipulate this protein response, the dynamic interactions among the individual protein molecules had to be identified. Murray et al. (1978) originally described the entire process of PMM formation as a "salting-in, hydrophobic out" phenomenon with initial protein solubilization followed by massive micelle formation as a result of intermolecular hydrophobic associations. As an extension of this original general premise, it would seem that these hydrophobic interactions could be influenced by electrostatic parameters resulting from the overall charge of the protein as determined by the degree of amino acid ionization and possible association of solubilizing electrolytes. In addition, the capacity for intermolecular hydrophobic associations could be

affected by the number of hydrophobic residues actually exposed on the surface of the protein; a parameter which appears to be a function of environmental characteristics(Chapter 3). Accordingly, this study was designed in an attempt to understand and clarify some of the noncovalent forces that have been implicated in protein micelle formation. Vicilin, isolated from Vicia faba, was selected as a study system as its ability to form micelles in a mixed protein system had been previously established by Murray et al. (1978). The capacity of vicilin to associate into a micelle arrangement was assessed in a variety of different electrolyte media, involving a spectrum of ions representing destabilizing and stabilizing factors according to the Hofmeister series. This micelle-forming capacity was related to the molal surface tension increment(σ) of the different electrolytes and to previously determined molecular characteristics of vicilin such as surface hydrophobicity and thermal stability in an attempt to assess the relative importance of electrostatic and hydrophobic interactions in the micelle phenomenon.

MATERIALS AND METHODS

Standard Methods for Micelle Observation

The following procedure was established as a standard method to assess micelle formation under all experimental conditions:

i. A concentrated protein solution(20 μL) was dispensed onto the surface of a clean glass slide.

ii. The protein solution was examined without a coverslip with a Zeiss Universal Research Microscope possessing a model C35M Carl Zeiss automatic exposure 35 mm camera. Any notable structures were photographed using Kodak Ektachrome 160 ASA film.

iii. Distilled water(20 μL) was then added directly onto the drop of protein solution. Precipitation responses were assessed by the unaided eye and recorded. A coverslip was added carefully to the diluted protein solution; post-dilution structures were monitored and photographed. Size ranges of individual micelles were determined with a stage micrometer.

iv. All micelle responses were observed using a minimum of dulpicate samples.

Influence of Protein Concentration on Micelle Formation

A basic consideration was the concentration of vicilin in the initial protein solution necessary for the observation of the type of micelle formation characteristic of a particular environment. As a preliminary approach, vicilin, in 0.2 M sodium acetate, pH 7.5, was exposed to 0.2 M levels of all

experimental media(Table 4.1) by extensive dialysis as described in Chapter 3. Vicilin, in each experimental medium, was then concentrated using a Minicon-B15 Macrosolute concentrator to two different protein levels of approximately 20 and 40 mg mL^{-1}. The capacity of vicilin to form micelles was assessed at both protein concentrations using the standard method previously given. In all electrolyte environments, there was no apparent difference in the type of micelle formation exhibited at the two concentration levels. To establish a critical protein concentration level more accurately, vicilin in two different environments - 0.2 M sodium acetate, pH 6.6(a "representative" environment for the anionic series) and 0.2 M lithium chloride (a "representative" environment for the cationic series), was concentrated to a range of protein levels from approximately 2 to 25 mg mL^{-1} using a Minicon-B15 Macrosolute concentrator. The capacity of each sample for micelle formation was assessed using the standard method. The minimum concentration of vicilin at which there was no further change in the type of observed micelle formation was established for both environments.

Influence of Dilution Conditions on Micelle Formation

A second basic consideration was the influence of the dilution ratio of distilled water to protein on the observed micelle response. Results of preliminary micelle experiments with vicilin(approximately 20 mg mL^{-1}) exposed by extensive dialysis to 0.2 M levels of all electrolyte media(Table 4.1) showed that a relatively broad range of dilution ratios would

TABLE 4.1. Salt concentrations used in the assessment of micelle formation by vicilin. The pH remained uncontrolled unless indicated.

Salt	Concentration(M)
Sodium acetate, pH 6.6	0.2, 0.5, 1.0
Sodium bromide	0.1, 0.2, 0.5, 1.0
Sodium chloride	0.2, 0.5, 1.0
Sodium citrate, pH 6.6	0.1, 0.2, 0.5, 1.0
Sodium iodide	0.1, 0.2, 0.5
Sodium phosphate, pH 6.6	0.1, 0.2, 0.5
Sodium sulfate	0.1, 0.2, 0.5, 1.0
Sodium thiocyanate	0.1, 0.2, 0.5, 1.0
Potassium chloride	0.2, 0.5, 1.0
Potassium sulfate	0.1, 0.2, 0.5
Lithium chloride	0.2, 0.5, 1.0
Lithium sulfate, pH 6.6	0.1, 0.2, 0.5

result in the characteristic micelle response for a particular environment. Constant patterns of micelle formation were observed for dilution ratios ranging from 1:2 to 4:1(water:protein). In order to identify the extensiveness of this dilution range with one salt medium, various controlled volumes of vicilin (approximately 25 mg mL^{-1}) in 0.2 M lithium chloride were combined with controlled volumes of distilled water. The micelle response was recorded for a number of dilution ratios from 1:10 to 50:1(water:protein).

Influence of pH on Micelle Formation

Although pH has been suggested to be a critical factor in micelle formation(Murray et al., 1981); precise pH requirements have not been defined. To establish if a critical pH value existed, vicilin was exposed by extensive dialysis to a series of 0.1 M phosphate buffers ranging in pH from 6.0 to 8.0. The buffers differed in pH by 0.1 units from 6.0 to 6.8 and by 0.5 units from 7.0 to 8.0. At each pH level, the capacity of vicilin to form micelles was assessed using the standard procedure. All samples were concentrated to an approximate protein level of 25 mg mL^{-1} prior to observation.

Influence of Various Electrolytes on Micelle Formation

In order to assess the influence of various salt environments on micelle formation, vicilin was exposed initially to a series of different types and concentrations of electrolytes(Table 4.1) by extensive dialysis. As a result of the effect of pH on vicilin conformational parameters observed in

Chapter 3, all environments with an initial alkaline pH were adjusted to pH 6.6 using an acid with an anion identical to that of the original electrolyte. Following dialysis, all samples were concentrated to a protein level of approximately 25 mg mL^{-1}. The capacity of vicilin to form micelles was then assessed using the standard procedure.

RESULTS

Characterization of the Micelle Response

The observation of micelle formation by vicilin in a variety of media revealed that a number of different micelle responses were possible. In addition to the individual micelles of various sizes originally described by Murray et al. (1978), the initial micelle populations often exhibited different degrees of inter-micelle association to result in a spectrum of structures ranging from heterogeneous aggregates of discrete micelles to diverse protein networks and/or amorphous protein masses or sheets. Despite the general variability observed, it became evident that these different micelle responses could be arranged subjectively into categories identified by a defined set of descriptive characteristics. A numerical scheme was subsequently devised with each number representing a specific category or pattern of micelle formation(Table 4.2). Corresponding photomicrographs representing various aspects of each characterization level are given in Figures 4.1 to 4.7. Although this scheme was developed from results derived from both preliminary experiments and experiments during the course of this study, it seemed appropriate that this descriptive system be presented initially without elaboration on the various environmental conditions in which these different micelle responses occurred. As micelle observations were ultimately qualitative, the capacity to identify and utilize a characterization scheme facilitated the comparison of subsequent experimental results.

TABLE 4.2. Characteristics of the various types of micelle formation observed with vicilin in different environmental conditions. Each type of formation has been assigned a numerical value, referred to as a micelle rating.

Characterisics	Micelle Rating
No micelle formation.	0
Background haze, no detectable individual micelles.	1
Small single micelles(approximately 2μm in diameter).	2
Small micelles in aggregates.	3
Small micelles with formation of granular networks.	4
Small, intermediate and large micelles (approximately 2 to 20 μm in diameter). Mainly discrete structures; possible limited coalescence.	5
Small, intermediate and large micelles. Extensive coalescence, may have homogeneous and granular networks.	6
Small, intermediate and large micelles. Extensive coalescence to form homogeneous networks and eventual protein sheets.	7

Figure 4.1. Photomicrographs of micelle responses corresponding to ratings 1 and 2. Bar represents 25 μm.

A. Background haze, rating 1.

B. Small discrete micelles, rating 2.

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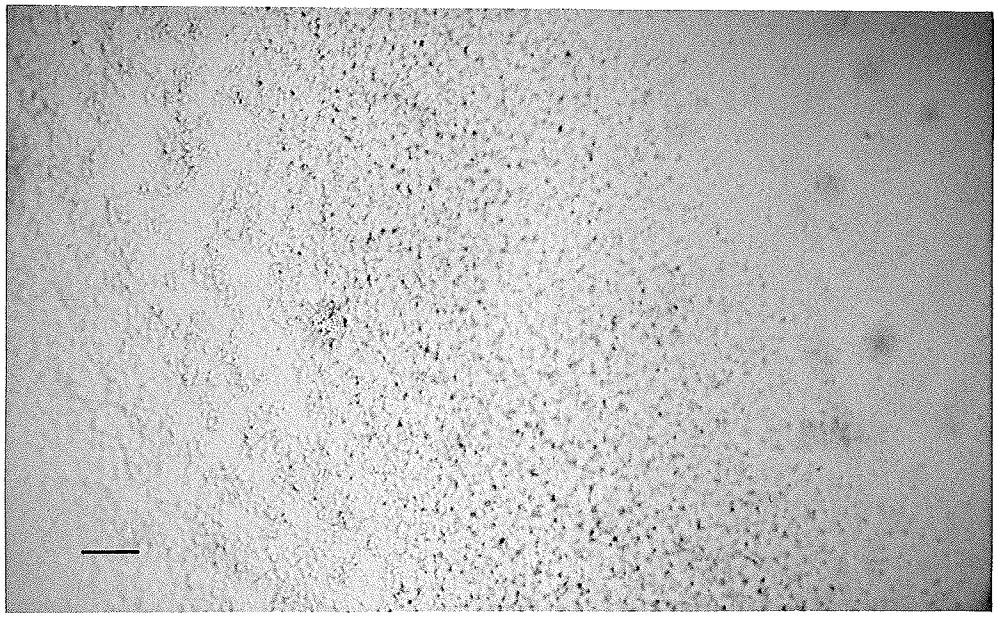

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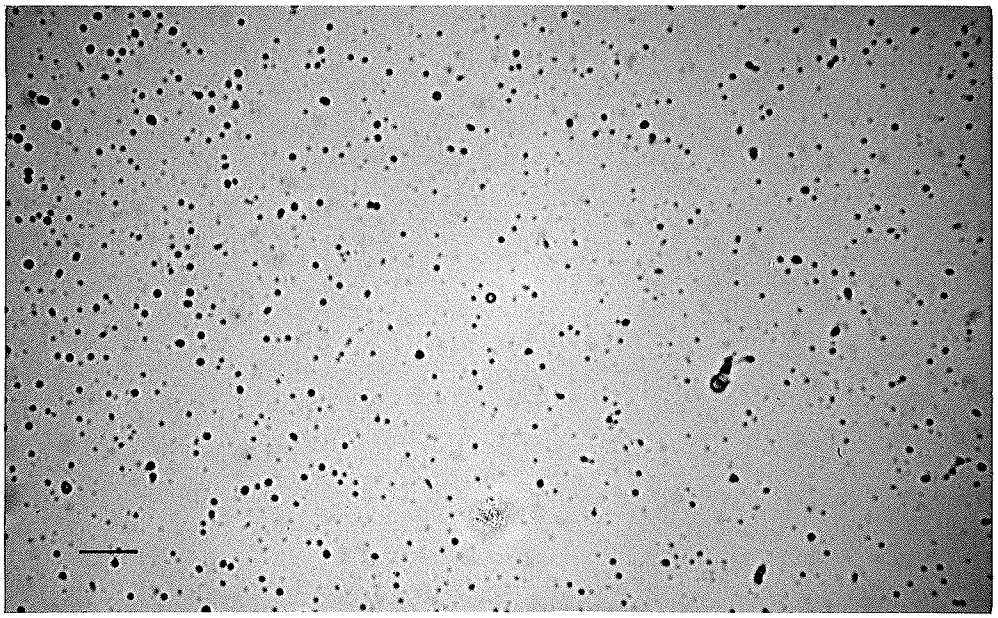

B

Figure 4.2. Photomicrographs of micelle responses correspond-to rating 3. Bar represents 25 μm.

A. Small micelles, limited aggregation.

B. Small micelles, extensive aggregation.

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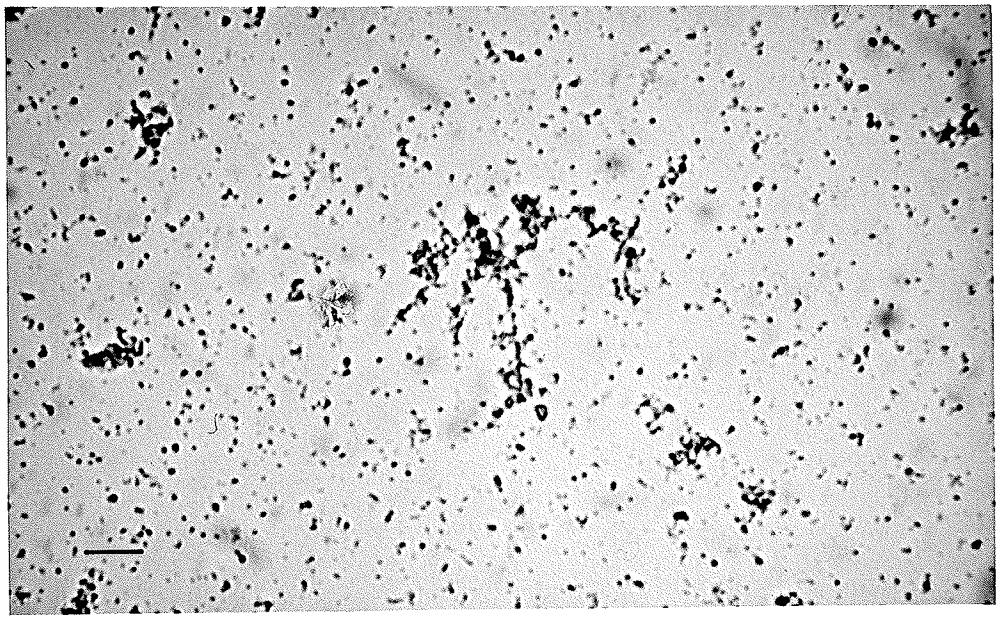

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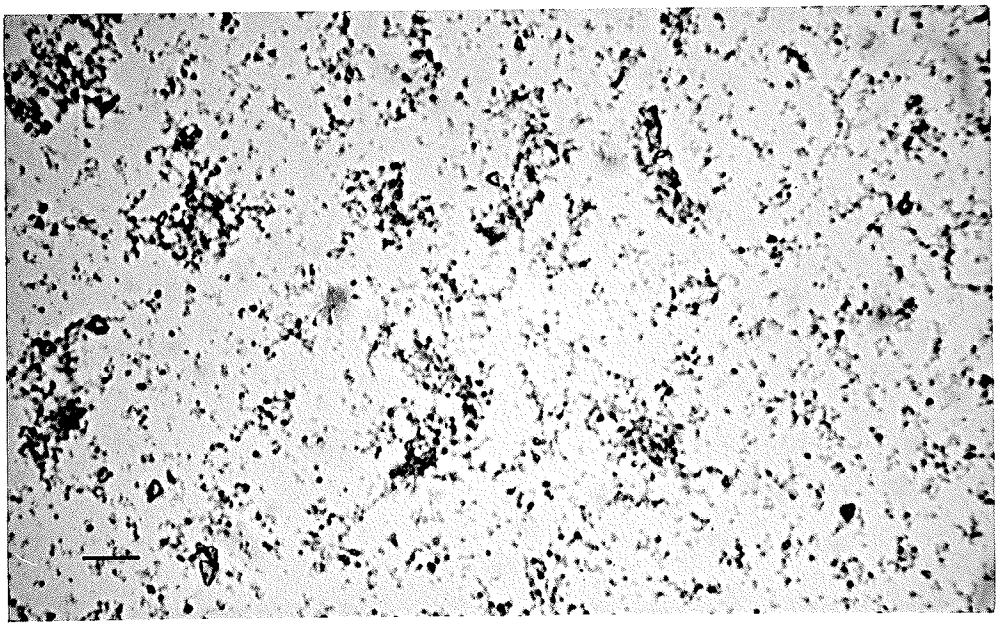

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Figure 4.3. Photomicrographs of micelle responses correspond-
to rating 4. Bar represents 25 μm.

A. Small micelles, limited granular networks.

B. Small micelles, extensive granular networks.

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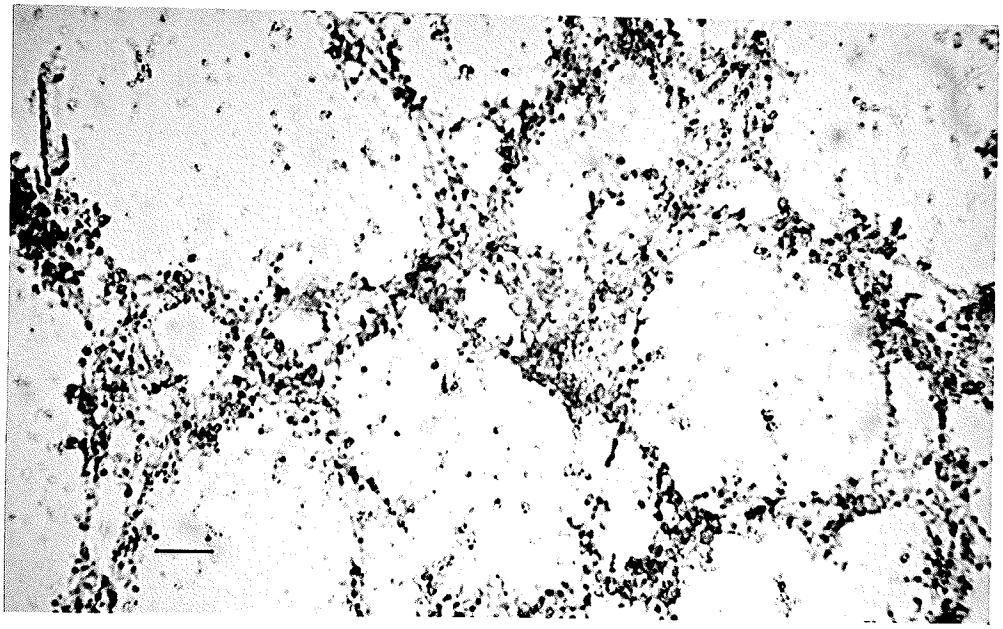

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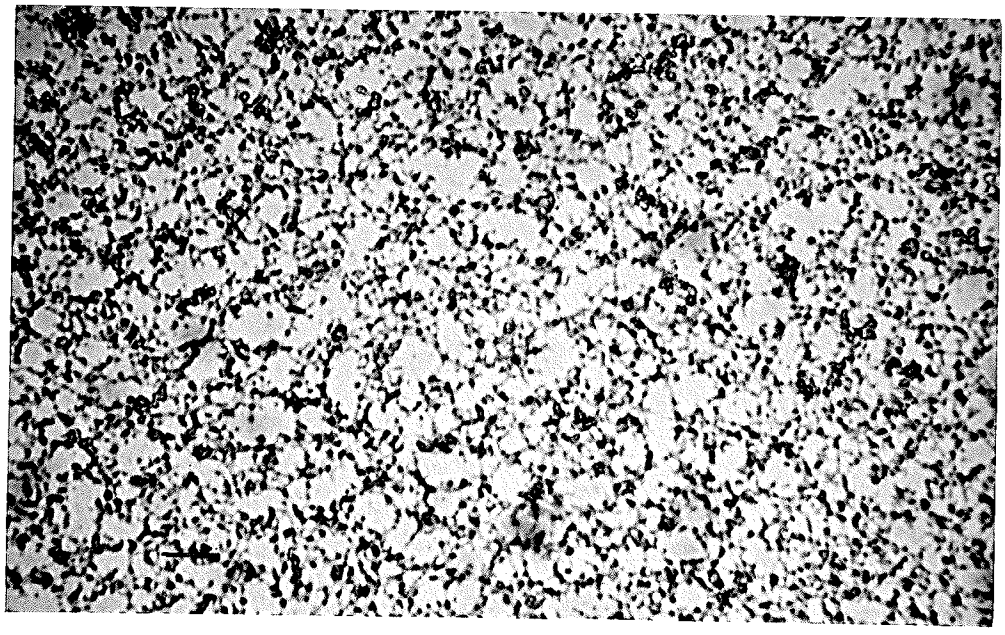

B

Figure 4.4. Photomicrographs of micelle responses correspond- to rating 5. Bar represents 25 μm.

A. Small and intermediate discrete micelles.

B. Small, intermediate and large discrete micelles.

C. Limited homogeneous coalescence.

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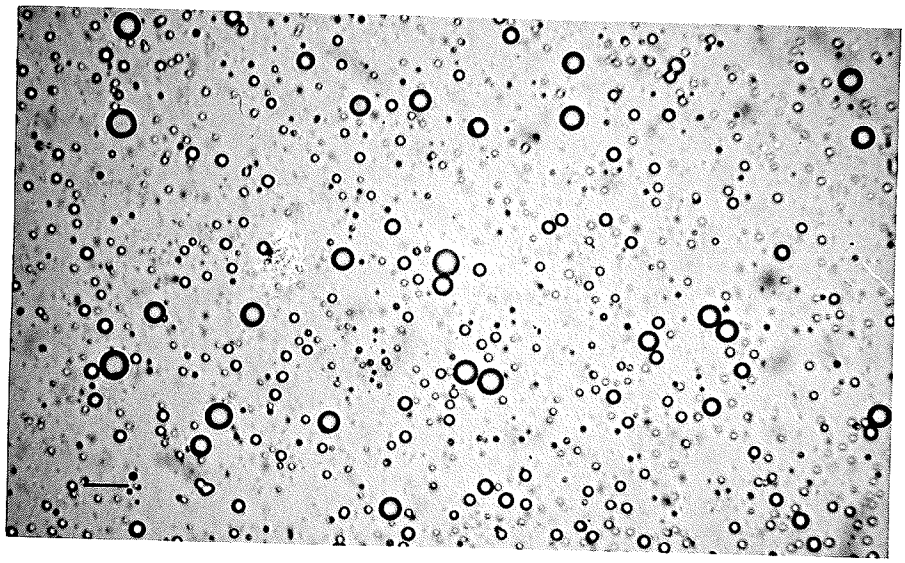

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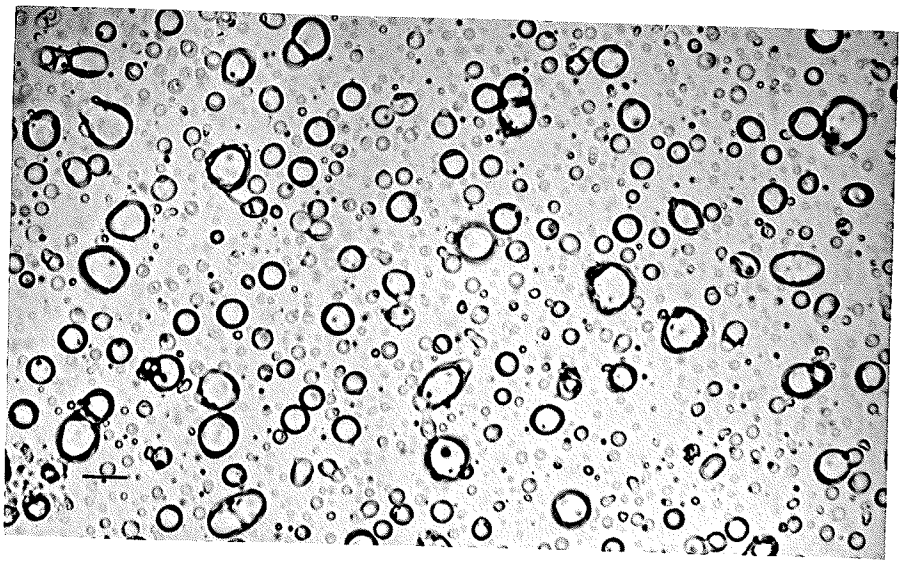

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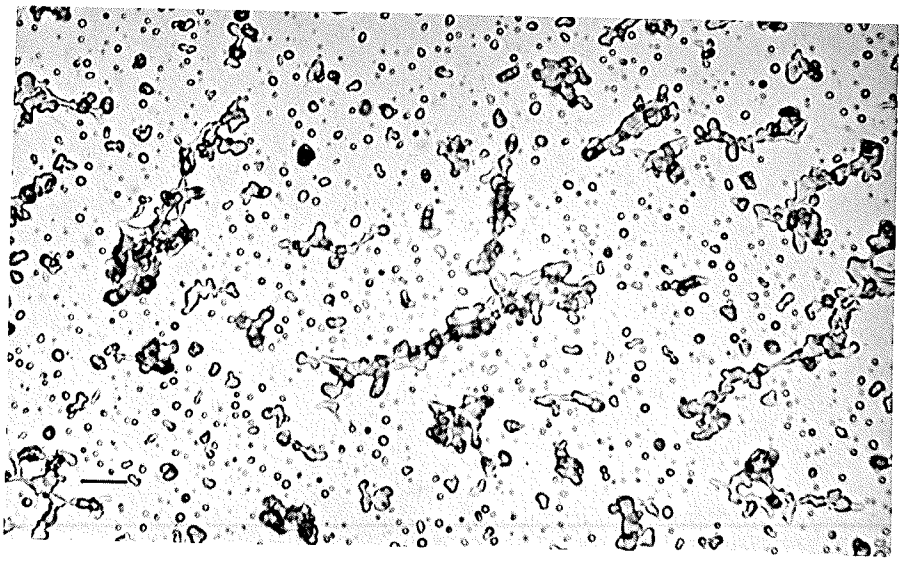

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Figure 4.5. Photomicrographs of micelle responses corresponding to rating 6. Bar represents 25 μm.

A. Granular and homogeneous coalescence.

B. Homogeneous coalescence.

C. Extensive homogeneous coalescence.

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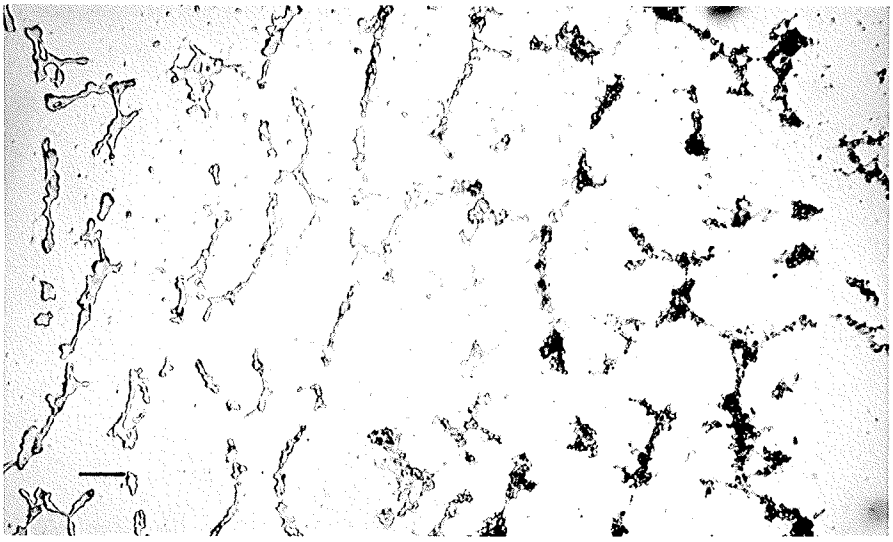

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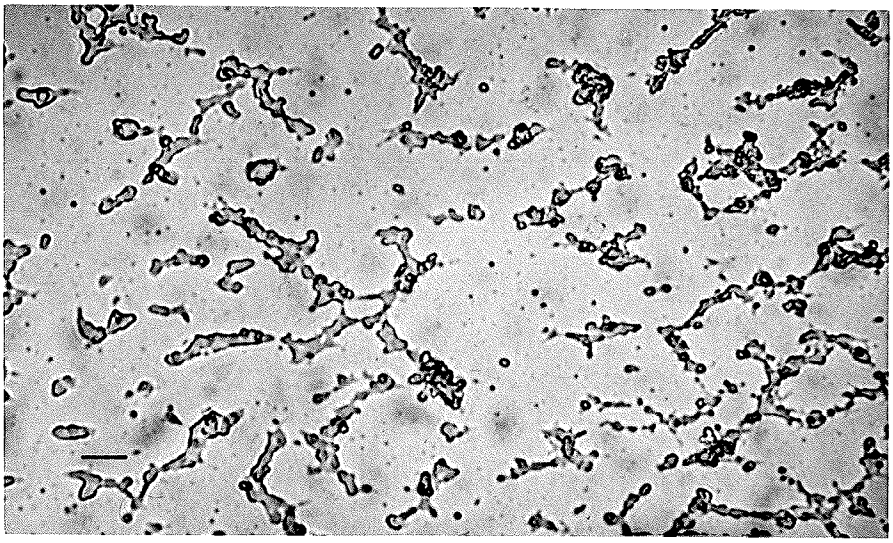

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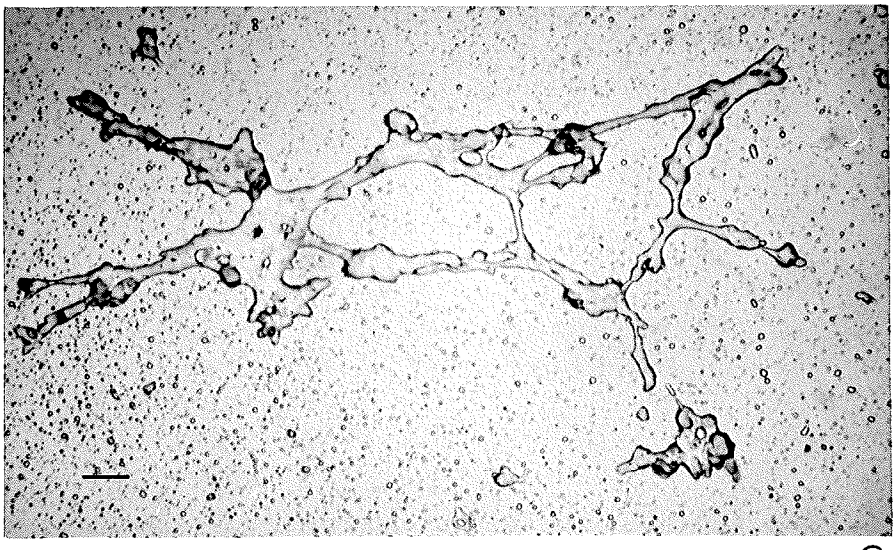

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Figure 4.6. Photomicrographs of micelle responses corresponding to rating 7. Bar represents 25 μm.

A. Extensive network formed by homogeneous coalescence.

B. Further coalescence into amorphous protein masses.

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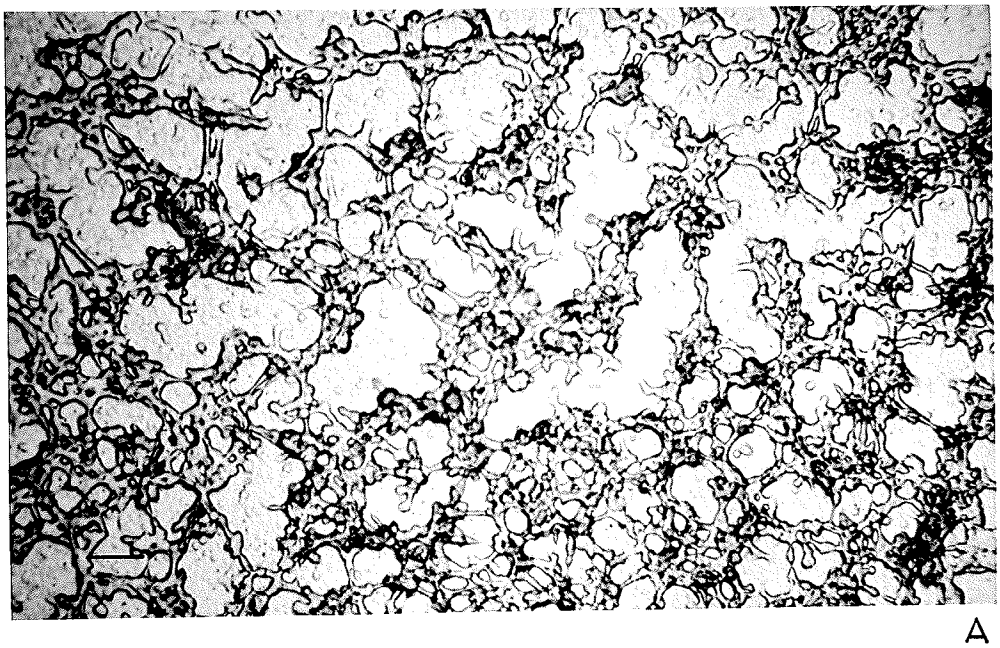

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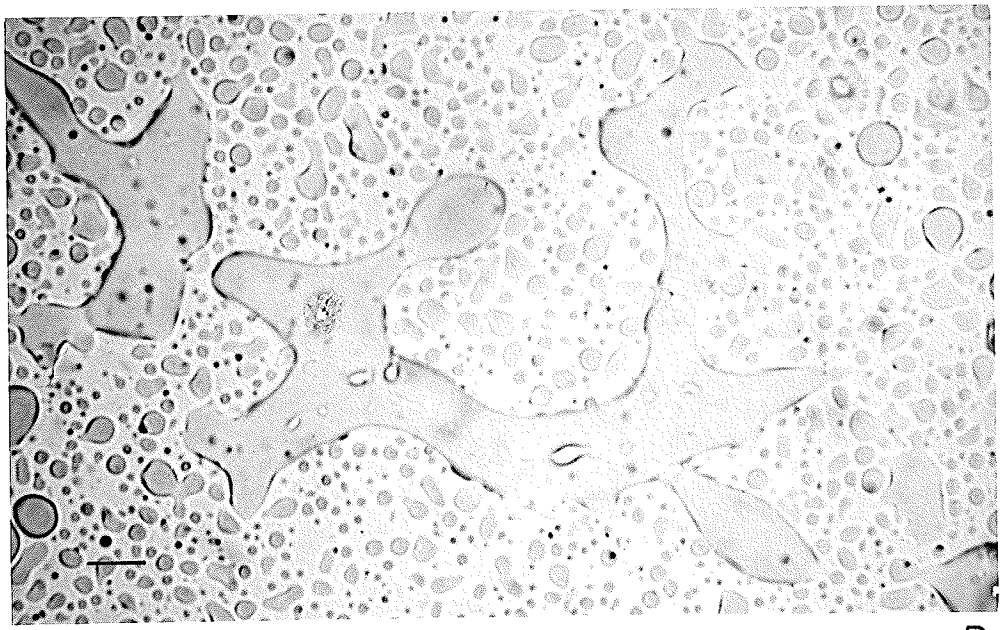

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Figure 4.7. Photomicrographs of micelle responses corresponding to rating 7. Bar represents 25 μm.

A. Protein coalescence into extensive amorphous masses.

B. Further coalescence to form protein sheet.

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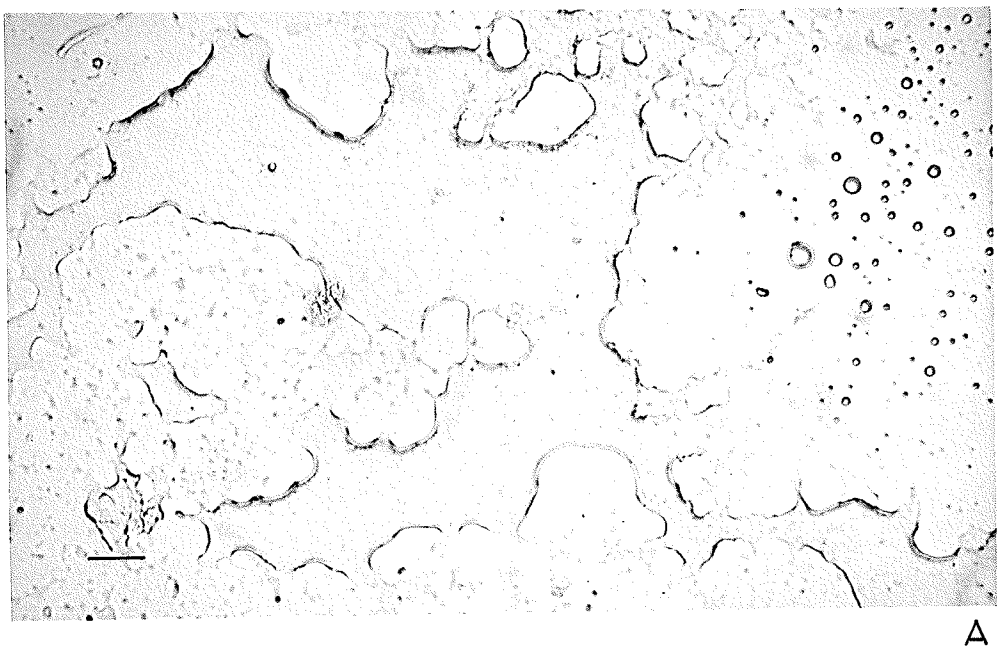

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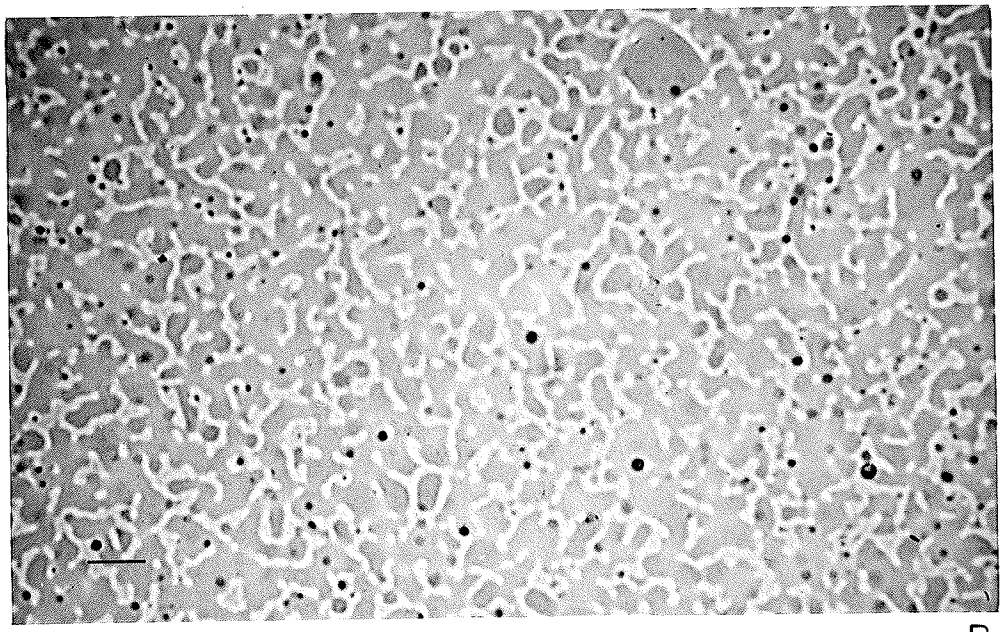

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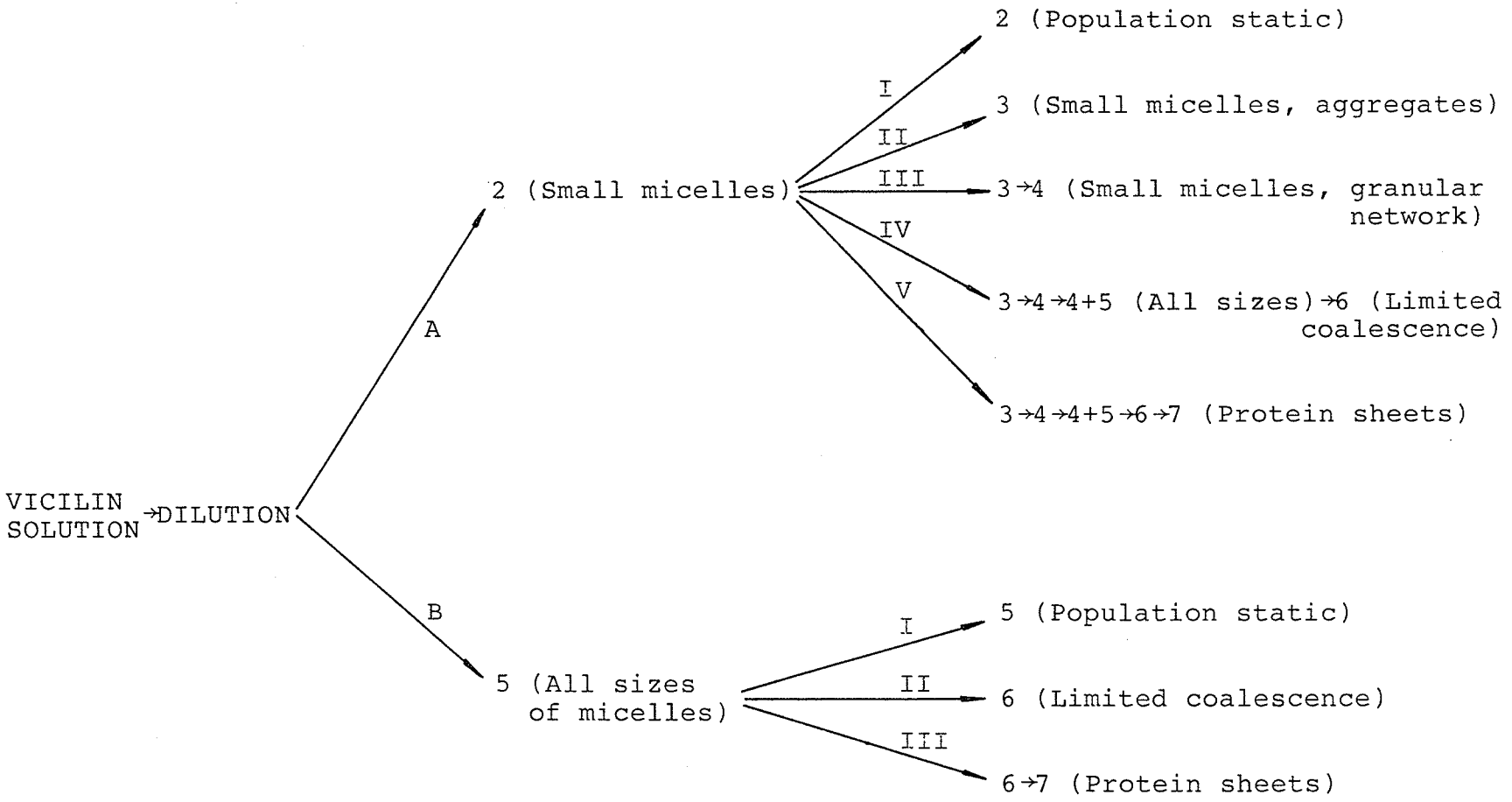
Although the descriptive parameters for each numerical "micelle rating" are presented in Table 4.2, several terms warrant futher clarification. In ratings 3 and 4, small micelles(approximately 2 μm in diameter) were present either in aggregates(3) or in fine granular networks(4). The term "aggregate" implies association of the individual micelles but not apparent inter-micelle coalescence(Figure 4.2 A, B); the phrase "granular network" describes a three-dimensional arrangement formed by partial inter-micelle coalescence in which small micelle structures were still evident. With rating 5, small micelles coalesced to form discrete micelles of larger sizes, designated as intermediate and large based on approximate diameters of 10 and 20 μm(Figure 4.4 A, B). Also for rating 5, coalescence was limited in that a significant static population of small micelles remained; in addition, there was only minimal inter-micelle association to form more elongate, non-spherical structures(Figure 4.4 C). Rating 6 was characterized by "homogeneous" coalescence. In contrast to the granular phenomemon(rating 4), this coalescence appeared to involve complete assimilation of the micelles such that any impression of individual micelles was lost(Figure 4.5). As described in rating 7, the homogeneous coalescence of rating 6 was frequently followed by formation of extensive networks(Figure 4.6 A) - structures which seemed more substantial than the apparently delicate networks characteristic of rating 4(Figure 4.3 A, B). Varying degrees of continued coalescence inevitably followed these homogeneous

networks to result in amorphous patches of aggregated protein(Figures 4.6 B, 4.7 A) or nearly complete protein sheets(Figure 4.7 B).

Although the numerical scheme of micelle ratings was primarily designed to be a characterization approach, it was also intended to have some subjective hierarchical connotations. The hierarchical implications were a result of observations relating to the development of specific micelle responses; often a definite series of structures was evident depending on the environmental conditions present. The different pathways observed are outlined in Figure 4.8. In general, any initial micelle response was characterized by one of two patterns - either a population of small discrete micelles(rating 2, route A) or a heterogeneous population of variable-sized discrete micelles(rating 5, route B). In some cases, further molecular interaction ceased at this point and the two types of populations became static. In other situations, further coalescence occurred, resulting in a variety of end-points depending upon the degree of continued association. These possible pathways have been defined as AI, AII, AIII, AIV, and AV or BI, BII, and BIII in Figure 4.8. In terms of the hierarchical assessment of the stages comprising these pathways, one of the first considerations was the ultimate final result of extensive micelle interaction regardless of the intermediate structures. For both A and B alternatives, this was identified as the formation of amorphous protein sheets and was subsequently described by the highest numerical rating of 7. A slightly

Figure 4.8. Observed pathways in the formation and interaction of micelles. Protein concentration of pre-dilution vicilin solution was approximately 25mg mL^{-1}. Dilution ratio was 1 water:1 protein. Numbers indicate micelle ratings as given in Table 4.2.


VICILIN SOLUTION
DILUTION
A
B
2 (Small micelles)
I
II
III
IV
V
2 (Population static)
3 (Small micelles, aggregates)
3→4 (Small micelles, granular network)
3→4→4+5 (All sizes)→6 (Limited coalescence)
3→4→4+5→6→7 (Protein sheets)
5 (All sizes of micelles)
I
II
III
5 (Population static)
6 (Limited coalescence)
6→7 (Protein sheets)

weaker interaction was that designated by rating 6 in which homogeneous coalescence was extensive but terminated prior to formation of amorphous aggregates. Similar to the approach for ratings 6 and 7, ratings 2 through 4 were also established using the degree of molecular interaction as an indicator of the strength of the micelle response. Small discrete micelles(rating 2, Figure 4.1 B) appeared to be less reactive than small micelles in aggregates(rating 3, Figure 4.2). The aggregates of rating 3, in turn, appeared to be less interactive than the micelles of rating 4(Figure 4.3) in which actual inter-micelle coalescence occurred. The assignment of ratings 2, 3, 4, 6 and 7 to various micelle responses appeared logical in terms of sequential molecular interaction; however, the position of the phases referred to as 1 and 5 was less easily identified. A particulate microscopic field in which no individual structures were discernible(Figure 4.1 A) was eventually described as rating 1. This response was only observed with specific conditions and did not appear to be an essential part of the hierarchical scheme. It can, however, be considered to represent some type of protein aggregation phenomenon.

Designation of a numerical value to the micelle response characterized by a heterogeneous mixture of small, intermediate and large micelles was more difficult. It was initially questionable as to whether an assessment of the extensiveness of this type of reaction in comparison with an elaborate granular network(rating 4, Figure 4.3 B) was valid; different types of associative mechanisms seemed to be operative. With

further observations, however, it appeared that the presence of multiple-sized micelles was a prerequisite for progression to the more extensive reactions(ratings 6 and 7, Figures 4.5, 4.6 and 4.7). Even in situations where the micelle pattern developed from an original base of small micelles(route A), a mixed population of various-sized micelles was usually observed prior to homogeneous coalescence and eventual protein sheet formation(routes AIII and AIV, Figure 4.8). On this basis, the specific response of various-sized discrete micelles was assigned the intermediate numerical rating of 5. Despite the position assigned to this micelle response in the rating scheme, it was not implied that various-sized micelles would ultimately coalesce; several examples were observed in which a static population was formed(route BI, Figure 4.8).

Protein Concentration and Dilution Parameters for Micelle Observations

In the experimental approach used to define and understand some of the environmental influences on micelle formation, it was important initially to establish certain parameters that would be controlled in the assessment of the micelle response. One consideration was the initial concentration of vicilin in the pre-dilution solution. As the main concern of this study was to examine the effect of environmental parameters on vicilin micelle formation, it was not desirable to have protein concentration as a limiting or influential parameter in the type of micelle response produced. Preliminary studies indicated that vicilin levels of 20 mg

mL^{-1} were adequate to establish a characteristic or maximum micelle response for a particular environment. Further concentration assessments with vicilin exposed to two specific environments(0.2 M lithium chloride and 0.2 M sodium acetate, pH 6.6) indicated that the minimum protein level required for a maximum micelle response differed between the two electrolytes. For vicilin in sodium acetate, pH 6.6, the maximum micelle response(rating 7, Figures 4.7 and 4.8) occurred at a protein level above 15.6 mg mL^{-1}; that for vicilin in lithium chloride(also rating 7) occurred at a level above 7.4 mg mL^{-1}(Table 4.3). As a result of the data from this detailed study and from preliminary experiments, all predilution protein levels were adjusted to approximately 25 mg mL^{-1} in order to eliminate vicilin concentration as a limiting factor in further micelle experimentation.

A second basic concern was the dilution ratio (water:protein) required for a maximum micelle response with vicilin in different environments. An initial guideline for this consideration was the dilution ratio of 3:1 (water:protein) used by Murray et al. (1978) in the original PMM procedure. Preliminary studies, designed to expand on this starting-point, were indicative of a relatively broad dilution range, 1:2 to 4:1 (water:protein), that would result in a constant characteristic micelle response for a specific environment. In a more detailed analysis of dilution parameters with vicilin exposed to one electrolyte environment(0.2 M lithium chloride), it was found that the most extensive micelle

TABLE 4.3. The influence of initial protein concentration on the capacity of vicilin to form micelles in the two salt environments.

Protein concentration (mg mL^{-1})	Characteristics of Micelle Response	Micelle Rating[1]
Sodium acetate(0.2 M, pH 6.6[2])		
5.8	Background haze.	1
6.8	Small micelles, some granular network.	4
7.5	As above.	4
11.8	Small micelles, more extensive granular network.	4
15.6	All sizes of micelles; extensive coalescence to form protein sheets.	7
27.9	As above.	7
Lithium chloride(0.2 M)		
3.6	All sizes of micelles, remain discrete.	5
4.8	All sizes of micelles, limited coalescence.	5
7.4	All sizes of micelles, extensive coalescence to form protein sheets.	7
9.6	As above.	7
12.2	As above.	7
17.8	As above.	7
21.5	As above.	7

[1] Description of ratings given in Table 4.2.

[2] pH adjusted to 6.6.

response(rating 7, Figures 4.6 and 4.7) was present for dilutions of 10:1 through to 1:4, water to protein(Table 4.4). With water excesses greater than 10:1, the micelle response gradually deteriorated. Only individual micelles(rating 5, Figure 4.4 B) were apparent at 25:1. These were replaced by small micelles at 30:1(rating 2, Figure 4.1 B) and by a transitory population of small micelles at 40:1 and 50:1. If water were a more limiting factor, the micelle response also deteriorated with only small micelles(rating 2, Figure 4.1 B) formed at a 1:10 water to protein ratio. Interestingly, as the water level was reduced below 1:1, large amounts of air were entrapped in the micelle structures. Examples of air entrapment in different micelle populations including single structures and coalesced networks are given in Figure 4.9. As a consequence of these experimental results, a dilution ration of 1:1 (water:protein) was selected for all further micelle assessments.

Influence of pH on Micelle Formation

The capacity of vicilin to form micelles varied with pH, as shown by the micelle ratings given in Table 4.5 and Figure 4.10. At pH 6.0, vicilin molecules aggregated to form numerous small and intermediate micelles(rating 5, Figure 4.4 A); however, there was no tendency for further interaction, individual micelles remained discrete. From pH 6.1 to 6.8, vicilin exhibited a strong micelle reaction culminating in protein masses and sheets(rating 7, Figures 4.6 B and 4.7 A). The progression to this end-point was rapid; however, a distinct

TABLE 4.4. Variations in the micelle-forming capacity of vicilin in 0.2 M LiCl with changes in the water:protein dilution ratio. Each vicilin solution prior to dilution contained 25 mg mL^{-1} protein.

Dilution Ratio (water:protein)	Characteristics of Micelle Response	Micelle Rating[1]
1:10	Many small discrete micelles.	2
1:4	All sizes of micelles, extensive coalescence to form protein sheets.	7
1:1	As above.	7
2:1	As above.	7
3:1	As above.	7
10:1	As above.	7
25:1	All sizes of micelles, limited coalescence.	5
30:1	Many small discrete micelles.	2
40:1	Initial formation of small micelles; unstable with time.	2 → 0
50:1	As above.	2 → 0

[1] Description of ratings given in Table 4.2.

Figure 4.9. Photomicrographs of micelle responses exhibiting multiple pockets of entrapped air. Bar represents 25 μm.

A. Large single structures.

B. Extensive protein mass.

NOTICE/AVIS

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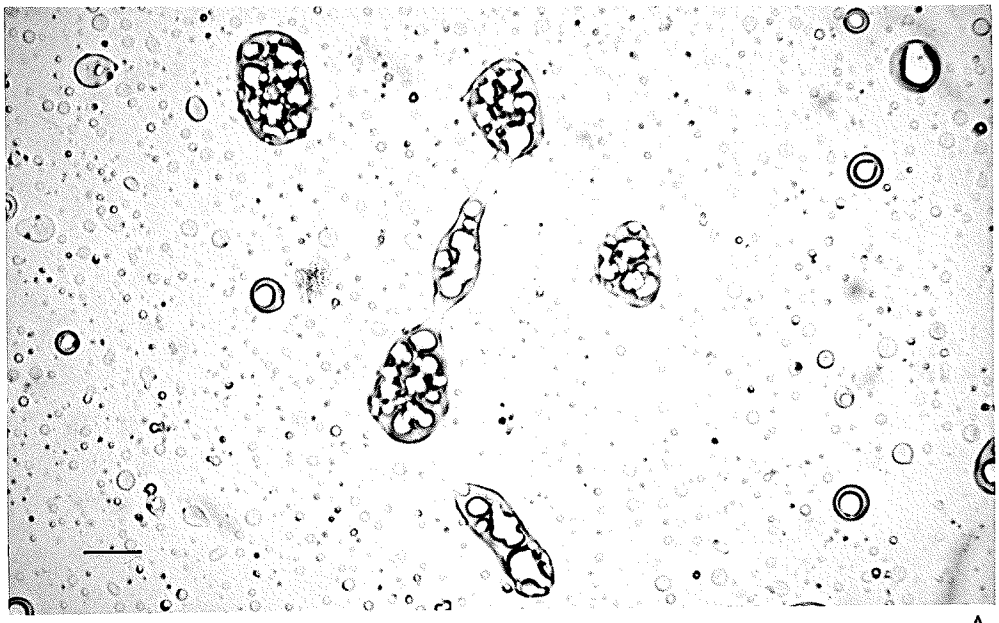
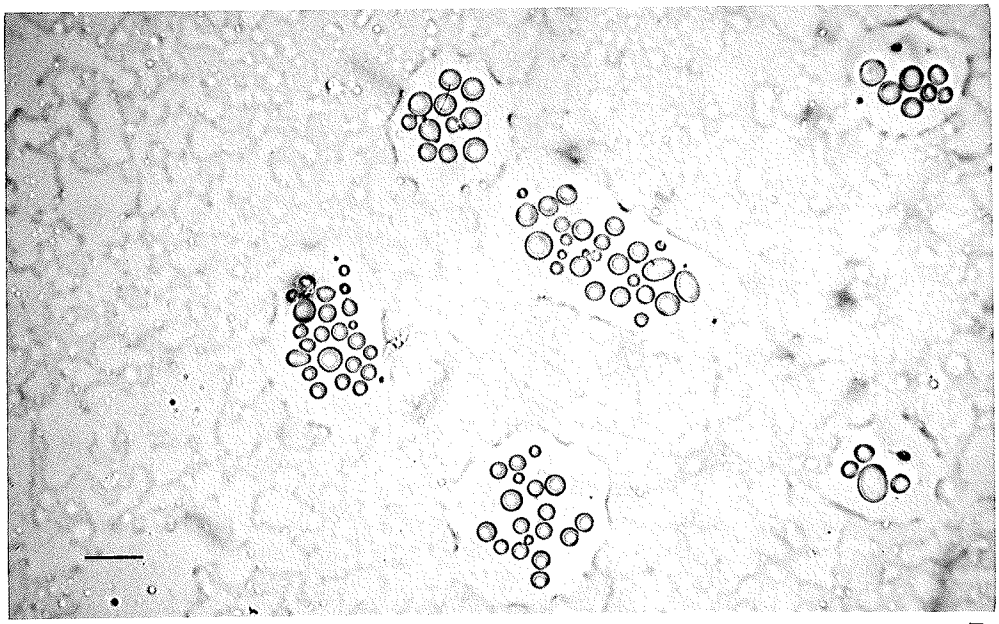


A



B

TABLE 4.5. Micelle-forming capacity, denaturation temperature (Td) and surface hydrophobicity (So) of vicilin exposed to a series of 0.1 M phosphate buffers ranging in pH from 6.0 to 8.0.

pH	Micelle Rating[2]		Td[3] (°C)	So[3]
	Pre-dilution	Post-dilution		
6.0	5 (Few)	5	86.9±1.1[a,b]	286±22[a]
6.1	0	7	86.8±1.0[a,b]	nd[4]
6.2	0	7	86.4±0.4[a]	nd[4]
6.3	0	7	86.3±0.5[a]	nd[4]
6.4	0	7	86.3±0.9[a,b]	296±17[a]
6.5	0	7	85.6±0.4[b,c]	nd[4]
6.6	0	7	84.6±0.5[d]	nd[4]
6.7	0	7	83.8±0.3[d,e]	nd[4]
6.8	0	7	83.8±0.3[d,e]	nd[4]
7.0	0	4	83.9±1.3[c,d,e]	246±14
7.5	0	2	82.0±0.6[f]	162±16[b]
8.0	0	2 (Few)	81.2±1.2[f]	158±12[b]

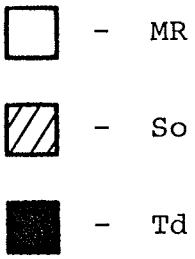
[1] Column values followed by the same letter are not significantly different ($P \leq 0.05$).

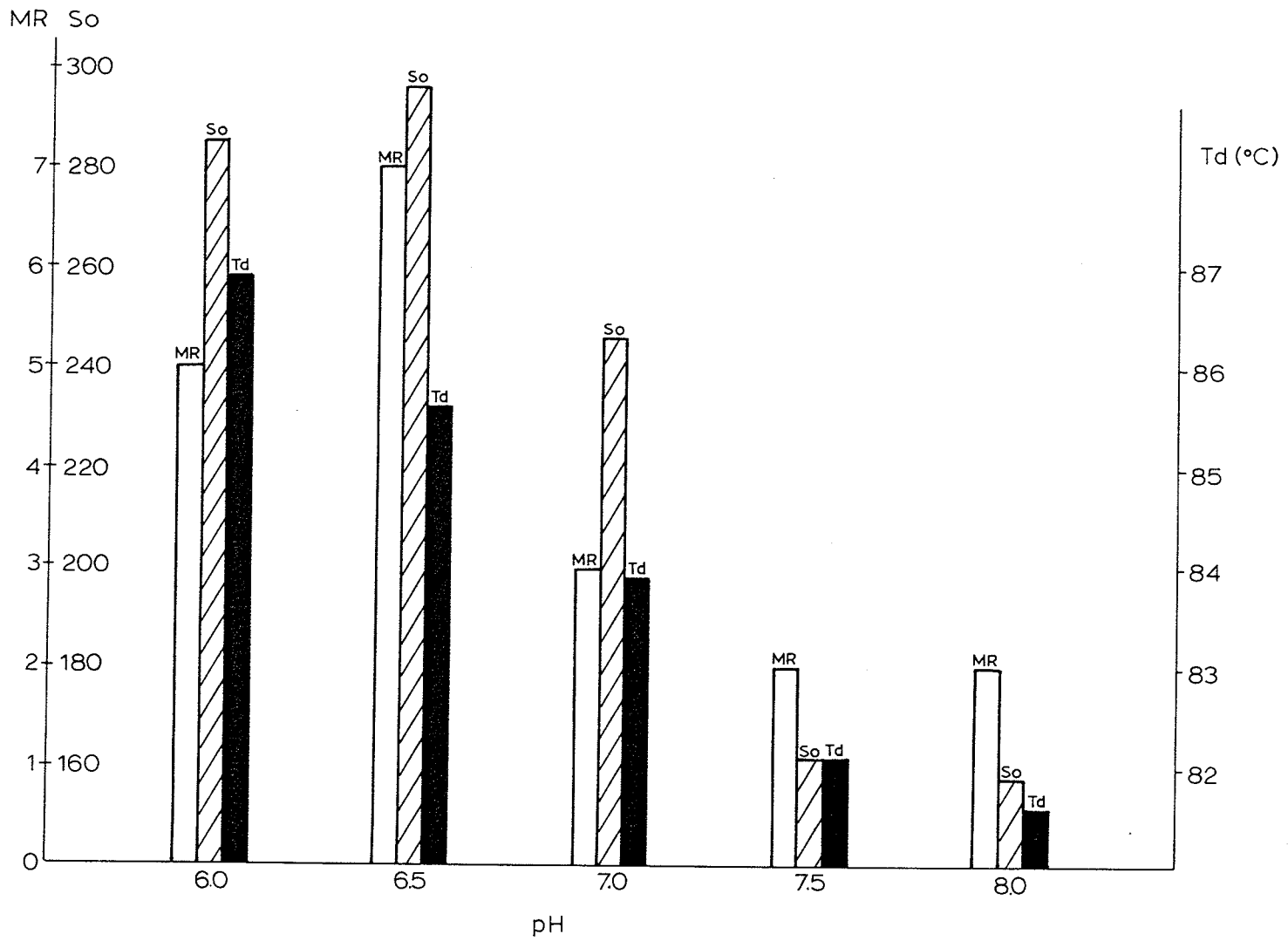
[2] Description of ratings given in Table 4.2.

[3] Values determined in Chapter 3.

[4] Value not determined.

Figure 4.10. Micelle ratings (MR), surface hydrophobicities (So) and denaturation temperatures (Td) for vicilin exposed to a series of 0.1M phosphate buffers ranging in pH from 6.0 to 8.0.




MR So
300
280
260
240
220
200
180
160
0
1
2
3
4
5
6
7
MR
So
Td
Td (°C)
87
86
85
84
83
82
6.0
6.5
7.0
7.5
8.0
pH

series of structures leading up to the maximum response was observed. The initial micelle reaction was characterized by small, intermediate and large micelles(rating 5, Figure 4.4 B). With time, these micelles coalesced to form homogeneous networks(Figure 4.5 B, C) which then united to form the final amorphous protein aggregates, a pathway corresponding to BIII, Figure 4.8. At pH 7.0, the micelle response showed some deterioration with the formation of small discrete micelles and fine granular networks(rating 4, Figure 4.3 A). Further deterioration in the micelle response was evident above pH 7.0. At pH 7.5, small discrete micelles were formed(rating 2, Figure 4.1 B); at pH 8.0 only a limited number of small micelles were observed. This influence of pH on the capacity of vicilin to form micelles in one electrolyte environment was supportive of the restriction imposed on pH variability in other electrolyte experiments. All media normally resulting in an alkaline pH were adjusted to pH 6.6 prior to micelle assessment.

On the basis of the micelle observations, the optimum structural characteristics of vicilin for micelle formation and subsequent interaction, in this environment, were identified to exist from pH 6.1 to 6.8. In order to relate the micelle ratings over the entire pH range(6.0 to 8.0) to some vicilin structural characteristics, specifically surface hydrophobicity(So) and denaturation temperature(Td)(determined in Chapter 3), Figure 4.10 was constructed. Comparison of the So data with the micelle observations indicated that a higher So value promoted a stronger micelle response. Inclusion of

the pH 6.0 data in this comparison may not represent a realistic evaluation of this proposed relationship between So and micelle-rating. Concentrated protein samples, at this pH level, exhibited some visible precipitation prior to dilution, as partially evidenced by the pre-dilution presence of single micelles(rating 5, Figure 4.4 B). As a result, the amount of soluble protein may have been reduced below the level required to give the maximum micelle response for this environment.

In addition to the changes in So with increasing pH, the Td of vicilin also decreased over this pH range from 86.9°C at pH 6.0 to 81.2°C at pH 8.0(Table 4.5). Although this progressive decrease in Td, indicative of general molecular destabilization, began at pH 6.5, it was only associated with a deteriorating micelle response from pH 7.0 to 8.0(Table 4.5, Figure 4.10).

Influence of Various Electrolytes on Micelle Formation

A. Influence of Specific Anions

The effects of various sodium-associated anions on the micelle-forming capacity of vicilin were examined. The micelle ratings corresponding to pre-dilution and post-dilution observations are given in Table 4.6. Although the basic premise of this study was to invoke a micelle response by sudden exposure of the vicilin molecules to an aqueous environment, it was observed that concentrated vicilin, in some environments, would aggregate to form a micelle population prior to dilution. As an initial consideration, it is interesting to

TABLE 4.6. Pre- and post-dilution micelle ratings[1] for vicilin exposed to different concentrations of a number of sodium salts. Salts are arranged in sequence according to their molal surface tension increments (σ).

Salt	σ[2]	0.1 M		0.2 M		0.5 M		1.0 M	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
NaSCN	0.60[3]	2	6	0	3	0	2	0	2
NaI	1.02[4]	2(Few)	4	2(Few)	7	2(Few)	2	nd[5]	
Na-acetate	1.27[4]	nd[5]		3	7	0	7	0	7→0[6]
NaBr	1.32[4]	5(Few)	5	3	7	0	7	0	7→0[6]
NaCl	1.64[4]	nd[5]		3	7	3	7	0	7→0[6]
Na_2HPO_4	2.02[3]	0	7	0	0	0	0	nd[5]	
Na_2SO_4	2.73[4]	3	6	3	2	2	0	2	0
Na_3-citrate	3.27[3]	0	2	0	2	1	0	1	0

[1] Description of ratings given in Table 4.2.

[2] Units are 10^{-3} dyn g cm^{-1} mol^{-1}.

[3] Melander and Horvath (1977).

[4] International Critical Tables (1929).

[5] Value not determined due to solubility limitations.

[6] Micelle populations unstable.

examine some of the conditions in which pre-dilution vicilin micelles occurred. In general, although these pre-dilution micelles existed for most anions at 0.1 and 0.2 M salt levels, the populations were not extensive and the degree of molecular interaction resulted in ratings of 1, 2 or 3(Figures 4.1, 4.2 and 4.3). There was only one exception - a rating of 5(Figure 4.4 B) for vicilin in sodium bromide.

As this phenomenon occurred mainly at 0.1 and 0.2 M salt levels, it would appear that electrostatic influences of the electrolytes in these concentrated protein solutions were inadequate to promote a total salting-in effect. As the salt concentration was increased the pre-dilution micelles were reduced, suggesting a more complete pre-dilution solubilizing influence of the salts on vicilin. In fact, at 1.0 M, only the sulfate environment exhibited pre-dilution micelle-type structures. Interestingly, these micelles were not stable to the dilution procedure. The background haze, designated as rating 1(Figure 4.1 A), for pre-dilution conditions at higher concentrations of citrate(0.5 and 1.0 M) could not be conclusively designated as a micelle response at the light microscope level. However, regardless of the structural format of this molecular interaction, this type of aggregation was not stable to dilution.

Two specific salt environments were consistantly free from pre-dilution structures indicating some salt-protein interactions which promoted continued solubility. These included 0.2, 0.5 and 1.0 M levels of the destabilizing NaSCN

which did give a micelle response with dilution at all concentration levels and 0.1, 0.2 and 0.5 M levels of sodium phosphate which only resulted in a micelle response at 0.1 M salt levels.

In terms of post-dilution results, the main emphasis of this study, the general effect of an increase in salt concentration from 0.1 to 1.0 M on micelle formation was a complete supression of the micelle response(rating 0). The only exception was that of vicilin exposed to 1.0 M NaSCN in which a few small discrete micelles were observed(rating 2, Figure 4.1 B). With respect to the effects of individual anions on micelle formation, it seemed most appropriate to compare their influences with reference to salt concentration levels and the molal surface tension increments(σ) of individual salts. Both of these factors were important in considering specific conformational parameters of vicilin molecules(Chapter 3). In addition, surface hydrophobicity values and denaturation temperatures for vicilin(as determined in Chapter 3) were compared to various micelle responses; specific emphasis was placed on So due to the implied significance of hydrophobic interactions in vicilin micelle formation(Murray et al., 1978). Comparison of the micelle responses for different anions at 0.1 M, a salt concentration where electrostatic influences are important for most salts, revealed relatively erratic patterns with no apparent relation to the σ of the salt involved(Table 4.6). In terms of individual salts, vicilin exposed to NaSCN, sodium phosphate and Na_2SO_4 showed strong micelle reactions with rat-

ings of 6, 7 and 6, respectively. In all three situations, the micelle response appeared to develop according to routes BII and BIII with a heterogeneous micelle population as the initial pattern(Figure 4.8). The micelle ratings for these environments were associated with relatively high So values for vicilin in Na_2SO_4(220) and sodium phosphate(219); however, the So value of 179 for NaSCN was significantly lower(Figure 4.11). In fact, in comparison with the other anions at this concentration level, vicilin in 0.1 M NaSCN appeared somewhat destabilized with a reduced Td value of 79.0°C(Figure 4.11). With 0.1 M NaI and NaBr, the micelle responses were slightly reduced(ratings 4 and 5, Figures 4.3 and 4.4). These observations, however, may not be realistically comparable to those for vicilin exposed to other salts at 0.1 M. With both NaI and NaBr, visible precipitation was noted in the concentrated vicilin solutions prior to dilution. As a result, these micelle responses may be a function of the concentration of soluble protein available for interaction.

The greatest difference in micelle patterns at 0.1 M occurred with vicilin in sodium citrate(μ = 0.45),the only salt assumed to be exerting predominantly lyotropic effects at this concentration level. Only a population of small discrete micelles was observed(rating 2, Figure 4.2 A); in fact, it was difficult to assess a differential between pre- and post-dilution conditions. The So value for vicilin in this environment was low(191); however, this value was higher than the So for vicilin in NaSCN(179) where a strong micelle

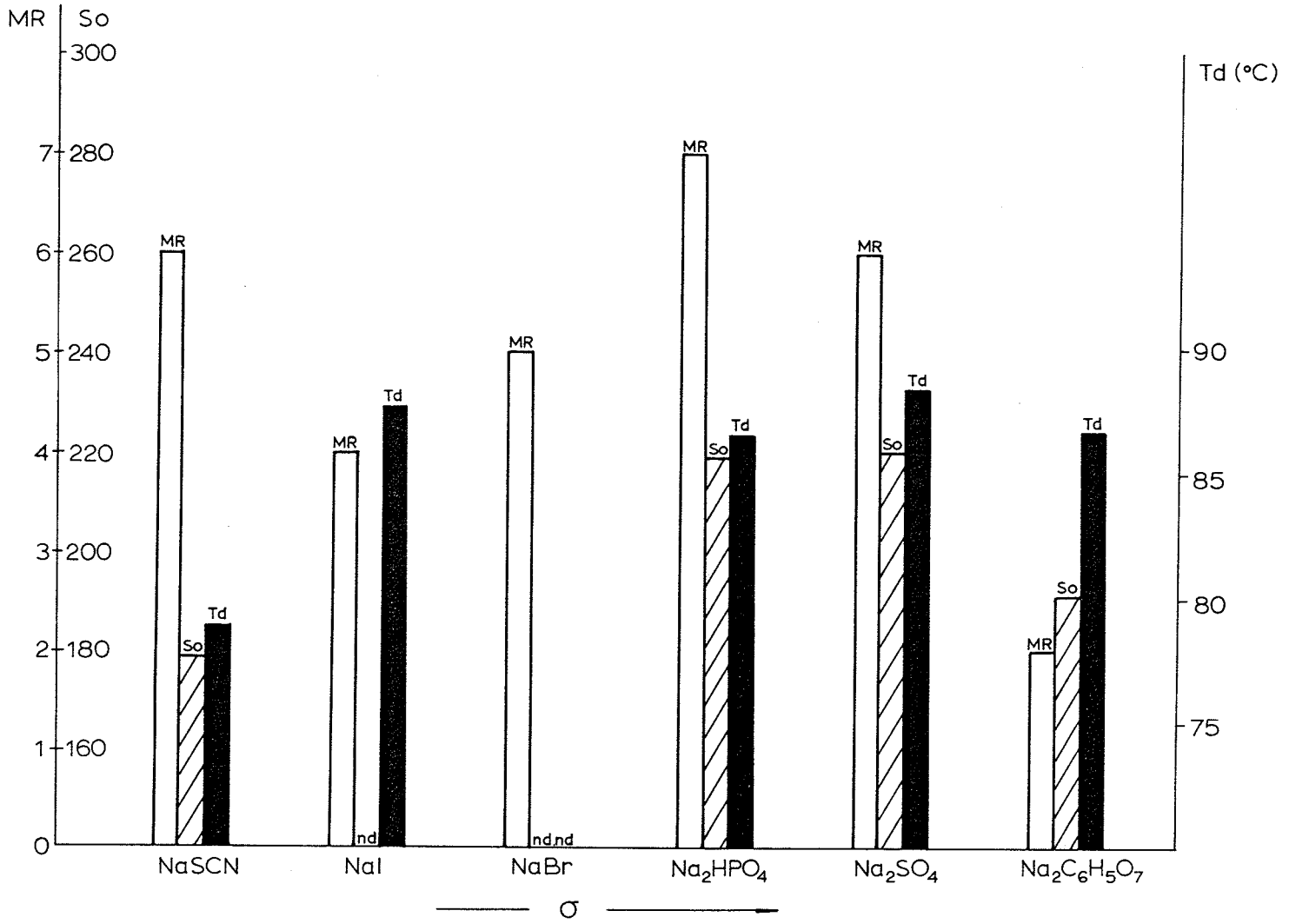
Figure 4.11. Micelle ratings (MR), surface hydrophobicities (So) and denaturation temperatures (Td) for vicilin exposed to a series of 0.1M sodium salts. All values for So and Td were determined in Chapter 3.


- MR


- So


- Td

nd - Values not determined due to quenching effect of anion (So) or solubility limitations (Td)


MR
So
300
7
280
6
260
5
240
4
220
3
200
2
180
1
160
0
Td (°C)
90
85
80
75
MR
So
Td
nd
nd,nd
NaSCN
NaI
NaBr
Na_2HPO_4
Na_2SO_4
$Na_2C_6H_5O_7$
σ

response(rating 6, Figure 4.5) was observed(Figure 4.11). As the So values were similar, the different micelle responses in these two environments may be related to the more highly stabilized vicilin molecules in the citrate medium. This stability was inferred from the significantly higher Td value for vicilin in sodium citrate(86.8°C) as compared with that for vicilin in NaSCN(79.0°C)(Figure 4.11).

In summary, at salt concentrations of 0.1 M, the hierarchy of anions in terms of their ability to elicit a strong micelle response can be given as follows:

phosphate > sulfate, thiocyanate > bromide, iodide > citrate

A general trend in micelle response with respect to the lyotropic series of anions became more apparent at 0.2 M salt concentrations; groups of anions with similar σ values tended to exhibit comparable reactions(Table 4.6, Figure 4.12). A strong micelle response(rating 7, Figures 4.6 and 4.7) was observed for vicilin exposed to the univalent anions - iodide, acetate, bromide and chloride(Table 4.6). In addition to exhibiting massive protein interactions, each micelle response was characterized by multiple pockets of entrapped air(Figure 4.9 B). Although the same associative end-point was established by vicilin in these four anionic environments, different developmental patterns were observed. For NaI, sodium acetate and NaCl, the micelle response seemed to develop according to route A(Figure 4.8), progressing from initial small micelles and aggregates. For NaBr, however, establish-

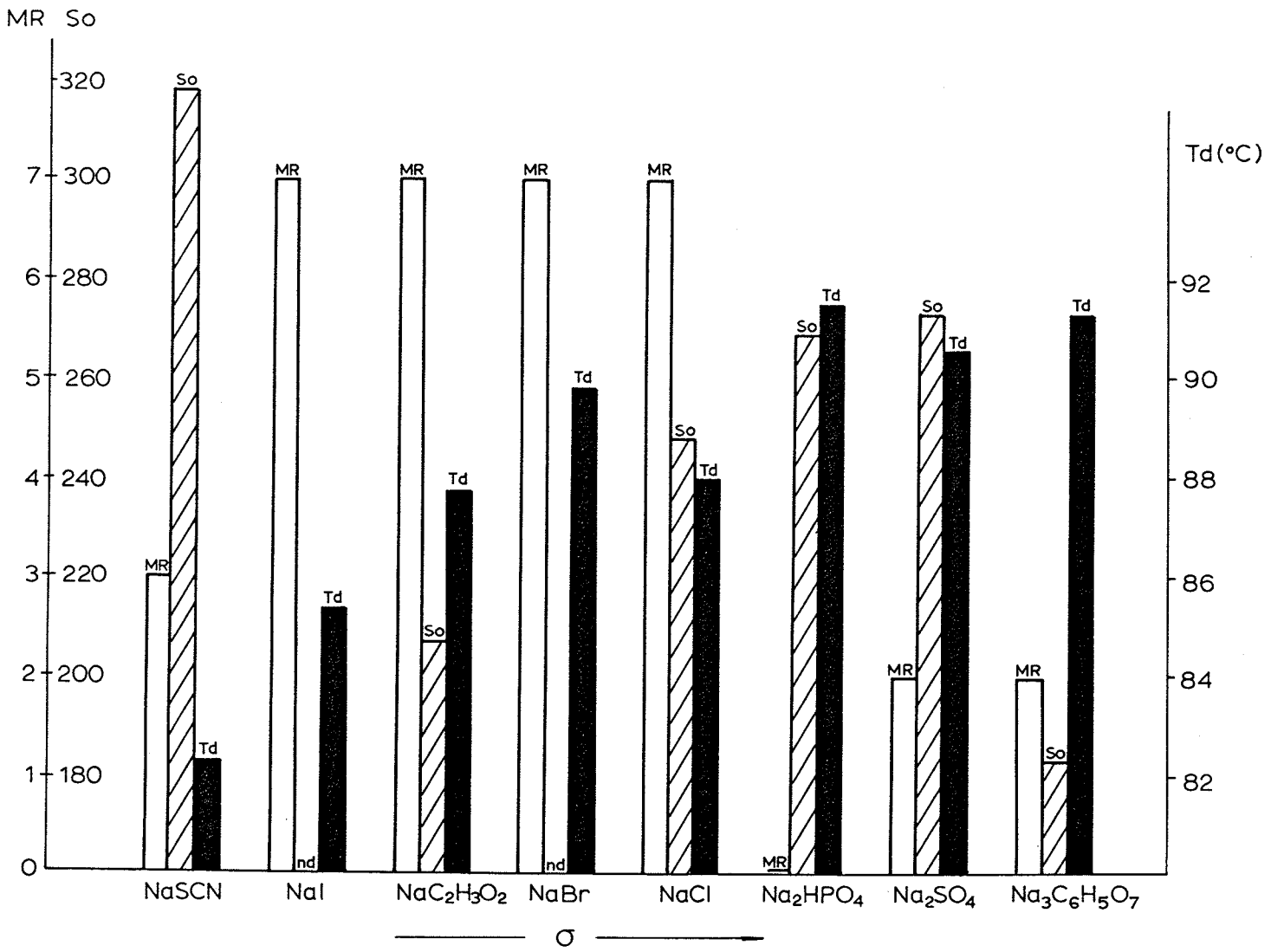
Figure 4.12. Micelle ratings (MR), surface hydrophobicities (So) and denaturation temperatures (Td) for vicilin exposed to a series of 0.2M sodium salts. All values for So and Td were determined in Chapter 3.


- MR


- So


- Td

nd - Value not determined due to quenching effect of anion.


MR So
320
300
280
260
240
220
200
180
0
7
6
5
4
3
2
1
Td(°C)
92
90
88
86
84
82
So
MR
Td
nd
NaSCN
NaI
$NaC_2H_3O_2$
NaBr
NaCl
Na_2HPO_4
Na_2SO_4
$Na_3C_6H_5O_7$
σ

ment of the maximum protein interaction appeared to follow route B(Figure 4.8), with the initial dilution response characterized by a heterogeneous population of various-sized micelles. For comparative purposes, it should also be noted that for vicilin exposed to NaI and NaBr, the micelle response at 0.2 M was stronger than that observed at 0.1M. A similar comparison could not be made for 0.1 M sodium acetate and NaCl as a result of reduced protein solubilities in these media. In fact, the observed differential in micelle ratings between 0.1 and 0.2 M levels of NaI and NaBr may also have resulted from reduced vicilin solubility in the 0.1 M environments.

In an attempt to establish molecular criteria for micelle establishment, some structural properties of vicilin were related to the protein micelle responses for these univalent anionic media(Figure 4.12). Surface hydrophobicity values for vicilin in sodium acetate and NaCl were relatively high, with absolute values of 207 and 248, respectively(Figure 4.12). Unfortunately, comparable values for vicilin exposed to NaI and NaBr were not determined due to the quenching nature of the anions. In terms of thermal properties, the Td values for vicilin in sodium acetate, NaBr and NaCl were similar; that for vicilin in 0.2 M NaI was significantly lower than the values for the latter two salts(Figure 4.12). As the Td values for vicilin in NaI and sodium acetate were not significantly different, it is difficult to comment on the relative destabilizing influences of NaI at this concentration level.

Significant deterioration in the micelle response was

observed for vicilin in 0.2 M levels of NaSCN, sodium phosphate and Na_2SO_4(ratings of 3, 0 and 2, respectively) in comparison with the 0.1M results. A reduced micelle reaction(rating 2, Figure 4.1 B) was also observed for vicilin in 0.2 M sodium citrate; this, however, was similar to that at 0.1 M. In terms of relating these responses to the structural characteristics of vicilin, the low micelle rating for vicilin in NaSCN seemed to be associated with the general NaSCN destabilization effect. Vicilin in this medium was characterized by a significantly decreased Td value of 82.3°C(Figure 4.12). The reduced micelle response for vicilin in sodium citrate was associated with a slightly reduced So value of 183. However, an apparent contradiction in terms of a relationship between So and micelle response was exhibited by vicilin in sodium phosphate and Na_2SO_4. The micelle ratings for these media were low; however, the So values were high - 269 for vicilin in sodium phosphate and 273 for Na_2SO_4. This was especially apparent in a comparison with the So values for vicilin in sodium acetate(207) and NaCl(248), media which elicited a strong micelle response. In terms of Td values, those for vicilin exposed to sodium phosphate, Na_2SO_4 and sodium citrate were similar and significantly higher than those for the univalent anions producing a strong micelle reaction(Figure 4.12). Consequently, at the 0.2 M concentration level, the significant contrasting features between the groups of anions that either promote or discourage a micelle response did not appear to be the resulting So values or the protein conforma-

tional state as reflected by Td values. Instead, the greater ionic strength associated with the multivalent anions appeared to be important. In fact, the negative influence of the multivalent anions on micelle formation was much more apparent at 0.2 M salt levels than 0.1 M. As such, quite a different hierarchy of anions in terms of their effectiveness in promoting a strong micelle response is established:

iodide, acetate, bromide, chloride > thiocyanate > sulfate, citrate > phosphate

At 0.5 M, where lyotropic salt effects are important for all salts(von Hippel and Schleich, 1969), a definite relationship was apparent between micelle-forming capacity of vicilin and the lyotropic influence of the individual salts, as assessed by σ, the molal surface tension increment(Table 4.6). Vicilin exposed to both NaSCN and NaI, destabilizing salts with low σ values, showed a weak micelle response(rating 2, Figure 4.1 B). Interestingly, this was a dramatic change for the NaI medium; at 0.2 M NaI, the micelle response for vicilin was strong(rating 7, Figures 4.6 and 4.7). The low micelle ratings at 0.5 M were associated with an exaggerated So value of 373 for vicilin in NaSCN and reduced Td values of 80.8°C and 86.1°C for vicilin in NaSCN and NaI, respectively(Figure 4.13). With NaSCN in particular, these parameters were indicative of a destabilized molecule with an altered conformation.

For vicilin in 0.5 M sodium acetate, NaBr and NaCl, moderately-stabilizing salts with intermediate σ values, the

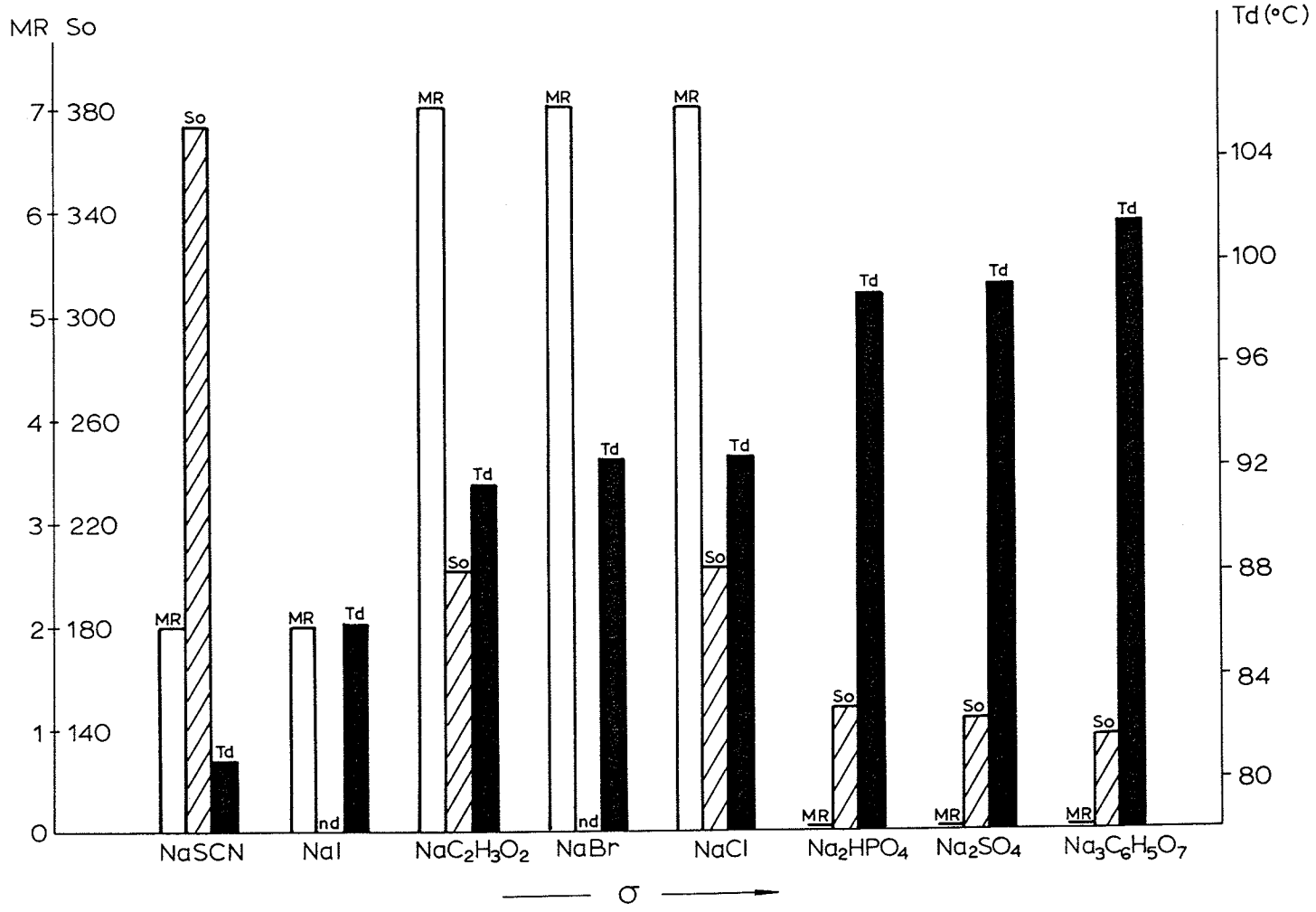
Figure 4.13. Micelle ratings (MR), surface hydrophobicities (So) and denaturation temperatures (Td) for vicilin exposed to a series of 0.5M sodium salts. All values for So and Td were determined in Chapter 3.


- MR


- So


- Td

nd - Value not determined due to quenching effect of anion.


MR So
Td (°C)
7
6
5
4
3
2
1
0
380
340
300
260
220
180
140
104
100
96
92
88
84
80
MR
So
Td
nd
NaSCN
NaI
$NaC_2H_3O_2$
NaBr
NaCl
Na_2HPO_4
Na_2SO_4
$Na_3C_6H_5O_7$
σ

micelle response was extensive(rating 7, Figures 4.6 and 4.7), similar to the results for these salts at 0.2 M. In addiition, the patterns of development for the maximum micelle response were similar to those described at 0.2 M concentrations - route A for sodium acetate and NaCl, route B for NaBr(Figure 4.8). These strong associative reactions for vicilin were correlated with the molecular So values of 201 for sodium acetate and 202 for NaCl(Figure 4.13); values apparently adequate for extensive micelle formation.

The strong micelle responses for the preceeding group of anions were in direct contrast to the complete absence of micelle formation(rating 0) by vicilin exposed to sodium phosphate, Na_2SO_4 and sodium citrate, highly stabilizing salts with elevated σ values(Figure 4.13). In these media, vicilin was characterized by low So values of 148 for sodium phosphate, 164 for Na_2SO_4 and 137 for sodium citrate. Significantly elevated Td values for vicilin in these same media(98.8°C for sodium phosphate, 99.1°C for Na_2SO_4 and 101.5°C for sodium citrate) were evidence of highly stabilized vicilin molecules(Figure 4.13).

In summary, at 0.5 M, the hierarchy of anions in terms of their relative effectiveness in promoting micelle formation is similar to that at the 0.2 M level, with the exception of the position of the iodide anion:

acetate, bromide, chloride > thiocyanate, iodide
> phosphate, sulfate, citrate

At 1.0 M, there was no salt environment that was suitable for the establishment of any stable major micelle population. With vicilin in 1.0 M NaSCN, a few single micelles(rating 2, Figure 4.1 B) were formed; however, this did not constitute a strong micelle response. Similar to the 0.2 M and 0.5 M concentration levels, the NaSCN environment was associated with an elevated So value(384) for vicilin and a decreased Td of 75.9°C(Figure 4.14). With the moderately stabilizing salts, sodium acetate, NaBr and NaCl, the micelle response was initially strong but transitory, disappearing rapidly after formation. Interestingly, the So values for vicilin in these media, 200 for sodium acetate and 201 for NaCl, seemed adequate for micelle formation based on previous observations.

Vicilin exposed to 1.0 M Na_2SO_4 and sodium citrate did not exhibit any micelle formation. Similar to the 0.5 M results, the So values for vicilin in these environments were significantly reduced with values of 129 for Na_2SO_4 and 124 for sodium citrate(Figure 4.14). In addition, the elevated Td values, 109.3°C for vicilin in Na_2SO_4 and 114.6°C for sodium citrate(Figure 4.14), were reflective of highly stabilized vicilin molecules. In general, it would appear that salt concentrations of 1.0 M were not appropriate for micelle formation, irrespective of the specific salt environment.

B. Influence of Specific Cations

The influence of the three cations, sodium, potassium and lithium (associated with both the chloride and sulfate anions), on the capacity of vicilin to form micelles was

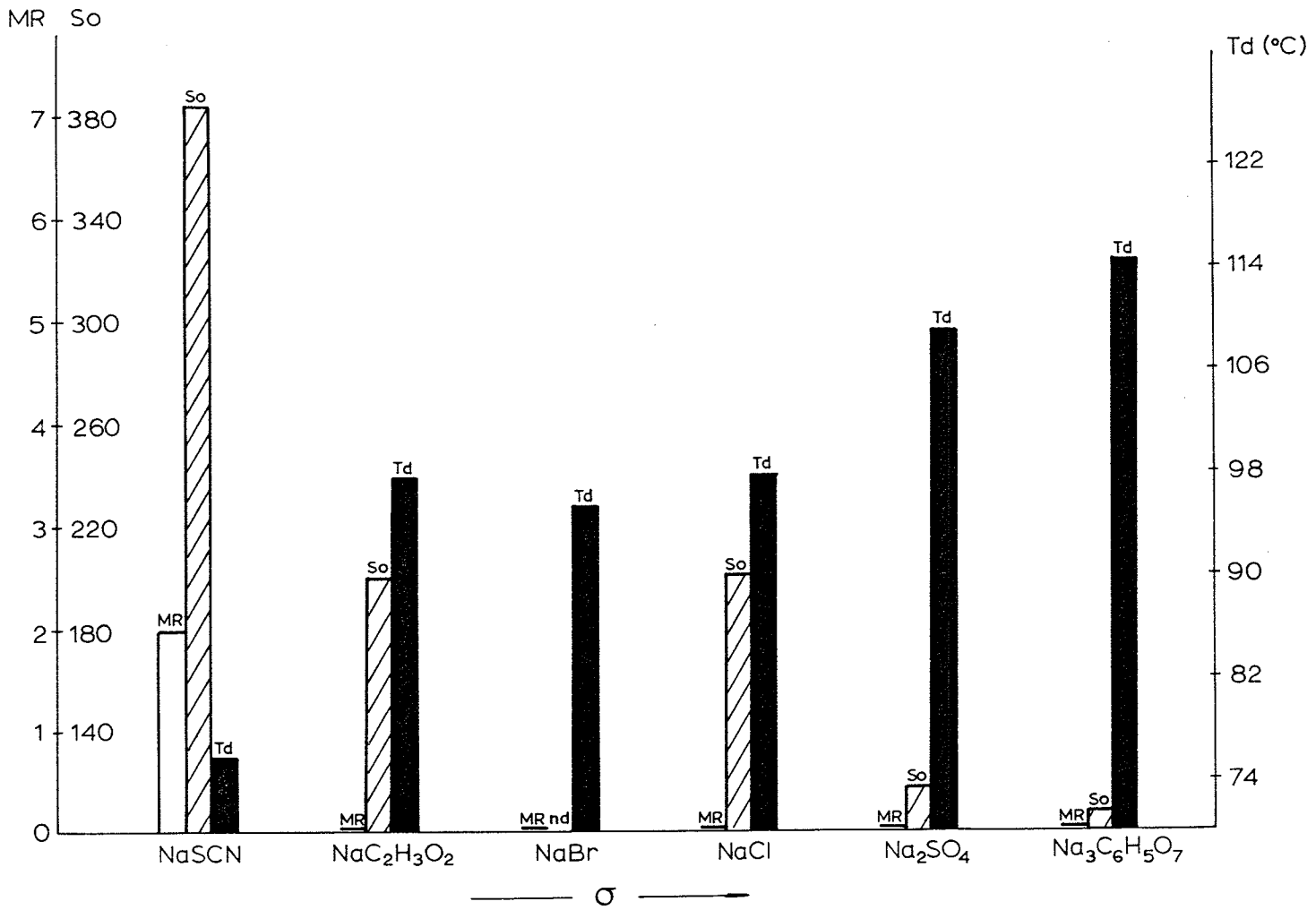
Figure 4.14. Micelle ratings (MR), surface hydrophobicities (So) and denaturation temperatures (Td) for vicilin exposed to a series of 1.0M sodium salts. All values for So and Td were determined in Chapter 3.


- MR


- So


- Td

nd - Value not determined due to quenching effect of anion.


MR So
Td (°C)
7
6
5
4
3
2
1
0
380
340
300
260
220
180
140
122
114
106
98
90
82
74
So
MR
Td
MR nd
NaSCN
$NaC_2H_3O_2$
NaBr
NaCl
Na_2SO_4
$Na_3C_6H_5O_7$
σ

assessed. Comparisons of these cations were made within each anionic group as a result of the implied effect of the associated anion on vicilin structural parameters(Chapter 3). With respect to the chloride salts, all three cations resulted in a strong micelle response(rating 7, Figures 4.6 and 4.7) at 0.2 and 0.5 M salt levels(Table 4.7). In addition, each of these micelle reactions was characterized by large amounts of entrapped air(Figure 4.9 B). The pattern for micelle response development differed somewhat among the three cations; that for sodium and lithium progressed via route A(Figure 4.8) with an initial formation of small aggregated micelles whereas that for potassium followed route B(Figure 4.8). In terms of molecular parameters associated with these micelle responses, the So values were variable but all were at a level of 200 or more(Figure 4.15). The Td values also differed; however, their relative values were indicative of stabilized vicilin molecules.

In comparison with 0.2 and 0.5 M chloride salts, the micelle responses at the 1.0 M level were quite different. For vicilin in 1.0 M NaCl, the micelle response was initially strong(rating 7) but completely unstable. In contrast, with vicilin in both 1.0 M KCl and LiCl environments, a significant stable population of single micelles was observed(rating 5). There was a differential in micelle size characteristics between the two salts - all sizes were observed with KCl(Figure 4.4 B), only small and intermediate sizes were noted with LiCl(Figure 4.4 A). The difference in the micelle

TABLE 4.7. Pre- and post-dilution micelle ratings[1] for vicilin exposed to different concentrations of several chloride and sulfate salts. Salts are arranged in sequence according to their molal surface tension increments (σ).

Salt	σ[2]	0.1 M		0.2 M		0.5 M		1.0 M	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
KCl	1.49	nd[3]		2	7	0	7	0	5
LiCl	1.63	nd[3]		3	7	3	7	0	5
NaCl	1.64	nd[3]		3	7	3	7	0	7→0[4]
K_2SO_4	2.58	3	3	2	6	2	0	nd[3]	
Na_2SO_4	2.73	3	6	3	2	2	0	2	0
Li_2SO_4	2.78	2	7	2	0	0	0	nd[3]	

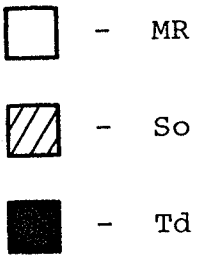
[1] Description of ratings given in Table 4.2.

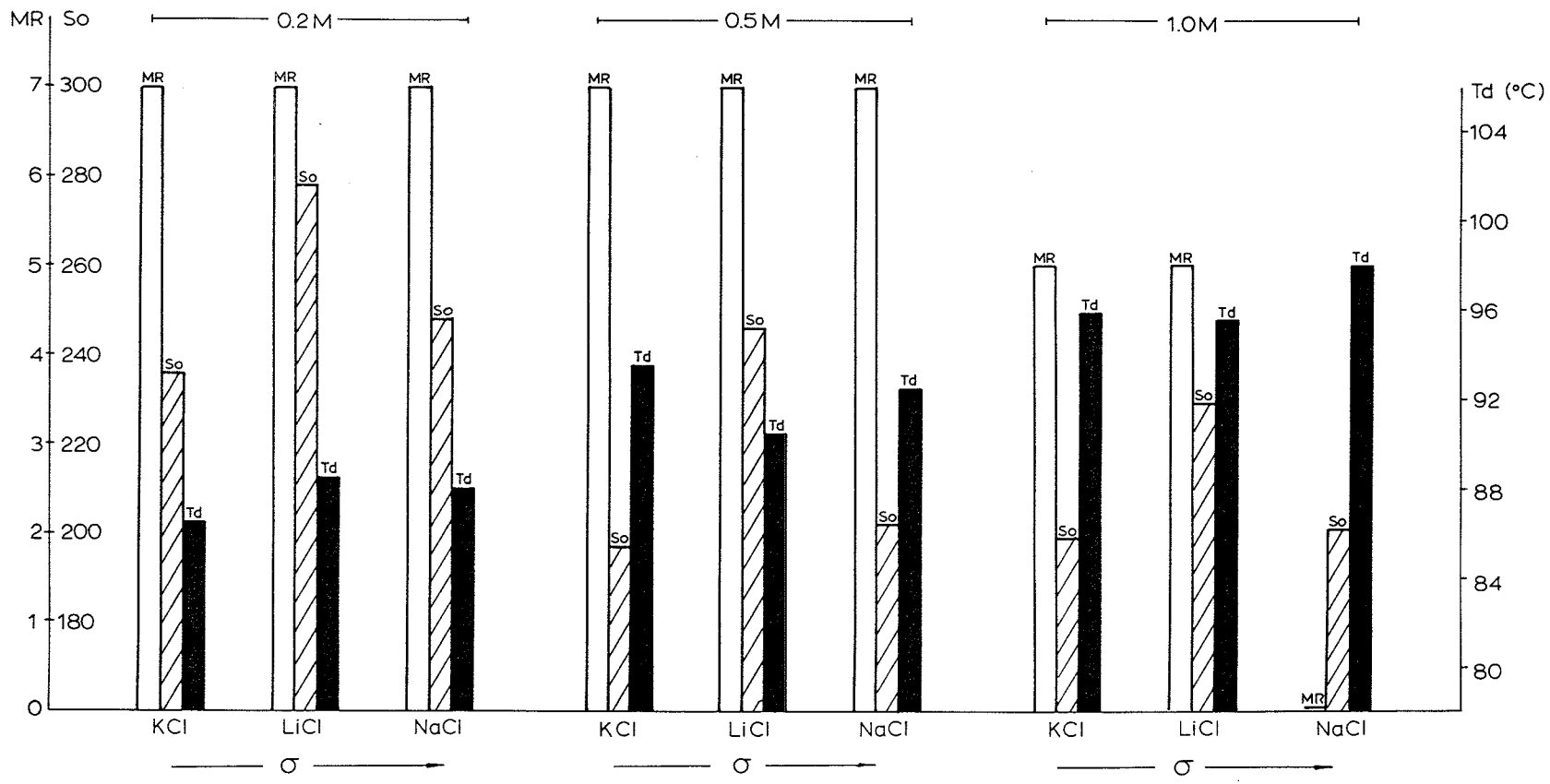
[2] International Critical Tables (1929). Units are 10^{-3} dyn g cm^{-1} mol^{-1}.

[3] Value not determined due to solubility limitations.

[4] Micelle populations unstable.

Figure 4.15. Micelle ratings (MR), surface hydrophobicities (So) and denaturation temperatures (Td) for vicilin exposed to 0.2, 0.5 and 1.0M levels of three chloride salts. All values for So and Td were determined in Chapter 3.



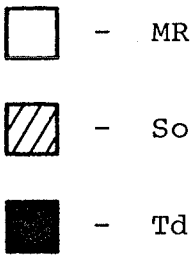

MR
So
0.2 M
0.5 M
1.0 M
Td (°C)
7
6
5
4
3
2
1
0
300
280
260
240
220
200
180
104
100
96
92
88
84
80
KCl
LiCl
NaCl
σ

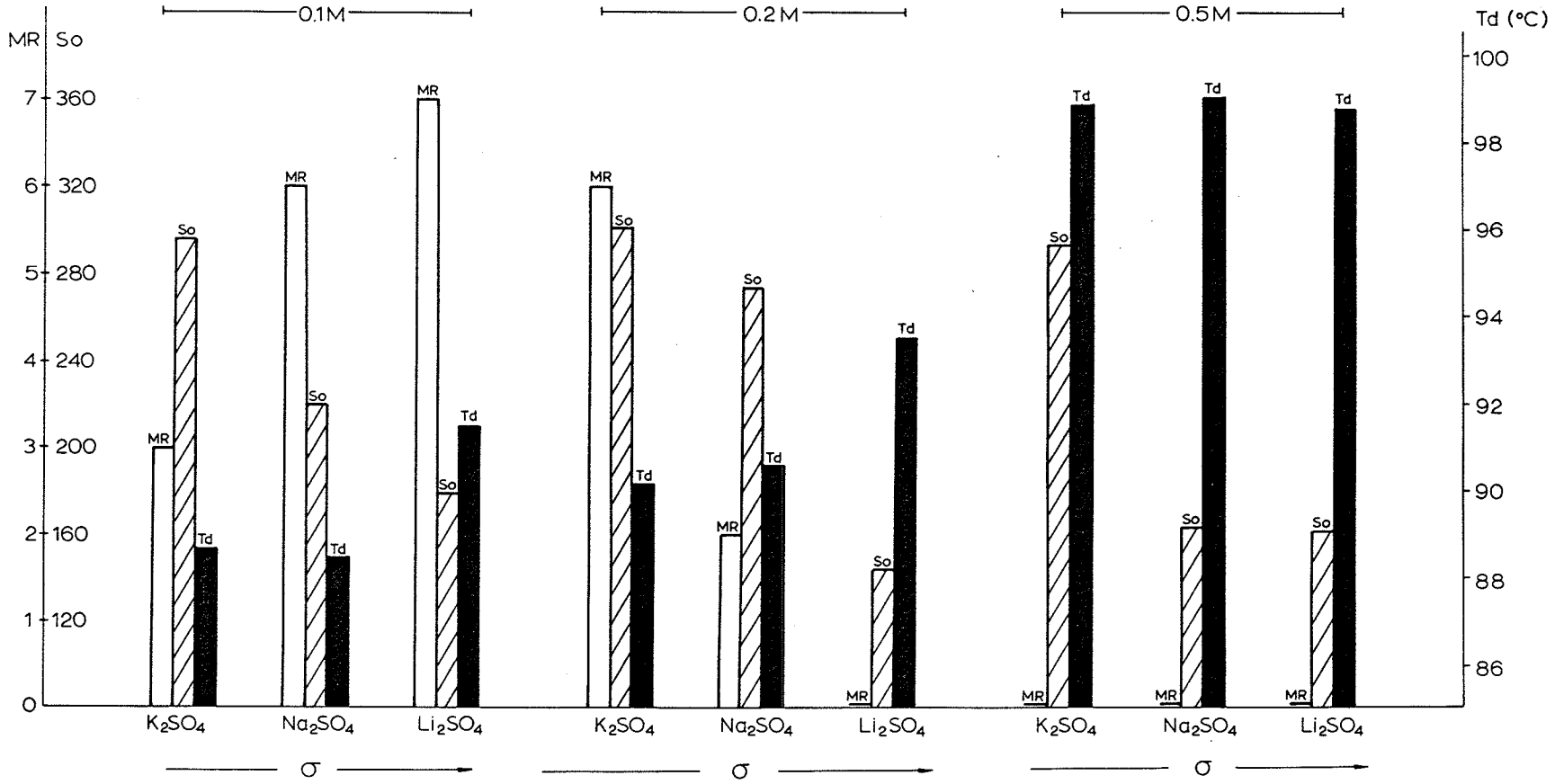
responses among these three environments did not appear to be related to either So or Td values for vicilin as there were no significant differences among either of these parameters for the three cations(Figure 4.15). From these results, a limited hierarchy in terms of cation effectiveness in promoting micelle formation can be given as:

potassium, lithium > sodium

With respect to the three cations associated with the sulfate anion, the micelle responses in general were much weaker than for the chloride salts. At 0.1 M, vicilin exposed to Na_2SO_4 and Li_2SO_4 exhibited a strong micelle response(ratings 6 and 7; Figures 4.5, 4.6 and 4.7). Similar to other massive interactions, both of these end-products were characterized by pockets of entrapped air(Figure 4.9). The pattern of development for the micelle response in Li_2SO_4 was characterized by an initial heterogeneous population of micelles(route BIII, Figure 4.8) similar to that previously described for Na_2SO_4. In contrast to these two media, the micelle response for vicilin in 0.1 M K_2SO_4 was relatively weak with only aggregates of small micelles formed(rating 3; Figure 4.2 A). Surface hydrophobicity values for vicilin in these three environments were quite variable(Figure 4.16). The weak micelle response for vicilin in 0.1 M K_2SO_4 was associated with an extreme So value of 296; the strong micelle response in 0.1 M Li_2SO_4 corresponded to a low So value of 179. The relatively strong micelle pattern for vicilin in

Figure 4.16. Micelle ratings (MR), surface hydrophobicities (So) and denaturation temperatures (Td) for vicilin exposed to 0.1, 0.2 and 0.5M levels of three sulfate salts. All values for So and Td were determined in Chapter 3.




0.1M
0.2M
0.5M
MR
So
Td (°C)
0
1
2
3
4
5
6
7
120
160
200
240
280
320
360
86
88
90
92
94
96
98
100
K_2SO_4
Na_2SO_4
Li_2SO_4
σ

0.1 M Na_2SO_4 was associated with a more expected So value of 220. The similar Td values for vicilin exposed to these three media were indicative of stabilized vicilin molecules(Figure 4.16).

At 0.2 M, the micelle response for vicilin exposed to K_2SO_4 was much stronger(rating 6; Figure 4.5) than that observed for 0.1 M concentrations. This observation was associated with an extreme So value(301), similar to that found at 0.1 M levels. In direct contrast, the micelle response in the other two environments showed extensive deterioration with small micelles(rating 2, Figure 4.1 B) formed in Na_2SO_4 and an absence of micelle formation in Li_2SO_4. The corresponding So values for vicilin exposed to these two media appeared somewhat contradictory in view of the micelle results. Although the micelle results were similar, a low So value(144) was observed for vicilin in Li_2SO_4, whereas a high value(273) was noted for vicilin in Na_2SO_4(Figure 4.16). As another dimension, the Td values for vicilin in the three media were generally reflective of stabilized protein molecules(Figure 4.16).

At 0.5 M, the conditions were not appropriate for micelle formation in any of the three environments. For vicilin in Na_2SO_4 and Li_2SO_4, the So values were low - 164 and 162, respectively. Although the So value for vicilin in K_2SO_4 was 285, micelle formation still did not occur(Figure 4.16). In all three environments, the similar Td values were indicative of highly stabilized molecules(Figure 4.16).

With the sulfate-associated cations, an hierarchy in

terms of effectiveness in promoting micelle formation was not apparent due to the lack of a definite pattern in the micelle results. The absence of an hierarchical arrangement for the sulfate salts, in comparison with that established for the chloride-associated cations, emphasized the general influence of the anion in cation-related effects.

DISCUSSION

Certain proteins, despite their structural limitations in terms if molecular design, have the capacity to form micelles under appropriate environmental conditions (Murray et al., 1978; Simons et al., 1978; Evans and Philips, 1979). One of the key factors in this phenomenon is the significance of the environmental situation; protein molecules are dynamic flexible structures with the ability to exhibit different surface properties as a function of their surroundings (Cooper, 1982). Molecular self-association into a micelle configuration appears to require a specific intramolecular hydrophilic-hydrophobic organization (Tanford, 1972). As a result, slight modifications in the protein environment may render molecular conformation, in terms of surface hydrophobic and hydrophilic residue arrangement, unsuitable for micelle formation.

Murray et al. (1978) found that the main protein components of fababean concentrate were in appropriate molecular configurations for micelle formation if 0.3 M NaCl was used as the solubilizing environment. Massive inter-protein interactions to form micelle structures, however, only occurred with the sudden introduction of a controlled volume of water. As a result, the driving force for the associative reactions was attributed to protein hydrophobic interactions. In recognition of the relatively delicate nature of noncovalent forces in general, it was assumed that many factors could possibly influence this phenomenon. Considerations included initial

protein concentration, volume of diluant, pH of the system and properties of the electrolyte medium. All of these represented significant parameters in terms of potential influence on either the structural characteristics of the interacting proteins or on the actual noncovalent forces involved. Despite the identification of important factors associated with the entire experimental system, a detailed understanding of micelle formation in terms of protein structural properties and environmental contributions was hampered by the multicomponent protein fractions used by Murray et al. (1978). As a result, the initial part of this study (Chapter 1) was designed to isolate one of the fababean storage proteins and assess some of its structural properties (Chapters 1, 2 and 3) prior to considering its capacity for micelle formation. Vicilin was the protein of choice as it was the easier of the two storage proteins to isolate using a mild extraction procedure.

With hydrophobic interactions considered to represent the fundamental driving force in the establishment of a micelle, it was initially conceived that the self-associating molecules should be highly hydrophobic structures. In comparison with other globular proteins described by Bigelow (1967), vicilin, a molecule capable of extensive micelle formation, was found to be characterized by a relatively low Bigelow hydrophobicity (901.43 cal/AA residue, Table 1.8) and a relatively high charge ratio (0.38, Table 1.8). In addition, vicilin also exhibited a relatively low surface hydrophobicity, with So

values ranging from a minimum of 124 to a maximum of 384 (Figure 4.14), depending upon the environmental media. Interestingly, despite the implied inter-relationship between Bigelow hydrophobicity and So values for vicilin, a direct correlation between these two parameters has not been established for native globular proteins in general (Nakai, 1983). In fact, So values have shown considerable variation among apparently similar proteins (Nakai, 1983).

From an initial assessment, the hydrophobicity and charge characteristics of vicilin would seem detrimental to massive inter-protein hydrophobic associations. In retrospect, however, it should be emphasized that the establishment of a micelle depends on a distinct intramolecular hydrophilic-hydrophobic balance (Tanford, 1972). For example, Simons et al. (1978) suggested that bacteriorhodopsin, an integral membrane protein, would not self-associate into a micelle structure as a result of its high molecular hydrophobicity. With extreme hydrophobicity, protein molecules cannot form spherical aggregates with an extensive enough hydrophilic surface to retain a micelle arrangement. As a result, it would appear that the "ideal" globular protein for micelle establishment is not one that is either extensively hydrophobic or hydrophilic, but rather one similar to vicilin, in which there appears to be a specific balance of surface hydrophobic and hydrophilic residues. In addition, spatial orientation of the various surface residues may also be influential; for example, some polarization of hydrophilic and hydrophobic side-chains

could facilitate micelle formation. Finally, the environmental medium is critical in the establishment of optimum surface characteristics, considering the dynamic nature of the macromolecules involved.

Types of Micelle Responses with Vicilin

With the examination of the micelle response by vicilin in a variety of salt environments, it became apparent that vicilin was not only capable of micelle formation but also, the established vicilin micelles under specific conditions interacted to form a spectrum of different structures. The formation of elaborate networks and amorphous aggregates from original micelle structures often followed an identifiable pattern of events (Figure 4.9). From this viewpoint, vicilin was "rated" in terms of its ability to initially form micelles and to subsequently undergo further intermolecular interaction by the association of discrete micelles.

The general phenomenon of different types of micelles or more extensive structures, as products of inter-micelle association, is interesting from a theoretical consideration. Firstly, vicilin association into a micelle arrangement appears to be the result of molecular orientation with a thermodynamic driving force; in this case, the thermomodynamic impetus is considered to be associative hydrophobic interactions. The formation of a static population of discrete micelles is the consequence of a co-operative interaction of noncovalent forces. According to the principle of opposing forces (Tanford ,1973), attractive forces must be dominant for

the formation of a micelle arrangement whereas surface repulsive forces must be dominant for the micelles to be restricted to a spherical shape of a particular size. Micelles, in general, are not stoichiometric compounds, but aggregates capable of existing over a wide range of sizes. There is a limitation to the minimum size based on the ability to reduce the water-nonpolar interface; similarily, there is a maximum size restriction based on surface repulsive characteristics (Tanford, 1973). For vicilin, the environmental media had a definite impact on the development of these surface repulsive phenomena. With some electrolytes, only small individual micelles (rating 2, approximately 2 μm in diameter) formed; in others, a spectrum of discrete micelles were observed ranging from small to intermediate (10 μm in diameter) to large (20 μm in diameter) (rating 5). In fact, this differential in the initial micelle response with vicilin, specifically small versus variable-sized micelles, actually formed the basis for the two types of micelle pathways outlined in Figure 4.8. At this point, in some situations, the repulsive forces dominated resulting in static populations for both types of responses. In other environments, however, electrostatic repulsive forces appeared to be minimized allowing possible hydrophobic associations to occur until the repulsive forces eventually became predominant. In both pathways, it seemed possible not to have the development of a major repulsive situation; interaction could occur until there were extensive amorphous masses of protein with complete phase sep-

aration. This was considered to be the ultimate interactive response and was described by a rating of 7. Progression to this end-point using route B (Figure 4.8) with variable-sized micelles as the starting point seemed to be a relatively direct procedure. Coalescence of individual micelles resulted in a homogeneous network which was either static, as a consequence of opposing forces, or dynamic, resulting in complete aggregation. However, with the alternative route A, a larger number of static end-points were possible without complete progression to the ultimate reaction. Often the initial small micelles associated into aggregates (rating 3), without apparent coalescence. This phenomenon required the existence of attractive forces between discrete micelles that did not, however, overcome the magnitude of inter-micelle repulsive forces to allow coalescence. In order for continued interaction to occur, a microenvironment around the individual micelles had to be established which minimized these repulsive forces and maximized attractive associations. This occurred in some situations giving rise to other degrees of interaction which formed either static or dynamic populations. These interactive forms included granular networks (rating 4) with incomplete coalescence of small micelle aggregates, larger discrete micelles of varying sizes (rating 5), limited homogeneous networks (rating 6) involving complete but restricted coalescence of different-sized micelles, and finally, the ultimate extensive amorphous aggregations (rating 7). In many of the highly interactive pathways which terminated with a response rated as

7, the coalesced protein masses were characterized by multiple pockets of entrapped air. The significance of this observation was not understood; however, this phenomenon appeared to reflect and emphasize the rapid and continuing cohesive nature of the molecular interactions in some environments.

Due to of the complexities of the system, it is not possible, at this point, to define the precise characteristics of the micelle microenvironments that contribute to the elaboration of different types of interactive pathways. It is possible, however, to assess some of the protein-solvent, protein-electrolyte or solvent-electrolyte interactions that might occur for a certain static end-point to be reached. Inherent in this assessment is also a consideration of the role of non-covalent forces, specifically hydrophobic and electrostatic interactions, in the manifestation of these diverse observations. In this respect, the impact of a number of salt environments on these phenomena was considered from two viewpoints - firstly, the influence of a particular salt on selected conformational aspects of vicilin and its general capacity to form micelles; secondly, the effect of a salt medium on the degree and nature of inter-micelle association. In both of these approaches, the two levels of salt action had to be considered; specifically, electrostatic influences at low salt concentrations ($\mu < 0.5$) and lyotropic effects at higher salt concentrations ($\mu > 0.5$). In addition, the stabilizing and destabilizing effects of the salts were considered, basically from the perspective of relative position in the Hofmeis-

ter (lyotropic) series which, in turn, was correlated with σ, the molal surface tension increment (Melander and Horvath, 1977).

Pre-dilution Observations

Although micelle formation by fababean storage proteins in general was considered to be a post-dilution phenomenon(Murray et al., 1978), it was found that vicilin in some media, especially at low salt concentrations, formed pre-dilution micelle structures(Tables 4.6 and 4.7). Invariably, these populations were not extensive and the degree of inter-micelle interaction was minimal. This pre-dilution vicilin association might be viewed as a similar phenomenon to the detergent micelle, in which an increase in the concentration of the surfactant up to a critical micelle concentration (actually a range of concentrations) results in an abrupt change in solution properties corresponding to the appearance of molecular aggregates or micelles(Ben-Naim, 1980). Similarily, in the concentrated pre-dilution vicilin solutions with the protein molecules in close proximity, it would be thermodynamically feasible if intermolecular hydrophobic associations were to occur. In many environments(Tables 4.6 and 4.7) electrostatic influences were not great enough to discourage some intermolecular association. However, due to the generally small size of the micelles formed(rating 2) and a tendency not to interact beyond simple aggregation(rating 3), some surface repulsion such as that created by an electrical double layer must have existed among the micelles. Pre-dilution micelle

responses in K_2SO_4(0.5 M) and Na_2SO_4 and sodium citrate may have been a consequence of vicilin preferential hydration. This protein surface hydration phenomenon tends to promote intermolecular hydrophobic association which, at higher salt concentrations, would develop into complete hydrophobic precipitation.

Protein Concentration and Dilution Requirements for Micelle Formation

Although pre-dilution micelle formation occurred with concentrated vicilin exposed to some environmental conditions, extensive association of vicilin into numerous micelles and/or interactive micelle populations occurred only as a response to the sudden dilution of the concentrated protein solutions with water. As indicated by experimental results, it was not surprising that this massive, apparently hydrophobic, response was influenced by initial vicilin concentration. If pre-dilution vicilin solutions had a reduced protein content, the vicilin molecules would not be in close enough physical proximity to interact. Hydrophobic forces are not highly attractive; therefore, in order for hydrophobic residues to associate even in the absence of major repulsive forces, the molecules must be compatible in terms of spatial orientation. Interestingly, the vicilin concentration necessary for establishment of a maximum micelle response was influenced by the identity of the electrolyte medium. For example, a strong interactive micelle response(rating 7) was formed with a lower initial vicilin concentration in 0.2 M LiCl(7.4 mg mL^{-1}) than

in 0.2 M sodium acetate(15.6 mg mL^{-1}) (Table 4.3). Both the conformational properties of vicilin in these two media and the characteristics of the individual electrolytes would appear to be influential in creating this differential. As previously discussed, a strong micelle interaction is dependent on the balance of intermolecular attractive and repulsive forces. If this strong association is to occur at a lower protein concentration, protein and electrolyte properties must favor the predominance of attractive interactions. In 0.2 M LiCl, vicilin had a significantly higher So value(278, Figure 4.15) than vicilin in 0.2 M sodium acetate(207, Figure 4.12). Interestingly, the thermal stability of the protein appeared to be similar in the two environments, as evidenced by the comparable Td values(88.5°C for LiCl; 87.7°C for sodium acetate; Figures 4.12 and 4.15). As a result of the So differential, vicilin in LiCl had a greater potential reactive surface for intermolecular hydrophobic association. As the electrical double layer established by both electrolytes may be physically disturbed by the sudden dilution phenomenon, the greater exposure of hydrophobic residues with vicilin in LiCl may facilitate extensive intermolecular hydrophobic associations. As a result, micelle responses occurred readily and extensively at low protein concentrations. A strong interactive micelle population was also formed with vicilin in sodium acetate; however, as a possible consequence of the reduced molecular So, a higher protein level was necessary to establish the association phenomenon.

In addition to pre-dilution protein concentrations as an influential factor in vicilin micelle formation, the actual volume of aqueous diluant also affected the post-dilution micelle response. From a strictly physical point of view, large water:protein excesses (i.e. 50:1) imposed a tremendous spatial separation on the protein molecules making intermolecular interactions difficult. With vicilin in 0.2 M LiCl, large volumes of diluant (i.e. 30:1 to 50:1, Table 4.4) resulted in populations of small, usually unstable, micelles. These seemed to be localized reactions with protein interaction occurring before the full influence of the dilution phenomenon was in effect. In general, a highly aqueous post-dilution environment was not appropriate for a stable micelle population. On the other hand, if the water:protein dilution ratio were low (e.g. 1:10, Table 4.4), the aqueous dilution force was reduced. Small areas of local micelle formation occurred with vicilin in 0.2 M LiCl; however, the main impact of the dilution phenomenon was absent.

Influence of Limited pH Variation on Micelle Formation

As pH apparently influences some conformational aspects of vicilin(Chapter 3), it was not unexpected that these molecular ramifications would ultimately affect micelle formation. Consequently, pH became an important factor for consideration in micelle assessment,. Significant deterioration of the micelle response occurred from pH 7.0 to 8.0, the pH range in which variations in vicilin conformational parameters, especially So and Td values(Figure 4.10) were most apparent. Up to

pH 6.5, the So values were high and the micelle response was strong(rating 7, Figure 4.10). From these results, it would appear that manipulation of vicilin conformation is not optimal for micelle formation if hydrophobic interactions are suppressed due to a decrease in So values resulting from an increase in intramolecular electrostatic contributions. This observed importance of So is supportive of the original premise by Murray et al. (1978) that these micelle structures are a product of hydrophobic associative forces. In addition, the observed micelle populations from pH 7.0 to 8.0 were characterized by a progressive decrease in inter-micelle interaction. Increasing molecular negativity with increasing pH may result in extensive surface repulsion among established micelles. It should, however, be mentioned that although the entire assessment of pH influence on micelle formation was conducted at one salt concentration(0.1 M sodium phosphate), the balance between the univalent phosphate($H_2PO_4^-$) and the divalent phosphate(HPO^{-2}) would be expected to shift with an increase in pH from a predominant $H_2PO_4^-$ at pH 6.0 to a predominant HPO_4^{-2} at pH 8.0. As a result, electrostatic shielding may be more effective at the higher pH values, contributing to the overall changes in conformational properties and differences in the interactive nature of micelle populations. Although this potential exists, the low salt concentrations in both pre- and post-dilution situations may offset this effect. In addition, at a constant pH(6.6, Chapter 3), the univalent-divalent phosphate anion mixture(0.1 M) did not have a greater

stabilizing influence, as reflected by Td values, than other univalent or multivalent electolytes. As an extension, if the divalent anion were to have a stabilizing influence on vicilin as its relative concentration increased to pH 8.0, this effect was not sufficient to negate the destabilizing phenomena associated with pH adjustment, as evidenced by lower Td values (Figure 4.10).

Influence of Various Anions on Micelle Formation

The general influence of different sodium-associated anions on vicilin micelle formation was most easily approached from a comparison of various responses at a constant salt concentration. At the lowest salt level(0.1 M), sudden dilution of the concentrated vicilin solutions may result in a physical disturbance of the electrical double layer associated with the protein molecules. Therefore, intermolecular association of vicilin by hydrophobic associations would be thermodynamically feasible in response to the aqueous environment if the protein So values were adequate for this response. As a further consequence of the dilution effect, electrostatic influences on the micelle surface may be minimized such that inter-micelle repulsive forces are reduced and further interactions are favored. Such a behavior pattern may be associated with the extensive networks observed for vicilin in NaSCN, sodium phosphate and Na_2SO_4, where micelle ratings of 6, 7 and 6 respectively, were assigned(Table 4.6). For vicilin in sodium citrate, however, the ionic strength(μ = 0.45) was such that lyotropic salt influences are expected to predominate in the

pre-dilution conditions. As an apparent consequence of preferential hydration effects, the So value for vicilin was reduced(191, Figure 4.11) reflecting a decreased exposure of hydrophobic surface residues for inter-protein interaction. In addition, the reduction in salt level upon dilution may have invoked an electrostatic effect by the trivalent cations, resulting in protein solubilization as a consequence of an established electrical double layer. This combination of pre-dilution lyotropic influences and post-dilution electrostatic effects, associated with the sodium citrate system, appeared to have restricted initial micelle formation and reduced subsequent micelle interaction.

At 0.2 M salt levels, there was a major differential in the effect of univalent and multivalent ions on micelle formation. With all univalent anions except thiocyanate there was a strong micelle response; however, with the multivalent anions, the micelle response was either reduced or absent(Table 4.6). In terms of relating this to the conformational properties of vicilin in the individual media, the degree of surface hydrophobicity of vicilin was relatively similar among the different environments(Figure 4.12). However, the multivalent anions appeared to invoke a greater degree of stabilization in the vicilin molecules as evidenced by higher Td values(Figure 4.12). This stabilization may have been a consequence of different modes of salt action as a function of the ionic strength of the individual salts. For example, 0.2 M sodium phosphate($\mu \simeq 0.4$) may exert an electrostatic effect causing

greater stabilization of vicilin than the univalent salts by virtue of increased anionic association with the protein surface and more efficient charge-shielding. This ion-protein association may be at least partially stable to the physical influence of dilution, resulting in a continued electrical double layer that prohibits hydrophobic interactions and micelle formation. This may account for the 0 micelle rating associated with vicilin exposed to sodium phosphate. Alternatively, with 0.2 M Na_2SO_4(μ = 0.6) and sodium citrate(μ = 1.2), the stabilization of vicilin may be attributed to a lyotropic influence resulting in preferential hydration of the protein surface. The degree of vicilin preferential hydration, however, appears to differ significantly between the two media as reflected by the differential in the So values(273 for Na_2SO_4 and 183 for sodium citrate, Figure 4.12). From the So value for vicilin in Na_2SO_4, it may be inferred that the sulfate anion at 0.2 M(μ = 0.6) was still bound to the protein surface, rather than exerting a lyotropic effect resulting in preferential hydration. The transition point from a predominantly salt-protein interaction to a salt-solvent interaction is not easily defined; in addition, differences exist among the various electrolytes. Therefore, the reduced micelle formation(rating 2) observed for the sulfate and citrate environments may be attributed to electrostatic influences for vicilin in Na_2SO_4 and a reduced exposure of hydrophobic residues for vicilin in sodium citrate. Post-dilution conditions were also not optimal for micelle interac-

tion; a population of small micelles remained static in both salt environments. A post-dilution electrical double layer formed around the surface of the micelles may have represented the repulsive force generated in Na_2SO_4; a continued surface hydration of the micelles in sodium citrate may have discouraged hydrophobic residue exposure and subsequent inter-micelle interaction.

With the stabilizing univalent anions at 0.2 M, physical disruption of the pre-dilution electrical double layer associated with individual vicilin molecules may have been sufficient to result in the observed post-dilution micelle responses(Table 4.6). In addition, the seemingly unlimited nature of the inter-micelle associations may have been the result of a reduced electrostatic influence of these diluted salt environments on the charge characteristics of the micelle surface. That is, with dilution, the effect of the environmental electrolyte becomes similar to that observed at 0.1 M. The electrostatic influences of the 0.1 M univalent anionic media were not sufficient to promote extensive vicilin solubilization; in fact, difficulties with micelle characterization for vicilin in 0.1 M NaI and 0.1 M NaBr were attributed to pre-dilution protein precipitation.

The influence of the previously-identified destabilizing anions(thiocyanate and iodide, Chapter 3) on micelle formation is interesting. At 0.2M, the influence of NaI is similar to that of the stabilizing univalent anions; that is, micelle formation was promoted(rating 7, Table 4.6). Vicilin, in this

NaI environment, does not seem to be destabilized with respect to its thermal properties; in fact, the Td value observed was not significantly different from that of vicilin in sodium acetate(Figure 4.12). In contrast, vicilin exposed to 0.2M NaSCN appeared to be destabilized with conformational alterations reflected by the elevated So value and the decreased Td value(Figure 4.12). The altered vicilin conformation was associated with a decreased micelle response (rating 3) in comparison with the stabilizing anions. Two forces may be operative to result in this particular micelle reaction. Firstly, thiocyanate binds specifically to the protein (Arakawa and Timasheff, 1982) creating a solubilizing destabilizing influence. This binding may persist with dilution causing intermolecular electrostatic repulsion or may resume after dilution to create a charge repulsion among established micelles. Secondly, the vicilin So value in NaSCN was high (318; Figure 4.12); this would seem initially ideal for hydrophobic interaction and micelle formation. However, the extensive exposure of originally internal hydrophobic side-chains may disturb the orientation of the hydrophobic-hydrophilic residues such that the micelle arrangement is more difficult. On the other hand, exposure of some hydrophobic residues on the surface of the small established micelles for vicilin in 0.2 M NaSCN may contribute to their aggregation, as observed.

At 0.5 M salt levels, the effects of anions on micelle formation followed a similar hierarchy to that established at 0.2 M. Previously(Chapter 3), conformational characteristics

of vicilin at this salt level have been attributed to lyotropic salt influences; however, with this specific salt concentration, the influence of the univalent anionic salts on post-dilution micelle structures may be more electrostatic due to the reduced salt concentration in the diluted system. Interestingly, there seemed to be a definite qualitative relationship between the micelle-forming capacity of vicilin and the position of the anions in the Hofmeister series, as reflected by σ, the molal surface tension increment(Table 4.6). Quantitative correlations between σ and micelle-ratings, similar to those for the conformational parameters of vicilin(specifically Td, ΔH and So, as determined in Chapter 3), were not attempted due to the subjective nature of the micelle observations.

In terms of individual anions, the destabilizing influences of both thiocyanate and iodide, with low σ values, were evident with respect to the Td values for vicilin(80.8°C and 86.1°C) and the elevated So value(375) for vicilin in NaSCN(Figure 4.13). With the thiocyanate anion, especially, this may reflect a stronger manifestation of the effect observed at 0.2 M; that is, increased thiocyanate binding to vicilin, increased intermolecular electrostatic repulsion and a distorted hydrophobic-hydrophilic balance of surface residues. As a result, the general micelle response was reduced(rating 2, Table 4.6) and inter-micelle association was essentially absent, possibly as a consequence of continued thiocyanate binding to the surface of the established

micelles.

Conformational characteristics of vicilin in salt media with intermediate σ values(sodium acetate, NaBr, NaCl) appeared to be ideal for micelle formation. In addition, environmental conditions were appropriate for continued inter-micelle interaction. All three salts are known to promote some pre-dilution preferential hydration of the vicilin molecules. This hydration effect on vicilin conformation, however, may be reduced by specific binding of the anions to the protein surface(Arakawa and Timasheff, 1982). The possible counteracting influences of hydration and anion-binding resulted in what appeared to be adequate vicilin So values for initial micelle formation(201 for sodium acetate, 202 for NaCl; Figure 4.13). If the anion-protein relationship was initially disturbed by the dilution phenomenon, the reduced electrostatic repulsion in combination with an appropriate vicilin So could account for the observed micelle formation. Post-dilution electrostatic interactions with the micelle surface were not prohibitive for inter-micelle associations; in fact, massive coalescence and aggregation(rating 7, Table 4.6) occurred in all three media.

With the salts having the greatest σ values(sodium phosphate, Na_2SO_4 and sodium citrate); vicilin was highly stabilized as evidenced by the Td values(98.8, 99.1 and 101.5°C, respectively; Figure 4.13). The corresponding So values were low(148, 164 and 137, respectively; Figure 4.13) as an apparent result of preferential hydration. The sudden dilution phe-

nomenon, however, did not result in micelle formation. As hydrophobic areas do not have a strong attractive capacity, the reduced surface exposure of hydrophobic residues for the pre-dilution hydrated vicilin did not seem to allow intermolecular association. Immediately after dilution, the multivalent anions may initially exert some electrostatic influences as a consequence of the reduced salt concentrations. These types of interactions, would only serve to further solubilize the molecules and discourage inter-vicilin associations. As the distribution of the ions stabilized in the diluted system, preferential hydration of the micelle surface may occur. This phenomenon is also not conducive to micelle formation as exposure of hydrophobic residues on the surface of the micelles may be suppressed.

In general, at 1.0 M salt concentrations, environmental conditions and their respective influences on vicilin conformation were not suitable for the establishment of a stable, interactive micelle population. In contrast to the 0.5 M concentration levels, both pre- and post-dilution salt effects are expected to be mainly a result of lyotropic influences. Approaching the results from a lyotropic hierarchical viewpoint, small discrete micelles(rating 2, Table 4.6) were formed by vicilin exposed to 1.0 M NaSCN, the salt with the lowest σ value. It would seem that the destabilized vicilin conformation, as evidenced by the elevated So value(384) and reduced Td value(75.9°C)(Figure 4.14), allowed minimal intermolecular interaction to occur in response to dilution. This

association may be partially a function of the increased hydrophobic surface and the disruption of the thiocyanate-protein interaction with dilution. However, resumption or continuation of thiocyanate-vicilin binding in the post-dilution environment would contribute to inter-micelle electrostatic repulsion.

For the salts with intermediate lyotropic effects(sodium acetate, NaBr, NaCl), an initial strong micelle reaction occurred; however, this was transitory and dissipated rapidly. A similar pre-dilution situation could be envisioned to exist as in the 0.5 M environment. That is, preferential hydration of the vicilin molecules in conjunction with some anion-binding to the protein surface resulted in relatively high vicilin So values(200 for sodium acetate, 201 for NaCl; Figure 4.14). The combination of the So features and the possible disruption of anion-vicilin interactions with dilution would allow initial micelle formation and subsequent interaction. A phenomenon for speculation, however, is the instability of the post-dilution protein aggregations. It might be assumed that rapid diffusion of the salt into the dilution area could re-establish anion-protein surface interactions. As a consequence, electrostatic repulsion among surface residues of the aggregated mass may have a destabilizing influence causing progressive deterioration of protein association with continued anion binding.

Vicilin exposed to Na_2SO_4 and sodium citrate(1.0 M), salts with extensive lyotropic effects, did not exhibit any

capacity for micelle formation(Table 4.6). A pre-dilution situation similar to the 0.5 M level may be present; specifically, preferential hydration of the protein surface caused an extreme reduction in surface exposure of hydrophobic residues and an extensive molecular stabilization as reflected by the decreased So and increased Td values, respectively(Figure 4.14). The reduced levels of surface hydrophobicity would discourage intermolecular association with dilution. Any post-dilution molecular aggregation would not be favored due to continued vicilin preferential hydration; this differs somewhat from the possible involvement of electrostatic salt-protein interactions in several of the diluted 0.5 M environments.

Influence of Various Cations on Micelle Formation

As the examination of the influence of cations was very limited, only several observations with respect to micelle formation can be made. Firstly, considering the chloride-associated cations(lithium, potassium, sodium) stronger interactive micelle responses(rating 7, Table 4.7) were observed for all environments at 0.2 and 0.5 M levels. However, an interesting differential occurred at 1.0 M. For LiCl and KCl, a stable static population of various-sized micelles was formed. This was in direct contrast to the transitory strong response observed with NaCl; a phenomenon previously attributed to post-dilution protein-chloride binding and subsequent destabilization of the aggregated molecules. As both KCl and LiCl exhibit similar σ values to that of NaCl, these salts are

expected to induce moderate preferential hydration of the pre-dilution vicilin. However, in contrast to NaCl, the cationic components, lithium and potassium, may exhibit binding to the protein surface(Eagland, 1975; Arakawa and Timasheff, 1982), resulting in incomplete exclusion of the salt from the protein surface and relatively high So values(229 for LiCl, 199 for KCl; Figure 4.15). The stable post-dilution micelle population for vicilin in KCl and LiCl may be attributed to resumed or continued lithium and potassium binding to residues on the micelle surface. As vicilin in this environment has an overall negative charge, a protein-cation association may promote a stabilizing influence. However, the electrostatic field eventually established at the micelle surface, appeared to set up a basic repulsive force among the individual micelles and further interaction was prohibited.

The different micelle responses for vicilin exposed to the sulfate-associated cations in comparison with the chloride salts seemed to illustrate the potential influence of the anionic salt component. Micelle responses could be observed at salt levels as low as 0.1 M as a result of the pre-dilution protein solubilizing influence of the divalent sulfate anion. The results at this salt concentration were similar for Na_2SO_4 and Li_2SO_4; that is, strong interactive reactions(ratings 6 and 7, Table 4.7). A physical disturbance of the electrical double layer by the dilution procedure in combination with post-dilution reduced salt concentrations appeared to minimize the electrostatic influences of the sulfate anion. However,

with vicilin exposed to K_2SO_4(0.1 M), the micelle response was quite reduced(rating 3, Table 4.7). This may be attributed to the detrimental effect of an excessively high So value(296, Figure 4.16), using a reasoning similar to that applied to vicilin in the presence of NaSCN. A disturbed hydrophobic-hydrophilic balance of surface residues may not be as conducive to an extensive micelle reaction.

At 0.2 M sulfate levels, the vicilin micelle responses were quite variable among the cations. The reduced micelle formation(rating 2, Table 4.7) for vicilin in Na_2SO_4 and the absence of a micelle response for vicilin in Li_2SO_4 may be attributed to the pre- and post-dilution electrostatic influence of the sulfate anion. The exceptional response was that of vicilin in K_2SO_4 - an extensive, reactive micelle population was observed(rating 6, Table 4.7). The high So value(301, Figure 4.16) for vicilin in this situation may be an asset rather than a detrimental feature, in that it may be able to offset the repulsive post-dilution electrostatic interactions associated with sulfate at this concentration level. Similarily, the relatively high So value for vicilin in 0.2 M Na_2SO_4(273, Figure 4.16) may have contributed to the limited micelle response in that salt environment despite the negative influence of the sulfate anion. On the other hand, vicilin in 0.2 M Li_2SO_4 did not form micelles and was characterized by a much lower So value(144, Figure 4.16).

The environment at 0.5 M was not appropriate for micelle formation with any of the sulfate salts. However, despite the

uniformity of the observation for the three cations, the actual mechanisms involved may differ. The pre-dilution So values for vicilin in Na_2SO_4 and Li_2SO_4 were low(162 and 164, Figure 4.16), as expected on the basis of the preferential hydration phenomenon. With dilution, the combined lyotropic and electrostatic influences of sulfate are assumed to be sufficient to minimize subsequent molecular interaction. In contrast, the So value for vicilin in K_2SO_4 was very high(293, Figure 4.16); however, a disturbed hydrophobic-hydrophilic balance of surface residues in conjunction with post-dilution electrostatic sulfate influences may contribute to the lack of micelle formation.

Conclusion

The protein vicilin has a molecular design that will allow self-association into a micelle arrangement; this, however, was influenced critically by environmental impact on the molecular surface properties. In addition, micelles were formed under different physical circumstances. Small micelles were often established in highly concentrated protein solutions; however, a more provoking stimulus was the sudden introduction of a controlled volume of water. In both situations, the micelle response was attributed to associative reactions mediated by hydrophobic forces.

With in the spectrum of environmental conditions examined in this study, some appreciation was derived for both the protein and electrolyte properties required for extensive micelle formation. Segregation of these two factors was diffi-

cult as a result of the extreme dependence of protein characteristics on environmental parameters. In general, however, vicilin was most interactive if the initial molecule was moderately stabilized, as assessed by thermal properties, with an "adequate" So value, usually in the range of 200 to 275. Highly stabilized vicilin molecules were frequently characterized by low So values(< 200). As a result, the relative exposure of hydrophobic residues appeared to be insufficient for extensive molecular interaction and strong micelle formation. Similarily, destabilized or partly-denatured vicilin molecules did not promote extensive micelle responses although So values were high(300). This observation was partially attributed to an imbalance in hydrophobic-hydrophilic intramolecular relationships.

Four main environmental considerations were recognized to be significant in the establishment of a moderately stabilized vicilin with a specific So on one hand, and a minimum of environmentally-induced repulsive forces on the other. These included the pH of the system and the concentration, charge characteristics and the molal surface tension increment of the electrolyte medium. Firstly, the pH had to be low enough to reduce intramolecular electrostatic repulsion yet high enough to allow protein solubilization. In this respect, a pH range of 6.1 to 6.8 was identified as appropriate. Secondly, the concentration of the electrolyte had to be minimized; however, the exact levels were dependent on the properties of the electrolyte in question. In general, the minimum level of a salt

was influenced by vicilin solubility limitations. With respect to a maximum level, it seemed most appropriate to expose vicilin to an electrolyte concentration in which electrostatic rather than lyotropic effects were predominant. In conjunction with this aspect, a univalent anionic salt was preferred; the electrostatic influences of the multivalent anionic salts, even at low ionic strengths, were usually too extensive to allow maximum protein interaction. A strong micelle response appeared to be partially dependent on the physical disruption, by sudden dilution, of intermolecular electrostatic repulsive forces associated with the electrical double layer formed at low salt concentrations. With vicilin in most multivalent salt environments, these charge-associated repulsive interactions appeared to persist in the post-dilution phase. At greater ionic strengths(μ > 0.5), the multivalent anionic salts, with high σ values , invoked extensive preferential hydration of the protein resulting in reduced So values - a feature detrimental to micelle formation.

As a result, vicilin, solubilized in relatively low concentrations(0.2 and 0.5 M) of moderately stabilizing univalent anionic electrolytes, such as NaCl or sodium acetate, formed extensive interactive micelle populations following dilution. In these environments, the physical impact of the aqueous medium may have been sufficient to disturb the electrical double layer associated with vicilin, allowing the thermodynamically favorable aggregation of hydrophobic residues. The post-dilution microenvironment established by the new

electrolyte-protein-water mixture favored attractive protein associative interactions rather than intermolecular repulsion.

With these observations, micelle formation by vicilin has become more of a predictable, controlled event. It does not represent a random type of interaction, which might be expected on the basis of the structural peculiarities of a protein molecule. Instead, if the molecular parameters and environmental surroundings are appropriate, the micelle represents a thermodynamically feasible molecular arrangement. In many situations,the micelle also represented a dynamic aggregation starting-point rather than a static association end-point. If intermolecular repulsive forces were minimized, inter-micelle attractions resulted in a spectrum of diverse nonmicellar protein aggregations.

GENERAL CONCLUSION

A study system involving a single protein species was established by the selective isolation of the storage protein vicilin from the seed of the fababean, *Vicia faba minor* var. *Diana*. The ultimate purpose of this system was to consider certain noncovalent forces involved in the self-association of vicilin into a micelle arrangement. Prior to this consideration, however, some appreciation was derived for the general physical characteristics of vicilin, for the importance of noncovalent forces(primarily at the quaternary level) to the structural integrity of the protein and for certain conformational responses of the molecule to specific environmental changes. From this composite assessment, vicilin was characterized as a multimeric molecule(154,000 M.W.) formed by a noncovalent arrangement of four possible subunits. Association of these subunits into a stable molecule appeared to be relatively tolerant to electrostatic perturbation, as determined by sequential chemical modification. In fact, substitution of negatively charged moieties for nearly the entire complement of positive lysine residues, resulted in a somewhat destabilized, but not fully denatured, molecule as evidenced by the thermal properties of the labelled proteins. Molecular integrity, in this instance, may have been maintained by other stabilizing forces which counteracted the increased intramolecular electrostatic repulsion. Alternatively, the charge status of the lysine residues may not have been critical to subunit assembly.

From another perspective, the conformational properties of vicilin, in terms of thermal stability and relative exposure of surface hydrophobic residues, were influenced significantly by the electrolyte constituents of the environmental medium. The degree of molecular stabilization of vicilin exposed to high levels of various salts was correlated with the capacity of the salt to induce preferential hydration of the protein surface. This correlation, in turn, was extrapolated to signify a dependence of subunit associations on hydrophobic interactions. As a result, vicilin was considered to be a dynamic protein structure, arranged into a cooperative unit by noncovalent forces, with hydrophobic interactions appearing to predominate over electrostatic associations in the maintenance of molecular integrity.

Some of the molecular parameters established for vicilin were subsequently related to the capacity of this protein to self-associate into a micelle arrangement. From these relationships, the micelle response appeared to be ultimately dependent on a balance of noncovalent forces operative at several levels. For example, vicilin seemed to be an appropriate protein for micelle formation on the basis of the molecular surface properties, with specific reference to the distribution of hydrophobic and hydrophilic residues. Distortion of this hydrophobic-hydrophilic balance either by the destabilizing action of certain electrolytes to exaggerate the surface hydrophobicity or by preferential hydration of the protein surface to minimize hydrophobic residue exposure was detrimen-

tal to micelle formation. Extension of this concept of a balance of noncovalent forces to an intermolecular level was inherent in the realization that noncovalent intermolecular attractive forces had to predominate over repulsive interactions if micelle formation and subsequent association were to occur. The main attractive forces appeared to be hydrophobic in nature; the sudden introduction of a controlled aqueous environment was essential for massive micelle responses. As further reinforcement for this supposition, the exposure of a certain number of surface hydrophobic residues appeared to be critical for intermolecular interaction. For example, preferential hydration of the surface of individual molecules or established micelle structures, as a lyotropic salt influence, resulted in reduced exposure of hydrophobic residues and decreased intermolecular association. On the other hand, the main repulsive force seemed to be electrostatic, with the magnitude of the repulsion related to the relative ionization of various amino acid residues and/or interactions of specific ions with individual protein molecules at the micelle surface.

In summary, a balance of noncovalent forces was integral to micelle formation and subsequent interaction. Associating molecules require specific distribution of surface hydrophobic and hydrophilic residues; in addition, repulsive electrostatic forces must allow intermolecular association by means of hydrophobic interactions.

FUTURE RESEARCH CONSIDERATIONS

1. A fundamental concept in the formation of micelles by vicilin is that of a specific balance in terms of residue distribution on an intramolecular level as well as noncovalent attractive and repulsive forces on an intermolecular level. This general premise may be pursued from several perspectives:

a. On an intramolecular basis, the addition of complementary techniques, to those used in this study for the assessment of vicilin conformational characteristics would facilitate interpretation of certain structural parameters. For example, similar So values were reported for vicilin with very different degrees of lysine modification (eg. 11 and 63%); these values are not likely to represent the same conformational states. In addition, a decrease in So was observed with vicilin exposed to mildly destabilizing conditions; this is difficult to interpret without additional structural information. Clarification of results may be improved with a combination of CD, ORD and intrinsic fluorescence spectra for vicilin exposed to various environmental conditions. The eventual consequence of compiled structural information may be a refinement of the So measurement.

b. Also on an intramolecular level, the general integrity of the protein molecule may be described more effectively by further detailed interpretation of DSC information, possible only with improved data handling capabilities. For example, loss of cooperativity within vicilin appeared to occur with extensive lysine modification, as evidenced by an increase in

1/2bw values. A similar response was not observed with cytochrome c. The capacity to distinguish single or multi-state transitions in the denaturation process, as implied by the 1/2bw parameter, is possible if van't Hoff enthalpies can be determined from the DSC data.

c. The conformational status of vicilin with respect to a balance of specific surface residues and the subsequent capacity of the protein to form micelles was influenced by the electrolyte medium. At higher salt concentrations, where lyotropic effects were important, the ability of an ion to bind to the protein or cause preferential hydration of the molecular surface was a fundamental consideration in terms of conformational ramifications. The extent of ion-binding to vicilin was speculative based on previous protein studies. In order to investigate these suppositions, a series of preferential interaction studies by densimetric methods would be informative as to the degree of interaction between vicilin and the solute components of the solvent system.

d. Disruption of the balance of hydrophobic and hydrophilic surface residues in vicilin appeared detrimental to molecular self-association into a micelle arrangement. A more precise definition of this general concept may be possible if vicilin, with specific conformational disturbances, is assessed for micelle formation. Molecular manipulation may range from different levels of destabilization as induced by known protein denaturants (eg. urea) to various levels of stabilization by non-electrolytes (eg. sucrose) to more unpredic-

table conformational responses as a result of the incorporation of hydrophobic molecules into the protein media.

As another perspective, this critical balance concept may be investigated with other globular proteins. This could involve examination of micelle formation by proteins with very similar and very different hydrophobic-hydrophilic characteristics to those of vicilin.

e. The capacity of vicilin to associate is partially influenced by the overall charge of the protein. Assessment of the charge profile of vicilin in different environmental conditions may be possible with either potentiometric titration or with isoelectric focusing using immobilized pH gradients. This latter technique is a relatively new electrophoretic procedure which allows determination of protein isoelectric points in the presence of different salt conditions; more traditional isoelectric focusing is restrictive as a result of the requirement for a reduced ionic strength environment.

Charge assessment may be considered on another level; specifically, in terms of the electrostatic environment associated with the surface of established micelles. Electrostatic repulsive forces are considered to be major contributing factors to inter-micelle interactions; these forces, in turn, are highly influenced by the micelle environment. In order to quantitatively assess the electrostatic status of the micelle surface, a measurement of the zeta potential of discrete micelles would be a feasible approach. The understanding

and eventual manipulation of different micelle associative-patterns necessitates some estimation of these repulsive forces.

2. In terms of an ultimate consideration, the eventual goal of this study was to relate the role of noncovalent forces to the functional properties of proteins in food, with specific emphasis on further application of the micelle phenomenon. Eventual progression to this end-point from studies with a single protein system(vicilin) might involve assessment of micelle formation and interaction initially in a defined mixed-protein system(specifically legumin and vicilin) followed by a defined mixed-protein system with incorporation of some of the significant non-proteinaceous components of a food system (eg. sucrose and starch). Some understanding of both thermal properties and the interactive capacities of these study systems may eventually lead to controlled development of this particular type of protein-protein association in foods.

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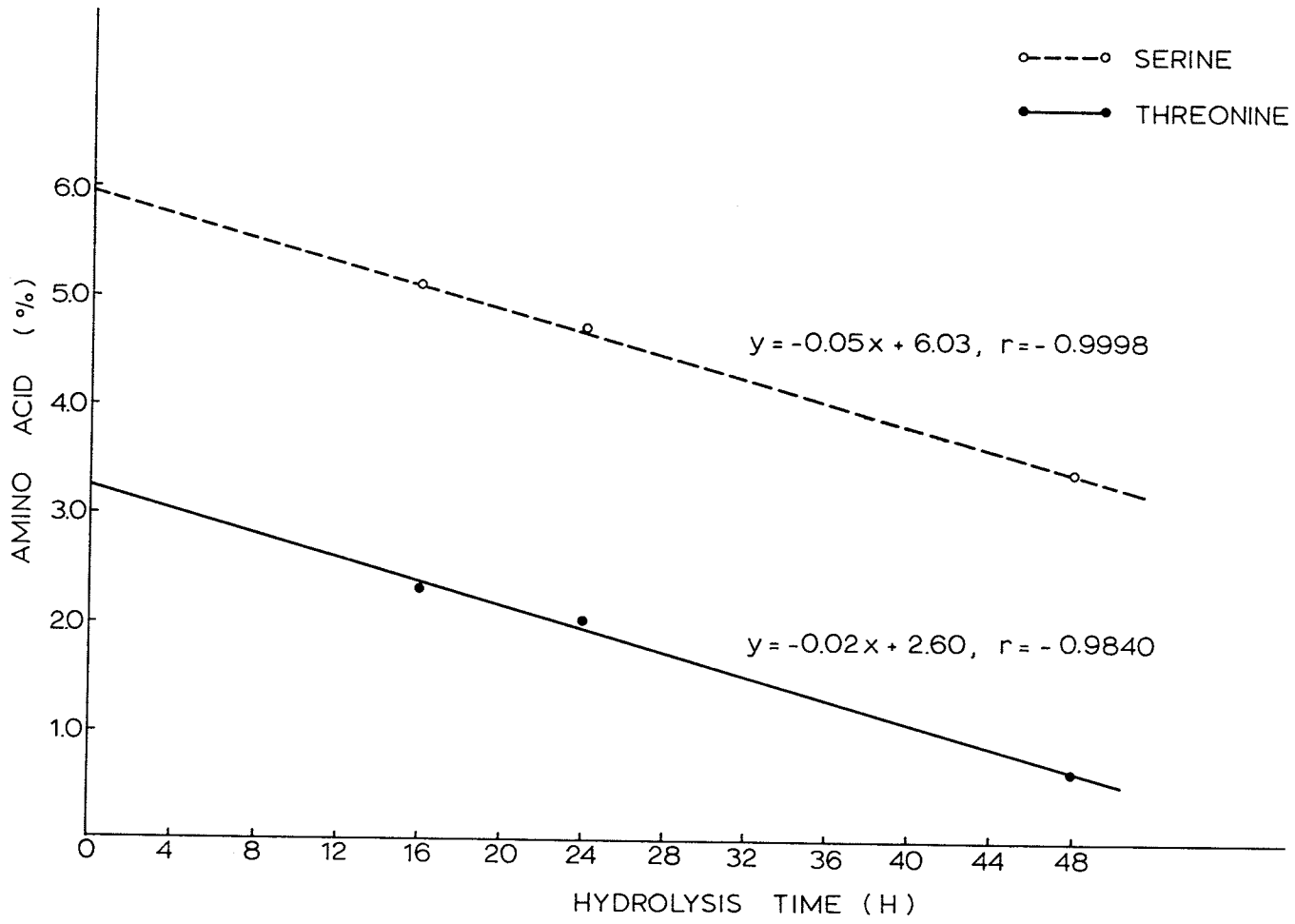
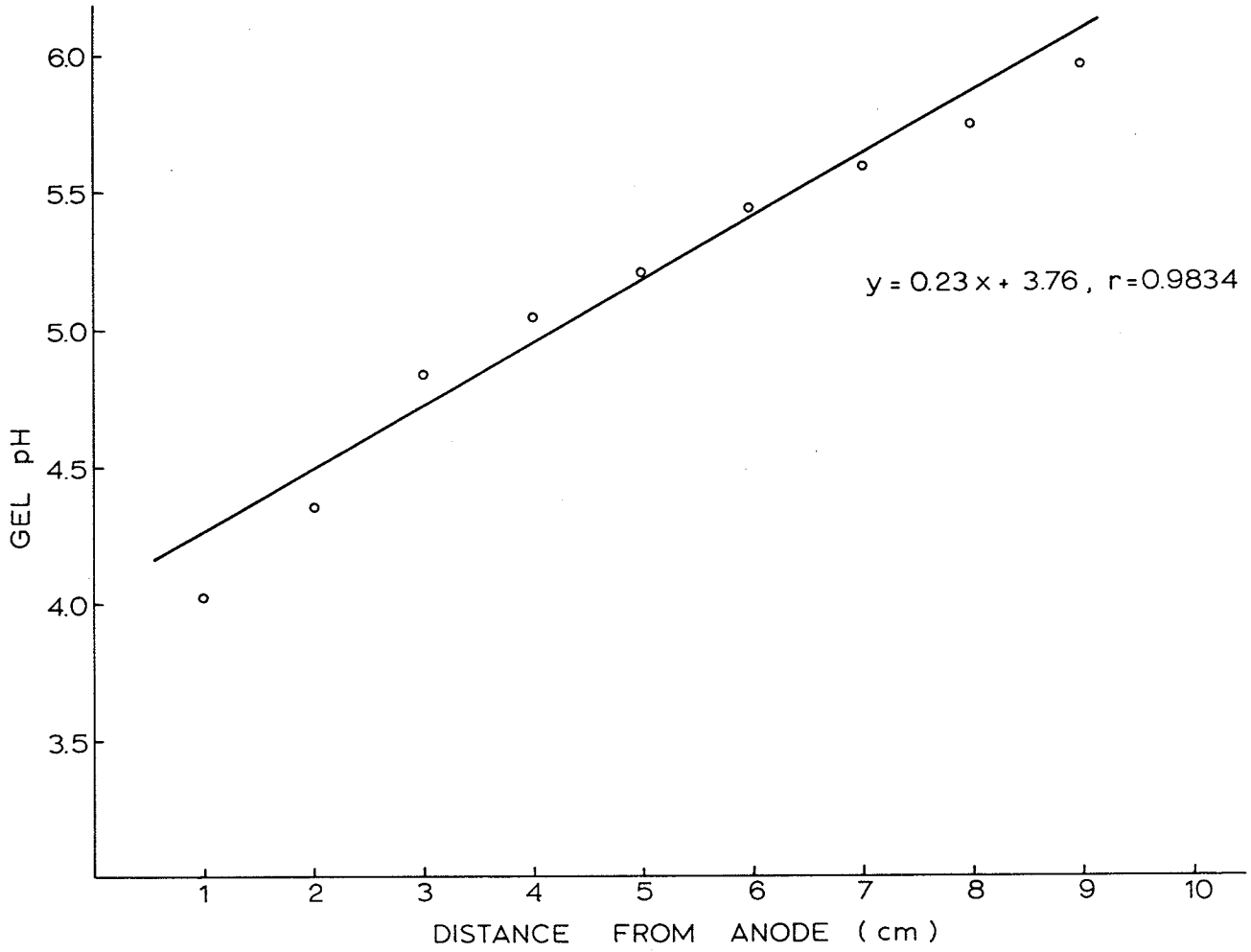
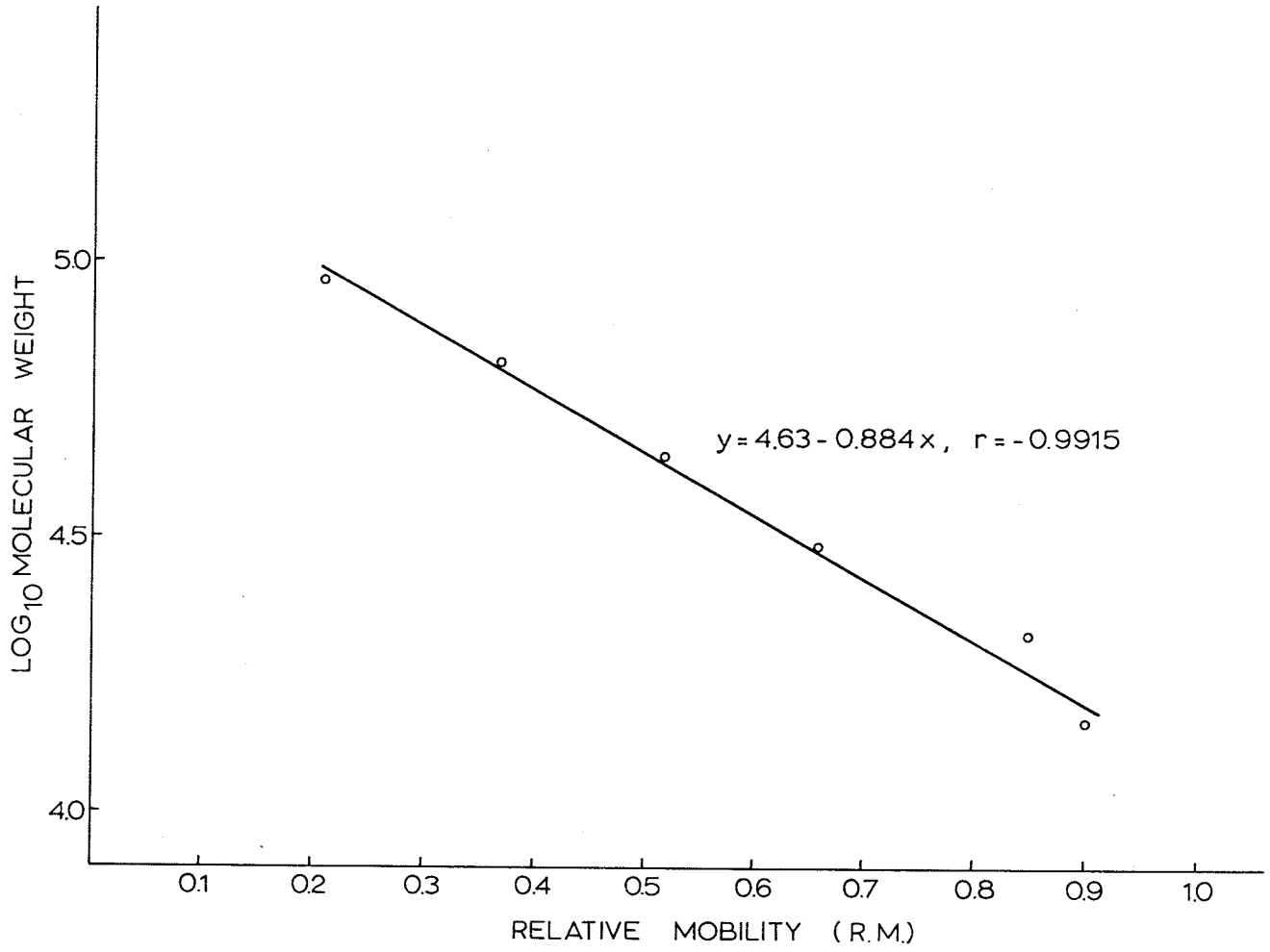
APPENDIX I. Levels of serine and threonine as a function of hydrolysis time during amino acid analysis of vicilin. Curves are extrapolated to zero hydrolysis time for the estimation of amino acid values in the original protein.


SERINE
THREONINE
y = -0.05x + 6.03, r = - 0.9998
y = -0.02x + 2.60, r = - 0.9840
AMINO ACID (%)
6.0
5.0
4.0
3.0
2.0
1.0
0
4
8
12
16
20
24
28
32
36
40
44
48
HYDROLYSIS TIME (H)

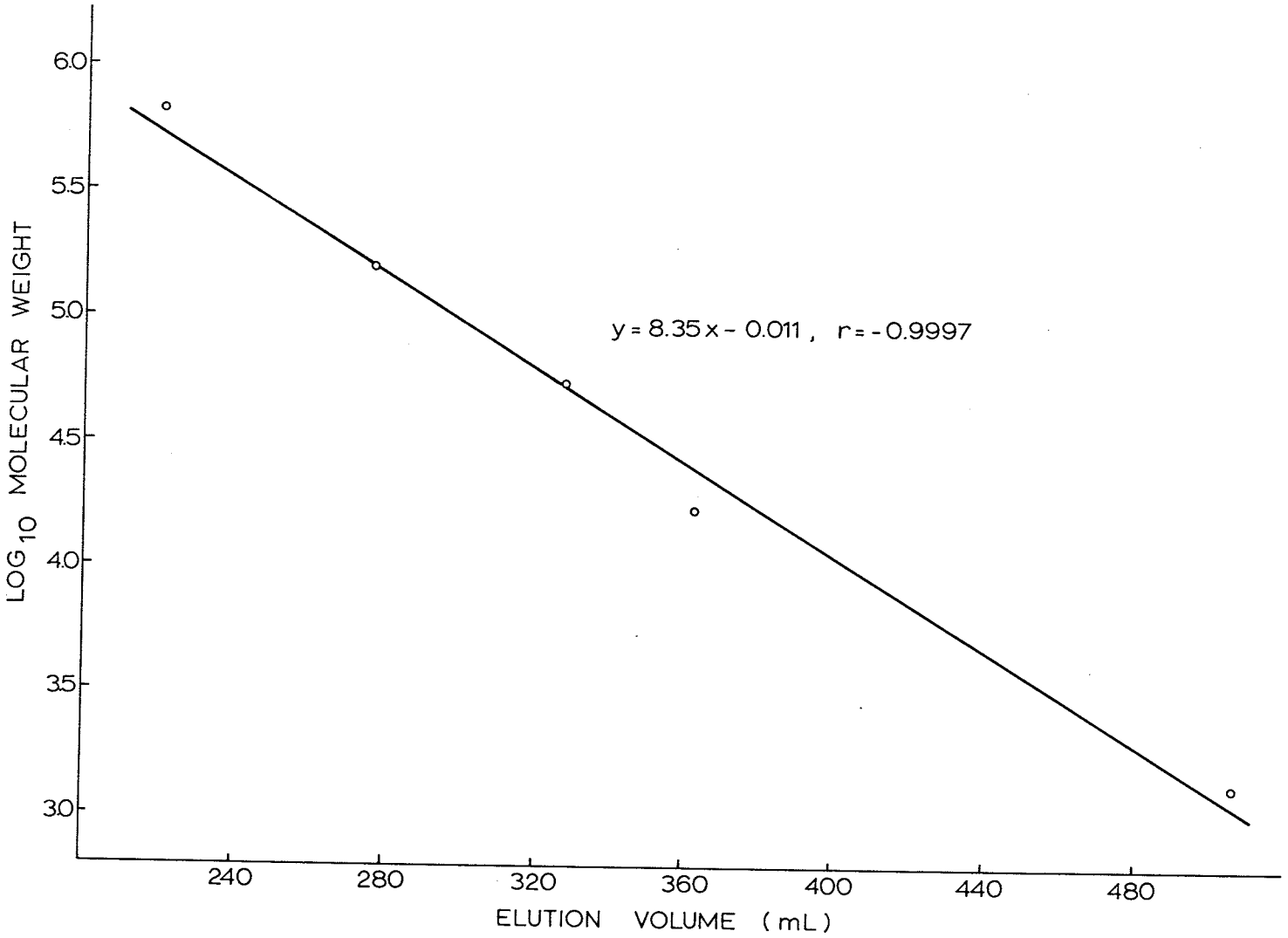
APPENDIX II. Calibration curve for isoelectric point determination of vicilin by isoelectric focusing.


6.0
5.5
5.0
4.5
4.0
3.5
GEL pH
y = 0.23 x + 3.76 , r=0.9834
1
2
3
4
5
6
7
8
9
10
DISTANCE FROM ANODE (cm)

APPENDIX III. Relative mobility (R.M.) of SDS-PAGE protein standards as a function of $\log_{10}$ molecular weight.


LOG10 MOLECULAR WEIGHT
5.0
4.5
4.0
y = 4.63 - 0.884 x , r = - 0.9915
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
RELATIVE MOBILITY (R.M.)

APPENDIX IV. Calibration curve for molecular weight determination of vicilin by gel filtration.


6.0
5.5
5.0
4.5
4.0
3.5
3.0
LOG 10 MOLECULAR WEIGHT
y = 8.35 x - 0.011 , r = - 0.9997
240
280
320
360
400
440
480
ELUTION VOLUME (mL)

APPENDIX VA. The Hofmeister (1888) series of electrolytes based on the relative effectiveness of different salts in salting-out euglobulins from agueous solution.

Most Effective

Na_3-citrate

↓

Li_2SO_4, Na_2SO_4, K_2HPO_4 Na_2HPO_4

↓

$(NH_4)_2SO_4$

↓

$MgSO_4$

↓

K-acetate, Na-acetate

↓

NaCl

↓

Least Effective $NaNO_3$

APPENDIX VB. Representative hierarchial arrangements of certain anions and cations in terms of their relative effectiveness in the stabilization of the melting temperatures of ribonuclease (von Hippel and Wong, 1964).

←Stabilizing Destabilizing→

$SO_4^{-2} < CH_3COO^- < Cl^- < Br^- < I^- < ClO_4^- < SCN^-$

$(CH_3)_4N^+$, NH_4^+, K^+, $Na^+ < Li^+ < Ca^{+2}$