THE EFFECTS OF ORGANIC PERTURBANTS ON THE STRUCTURE OF SOLUBLE COLLAGEN

Thesis Submitted to Rhodes University in Partial Fulfilment of the Requirements for the Degree of Master of Science

by

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January 1971

ACKNOWLEDGEMENTS

This investigation was carried out in the Protein Research Laboratory of the Leather Industries Research Institute, Rhodes University, Grahamstown. The author wishes to express his appreciation to Dr. S. G. Shuttleworth, Director of the Institute, for making the necessary facilities available.

Particular gratitude is expressed to:

the author's supervisor, Dr. D. R. Cooper, for his guidance and encouragement at all times, both prior to and throughout this study;

Dr. A. E. Russell for his help and interest, which was a continual source of inspiration;

Professor C. H. Price, Dean of the Faculty of Science and Head of the Department of Pharmacy, for his co-operation;

Mr. R. Kohl for technical assistance;

the Council for Scientific and Industrial Research and Rhodes University for financial aid;

and Mrs. I. Inggs for typing the final draft.

SUMMARY

Organic solvents were used in the present study as a means of investigating the non-covalent interactions involved in the maintenance and perturbation of the three-dimensional structure of the collagen macromolecule in solution. The two main types of non-covalent interaction under consideration are hydrogen bond formation and hydrophobic effects. Elucidation of the relative importance of these factors in the maintenance of the solution structure of proteins is an area of intensive investigation and fundamental significance to biochemistry as a whole. During the past decade, considerable progress has been made towards a clearer understanding of the forces involved, and a number of different theoretical and experimental approaches have emerged. Until about 1960, hydrogen bonding was widely believed to be the dominant non-covalent interaction responsible for the maintenance of secondary and tertiary structure in many proteins. Subsequently, an increasingly important role for apolar (hydrophobic) effects was suggested by a number of authors, and at present there is no satisfactorily definitive interpretation of the available experimental evidence.

The current work is based on a comparison of the effects of organic solvents on widely different substrates, namely collagen, cellulose, and the chromatographic reference material, catechin. The chromatographic mobility of catechin on cellulose may be regarded as a phenomenon which is mediated entirely by polar interaction mechanisms. The effects of various organic perturbants and of changing solvent/water ratios are readily interpreted on this basis. In the collagenous systems, however, certain results appear to require the introduction of concepts

other than those relating exclusively to polar bonding affinities. The experimental evidence shows that there are cases where the enhancement of the polar interaction potential of solvent/water mixtures, in relation to catechin-cellulose systems, is accompanied by an apparent reduction of polar interaction potential of the same solvent/water mixtures with respect to soluble collagen.

The anomaly outlined above will be discussed in terms of two fundamentally different theoretical assumptions. In the first of these, the mechanism of perturbant action in collagenous systems is regarded as essentially similar to that governing catechin-cellulose affinity patterns. Thus, interaction processes are all treated as polar phenomena, in which direct hydrophobic destabilization of the collagen triple helix plays no part. In an attempt to explain the effects of perturbants in both collagen and cellulose-containing systems in terms of the above assumption, two hypotheses are examined involving (1) direct polar interaction between perturbant molecules and functional groups of the protein; (2) the possibility of an enhanced polar interaction potential of water molecules, due to lowering of the environmental dielectric constant when organic solvents are added to the systems.

Within the other broad conceptual division, collagen and cellulose substrates are considered to respond in fundamentally different ways to the action of organic perturbants. As before, cellulose-catechin-solvent interactions are treated as entirely polar phenomena, and perturbant effects interpreted in terms of mechanisms such as direct solvation of the substrate, and the enhanced hydrogen bonding activity of water molecules. In contrast, perturbant lyotropic action with respect to soluble

collagen is viewed as the manifestation of a major contribution by hydrophobic interaction processes to macromolecular stability. Thus, solvents that competitively reduce the assumed entropic contribution to the stability of the collagen triple helix, are seen as potential destabilizers of the native state of the protein and inhibitors of the regeneration of co-operative structures during renaturation.

Both of the above approaches are critically assessed in the light of the present work and the dominant trends apparent in the recent literature.

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CHAPTER I

COLLAGEN AND ITS ENVIRONMENT

The environment within which a protein molecule exists exerts a profound effect on the three-dimensional structure of that molecule. The factors involved in the interaction between protein and environment are probably best interpreted as a delicate balance between complementary and opposing tendencies arising from weak, non-covalent forces which are extremely sensitive to any change in their surroundings. A valuable approach to the elucidation of the relative contributions of these non-covalent interactions to the maintenance of protein conformational stability is that involving selective perturbation of the solvent environment of the protein in such a way as to cause measurable variation in conveniently monitored parameters. Collagen is very useful in studies of this kind for a number of reasons. In addition to its ready availability and relative ease of purification, it is sufficiently stable to allow convenient manipulation under laboratory conditions. Its most outstanding value arises from the large variation shown in two readily determined parameters, specific rotation and viscosity. There exists an extensive and well-documented literature on the use of these and similar criteria in relation to conformational studies (e. g. Levedahl and James, 1961; Yang, 1961).

The role of collagen as the major structural component in all mammals and fishes and its unique contribution to the maintenance of mechanical form and tensile strength is analogous to the position occupied by cellulose in the plant kingdom (Bear, 1952). Collagen constitutes the major protein component of bone, tendon, skin, cartilage and other connective tissues; approximately 30% of the total organic matter and 60% of the protein of the animal organism is of a collagenous nature (Veis, 1964).

Collagen studies extend through many diverse fields of interest. Collagen is intimately involved in a number of physiological processes including bone deposition, ageing and scar-tissue formation

(Gillman, 1968; Glimcher and Krane, 1968; Sinex, 1968) and certain pathological conditions such as neoplastic growth, various forms of rheumatic inflammation, and vitamin deficiency symptoms (Bear, 1952). Commercially, collagen research arising out of the needs of the leather and the gelatin and glue industries has led to significant advances on both the fundamental and applied levels.

COLLAGEN STRUCTURE

Differentiation of structural elements in collagen extends through several orders of organization which may be distinguished as matrix collagen, intact collagen, collagen fibres, fibrils and filaments, and collagen molecules (Bear, 1952; Veis, 1967). Formal distinction among all levels is not necessary in the context of the present work which is concerned mainly with the behaviour of the collagen molecule in solution in response to environmental influences. However, reference will be made to the use of matrix collagen or collagen strips in certain chromatographic procedures: matrix collagen is defined here as the covalently bonded macroscopic network remaining after successive non-hydrolytic treatments of a suitable starting material with dilute organic acids, neutral non-denaturing salts and dehydrating solvents (after Veis, 1967).

The basic molecular unit of all collagens is the monomeric tropocollagen macromolecule (Veis, 1964), the nature of which has been intensively investigated in recent years. The single collagen molecule is an elongated cylindrical rod approximately 2800 Å in length and 15 Å in diameter (Boedtker and Doty, 1956; Hall and Doty, 1958). Individual macromolecules are composed of three polypeptide chains, each of molecular weight $\pm$ 100,000, and designated α-chains. The three chains each adopt a helical conformation and these helices are wound further around a common central axis. The three-stranded unit is referred to as the

superhelix or triple helix. Upon heating to a critical temperature, which varies according to the source and method of extraction of the particular collagen, the rigid three-stranded structure collapses to the random coil form referred to as gelatin. Covalent links between α-chains may occur producing, on denaturation, β and γ-components with molecular weights of about 200,000 and 300,000 respectively, corresponding to double and triple-stranded random coils (Piez *et al.*, 1963; Piez, 1965). Although the individual α-chains of native vertebrate collagens are not identical (Piez, 1965; Butler *et al.*, 1967; Stark and Kühn, 1968) it has been shown that it is possible to form triple helices *in vitro*, which are very similar to native collagen molecules, from identical α-chains (Tkocz and Kühn, 1968). There is also evidence to suggest that α-chains are further subdivided into distinguishable units (Gallop *et al.*, 1959; Blumenfeld *et al.*, 1965; Gallop, 1966).

Crystallographic studies indicate that the individual polypeptide chains of the triple helix are stabilized by one (Cowan *et al.*, 1955; Rich and Crick, 1955) or two (Ramachandran, 1967) hydrogen bonds for every three residues along each chain. Recent evidence (Engel *et al.*, 1966; Segal and Traub, 1969; Segal *et al.*, 1969; Traub *et al.*, 1969; Yonath and Traub, 1969) appears to support the Rich-Crick one-bonded model rather than the widely favoured two-bonded structure.

AMINO ACID COMPOSITION

The amino acid composition of collagen clearly sets it apart from all other proteins. It contains an unusually high proportion of glycine and proline and is the only mammalian protein containing large amounts of hydroxyproline. Vertebrate collagen has an extremely low sulphur content, this being solely in the form of methionine since no cystine is

present. However, an invertebrate collagen, isolated from the cuticle of Ascaris lumbricoides, has been shown to have a significant content of half-cystine residues and to exhibit a number of unusual chemical and physical properties (McBride and Harrington, 1967a, b). Another amino acid almost exclusive to the collagens is hydroxylysine.

The present state of development of amino acid composition studies of various collagens has been reviewed recently by Eastoe (1967). Typical mammalian collagens (Bowes et al., 1955; Eastoe, 1955; Piez et al., 1963) have the following amino acid residue ratios: glycine, 1 in 3; proline and hydroxyproline (combined), 2 in 9; alanine, 1 in 9. The sum of these four accounts for two-thirds of all residues present, the remainder being made up of the other fourteen amino acids that occur in collagen. The high content of the imino acids, proline and hydroxyproline, is to a considerable extent responsible for the unique properties of collagen. Firstly, the pyrrolidine ring residues bear no hydrogen atoms which could form inter-peptide hydrogen bonds, hence α-helical conformations cannot be assumed by the polypeptide chains. Secondly, the steric rigidity of the pyrrolidine nucleus is a major stability-mediating factor in the formation of the specific collagen-fold. Studies of the relation between imino acid content and thermal stability of vertebrate and invertebrate collagens (Lewis and Piez, 1964; Harrington and McBride, 1966; Harrington and Rao, 1967) have shown that there is generally a reduction in stability of collagens which have a relatively low proportion of pyrrolidine residues.

Total pyrrolidine content may not be the only factor affecting stability since there is evidence to suggest that residue sequence and the presence of residues such as serine may influence the properties of collagens (Rigby, 1967). Sequence studies on various collagens

(Bornstein, 1967; Hannig and Nordwig, 1967) have shown the primary structure of individual polypeptide chains to be differentiated into regions composed mainly of amino acids having non-polar side chains and sections in which there is a high concentration of amino residues with polar side chains. Nevertheless, the distribution of pyrrolidine residues through each polypeptide chain appears to be highly systematic and uniform (Harrington and von Hippel, 1961a), the imino residues nearly always occurring next to glycine. In calf-skin collagen proline and hydroxyproline are present in approximately equal amounts, which suggests that the sequence Gly-Pro-Hypro-Gly might be a major contributor to the primary structure. This has been substantiated by the identification of the sequences Gly-Pro-Hypro and Gly-(Hypro-Pro)-Gly (Kroner et al., 1955). In order to accommodate a single imino residue a polypeptide chain must rotate by -120°, hence two sequential imino residues would impart a left-hand rotation of -240° thus generating a fragment of the poly-L-proline II helix. The presence of a sufficient number of suitably disposed pyrrolidine residues may thus account for the formation of the poly-L-proline II helical structure of the individual polypeptide chains, and this process may be independent of inter-chain stabilization mechanisms. In view of the foregoing, it appears that the imino residues are of fundamental significance with respect to the equilibrium and kinetic behaviour of collagen. This topic will be discussed in greater detail in Chapter 3.

WATER STRUCTURE

The present study is concerned with the effects that changes in the solvent environment have on the denaturation and renaturation of the triple helix and the extent to which the maintenance of the native structure of collagen is due to hydrogen bond formation and to non-

covalent forces other than hydrogen bonds. Lyotropic agents have been widely employed as environmental regulators in studies on soluble and intact collagens. Alcohols, amines, neutral salts and other forms of perturbant are well known in this respect (Steven and Tristram, 1962; Schnell and Zahn, 1965; von Hippel, 1967; Herbage et al., 1968) and have also been extensively applied to investigations of other (non-collagenous) protein systems (e. g. Schrier and Scheraga, 1962; von Hippel and Wong, 1965; Timasheff and Inoue, 1968; Kaminsky and Davison, 1969; Katz and Denis, 1969; Sun, 1969; Salahuddin and Tanford, 1970; Timasheff, 1970). However, before proceeding to a more detailed examination of certain theoretical arguments emerging from studies of this nature, it is necessary to consider a fundamental concept that is central to the thermodynamic interpretation of protein conformational stabilization. The concept is that in which the existence of a degree of ordering or "structure" in liquid water is postulated. In terms of the Second Law of Thermodynamics, structural organization of water is of major importance with respect to hydrophobic stabilization mechanisms. The classical publication dealing with entropy-driven structure stabilization processes is that by Kauzmann (1959), the basic tenets of which have changed little in the past decade. Certain aspects and implications of the existence of structured water will be discussed briefly below. An attempt will be made to clarify treatments relating to bulk properties in terms of the corresponding molecular concepts. Thus proposals arising out of a consideration of such bulk parameters as entropy, free energy and dielectric constant will be used interchangeably with their molecular counterparts, where applicable, in order to demonstrate the essential compatibility of the two approaches.

The thermodynamic properties of water are greatly affected by its hydrogen bonding capacity. The presence of strong hydrogen

bonds in water explains such unusual properties as the high heat capacity, boiling point and melting point (Edsall and Wyman, 1958; Pauling, 1960; Eisenberg and Kauzmann, 1969). The introduction of non-polar molecules into an aqueous environment necessitates the disruption of water-water hydrogen bonds in the immediate vicinity of the non-hydrateable moiety. This results in a free energy increase due to the distortion of the favoured hydrogen-bonded arrangement of pure water (Tanford, 1970). This concept, arising partly out of the work of Frank and Evans (1945), has led to the development of models for the structure of water "clusters" surrounding ionic, polar and non-polar entities in aqueous solution (Hatefi and Hanstein, 1969; Hertz, 1970). The relevance of this approach to the study of the stabilization of protein molecules in aqueous solution has been extensively reviewed (Klotz, 1958; Némethy and Scheraga, 1962; Schachman, 1963; Némethy, 1967, 1969), the central thesis being that tertiary and higher structural organization in proteins is stabilized to a greater or lesser degree by the entropy gain arising from the exclusion of vicinal, ordered water when non-polar surfaces in aqueous solution come into close proximity. The return of the relatively highly structured vicinal water to the less ordered condition, or bulk structure, is regarded as the entropically favourable process. It has not yet proved possible (Schleich and von Hippel, 1970) to establish a consistent general relationship between water structure and macromolecular stability by direct methods. Nevertheless, evidence suggesting changes in water structure on the addition of certain small-molecule solutes, such as urea, guanidinium chloride and neutral salts, has recently been obtained using ultrasonic attenuation measurements (Hammes and Schimmel, 1967; Hammes and Swann, 1967; Hammes and Roberts, 1968). Other direct measurements indicating the presence of structural elements in aqueous solutions have also been reported (Assarsson and Eirich, 1968; Barsukova, 1968; Hoover, 1969; Krishnan and Friedman, 1969a, b).

An important aspect of the possible existence of structural elements in water near interfaces is the effect on dielectric constant in the ordered regions. Drost-Hansen (1969) has dealt at length with this question and argues that the experimental findings may be interpreted in terms of a lowering of dielectric constant in vicinal water due to restricted rotational freedom of water molecules in these regions. The resulting reduced orientation polarization would account for the lowering of the vicinal dielectric constant. If this interpretation is acceptable the implications with respect to hydrogen bonding and other largely electrostatic interactions in protein-solvent systems are considerable, since vicinal forces of attraction and repulsion would be enhanced relative to those existing in the body of the liquid. This possibility is discussed further in the following section in relation to a direct binding mechanism for lyotropic action.

THEORETICAL MODELS FOR LYOTROPIC ACTION

Two major theoretical approaches to the mechanism of lyotropic activity appear relevant to the present investigation. The first embodies concepts pertaining to the Second Law of Thermodynamics, as mentioned above, and has recently been invoked by Harrap (1969) to explain results obtained using soluble rat-tail tendon collagen and various alcohols and diols. Harrap found that monohydric alcohols such as methanol, ethanol and tertiary butanol caused a progressive lowering of the melting temperature, Tm, of rat-tail tendon collagen with increasing carbon chain length of the organic solvent. In contrast, the diols, ethylene glycol and propane-1,3-diol, produced considerable increases in Tm. Propane-1,2-diol, however, caused an initial marginal Tm decrease up to a concentration of about 10 molar, after which a slight increase

occurred. This slight increase at high molarities also appeared using methanol. Harrap interpreted the findings in terms of hydrophobic interactions and changes in dielectric constant, the former representing the classical approach that stems largely from studies on globular proteins (Tanford, 1962; Timasheff, 1970). Thus destabilization is regarded as being due to hydrophobic interaction between the hydrocarbon portions of perturbant molecules and non-polar side chains of the tropocollagen macromolecule. Increasing the hydrocarbon content of the environment would therefore render less favourable the entropic stabilization arising from mutual association of non-polar residue side chains, since there would be a competing tendency for apolar side chains to associate with non-polar molecules in the environmental atmosphere. At high concentrations of organic solvent, however, the general lowering of the dielectric constant of the system would produce an opposing trend due to enhancement of polar interactions within the macromolecule. Although all polar interactions would be enhanced, it is to be expected that the co-operative hydrogen-bonded system of the macromolecule would be stabilized to a greater extent than would random polar association between perturbant and protein and between water and protein. Hence, in those cases where the particular perturbant produced only a limited degree of hydrophobic destabilization, for example with methanol, a point would be reached where stabilization would become the dominant effect due to the enhancement of co-operative polar interactions within the protein molecule. The tendency towards trend reversal was also observed with the longer chain alcohols at lower concentrations in the order of decreasing dielectric constant of the pure alcohols. This is consistent with the requirements of the proposed model. In the cases of ethylene glycol and propane-1, 3-diol, Harrap considers that the hydrocarbon chain would be sterically shielded from hydrophobic interaction with the

apolar regions of the protein molecule by the terminally situated hydroxyl groups. In contrast, propane-1, 2-diol has a sterically more accessible methyl group which could interact hydrophobically with apolar portions of the collagen molecule. Harrap's conclusions may be summarized as follows: the major observed effects are rationalized in terms of (1) increased strength of the co-operatively hydrogen-bonded system of the macromolecule due to the lowering of dielectric constant upon addition of organic solvents and (2) a decrease in the contribution of hydrophobic interactions, between apolar side chains, to the stability of the tropocollagen monomer. Since these tendencies are of opposite sense the overall effect depends critically on the balance between them.

An alternative interpretation of lyotropic action has been formulated by Russell and Cooper (1969) with respect to experimental findings obtained using related systems. Renaturation of acid-soluble calf-skin collagen was monitored, in the presence of various polar organic solvents, by optical rotation and viscosity regain measurements. Regain was progressively retarded as concentration, hydrocarbon chain length and polarity of perturbants were increased, but retardation was less extensive with solvents having branched chain structures. The mechanism proposed does not provide a role for direct hydrophobic interactions as such, destabilization being regarded as a function of direct hydrogen bond formation between polar perturbant molecules and peptide carbonyl and imide groups. The resulting effects would be (1) competitive disruption of interchain hydrogen bonds and (2) reduction of the double-bond character of the peptide link, leading to increased rotational freedom of the main chain components (Mandelkern et al., 1962). The effects of increasing carbon chain length are interpreted by Russell and Cooper (1969) in terms of a shielding mechanism whereby the apolar portion of the perturbant

molecule competitively shields its polar group from interaction with environmental water molecules, thereby enhancing the potential for association between perturbant polar groups and peptide carbonyl and imide functions. The extent to which this shielding could be expected to take place would increase with increasing linear hydrocarbon chain length since the "sweep-out volume", that is the region of reduced water concentration, would increase proportionately. In contrast, branched chain structures would contribute to a relatively smaller extent to increasing the swept-out region in the immediate vicinity of the potential hydrogen-bonding group of the organic solvent molecule. Studies involving the synthetic homo-polypeptide poly-L-proline (Kurtz and Harrington, 1966; Veis et al., 1967; Strassmair et al., 1969), ultracentrifugation experiments using pigskin gelatin (Threlkeld et al., 1968), and shrinkage temperature, swelling and intrinsic viscosity measurements (Puett and Rajagh, 1968) have recently provided substantial evidence that comformational stability may be influenced by direct binding of perturbant molecules to functional groups of the polymer.

The concept of lowered dielectric constant of structured water vicinal to apolar surfaces (Drost-Hansen, 1969) is readily incorporated into the above model for direct binding. Thus the influence of mechanical shielding effects in lowering the local dielectric constant in the vicinity of a perturbant molecule may be supplemented by a similar reduction arising out of the phenomenon of water structure enhancement near a non-hydrateable interface. Longer hydrocarbon chains would be expected to induce a greater degree of vicinal ordering of water than would shorter chains or more compact isomers. It is conceivable that the influence on dielectric constant, of greater structural organization in vicinal water, may be the primary cause of enhanced perturbant-substrate

hydrogen bonding, rather than the mechanical shielding or sweep-out volume process.

In a recent publication Herskovits et al. (1970) have criticized the direct binding approach in terms of its energetic feasibility. Implicit in the direct binding concept is a requirement for the stabilization of hydrogen bonds between perturbant molecules and protein peptide groups as a result of the presence of the apolar portion of the perturbant molecule. Herskovits et al. discuss data which suggest that the energy required to account for denaturation purely in terms of such polar interaction mechanisms would have to be of the order of 2.3 kcal per mole of alcohol-peptide bonds. In addition, each further methylene group in the homologous series investigated would necessitate an energy increase of 0.6 to 0.7 kcal per mole. Comparing these requirements with the known enthalpies of dimerization of the carboxylic acid series, formic, acetic, propionic and butyric acids, these authors conclude that such a mechanism is unlikely since the reported enthalpies are in the region of 0 $\pm$ 1 kcal per mole and do not show any definite relation to hydrocarbon content (calculations were based on the work of Schrier et al., 1964). Herskovits et al. also note that the experimental observations of Susi and Ard (1966) show the enthalpies of dimerization of δ-valerolactam in water, methanol and ethylene glycol to be similar, indicating no selectively greater ability of these solvents to weaken the C=0...NH hydrogen bond of the lactam.

NON-PROTEIN SYSTEMS

The scope of the present study was extended to include a set of non-protein systems in which the mobility of a reference substance (catechin) on a cellulose substrate was shown to be a function of solvent composition. It is necessary to consider the validity of

comparing results obtained using these systems, with the very different situation which might be expected to obtain in relation to protein-solvent interactions. Recent work (Russell et al., 1967a, b, 1968a, b, c; Shuttleworth et al., 1968) on mobility patterns of vegetable tannins using cellulose and matrix collagen substrates is relevant to this question and will be reviewed here. Use was made of an ascending chromatographic technique to study the essentially reversible association between tannin and substrate and the effects of changing solvent composition on such interactions. The mobility profiles both on collagen and cellulose were markedly similar, overall mobility being somewhat lower on collagen. These authors suggest that a common mechanism of association of tannins with both substrates is operative, based on polar interactions between solvent, solute and substrate, the main characteristic being non-specificity and applicability to a variety of systems. The lower overall mobility of the wattle tannins on collagen than on cellulose is attributed to the expected stronger polar interactions between the polyphenolic tannin molecules and the carbonyl groups of the intact protein. In contrast, interactions between tannin molecules and cellulose would be weaker since the only available hydrogen-bonding sites on the cellulose are the hydroxyl groups. A further feature of the observed mobility patterns is the initial rise in mobility to a peak at intermediate organic solvent/water ratios, followed by a marked reduction in mobility towards the pure organic solvent extreme. The concentration at which maximum mobility occurs is lowered as carbon chain length of the organic solvent increases. Typical migration patterns are summarized in the following table, which shows the relationship between solvent carbon chain length and the mobility of catechin on collagen and cellulose. Rf maxima are shown relative to the concentrations at which they appear.

TABLE 1.1

Effect of increasing chain length of solvent on chromatographic mobility of catechin on cellulose and collagen

Solvent	Rf(maximum)		Solvent concentration at maximum Rf (volumes %)	
	cellulose	collagen	cellulose	collagen
Methanol	0.70	0.35	60	75
Ethanol	0.80	0.50	55	65
n-Propanol	0.95	0.60	45	55

Data from Russell *et al.*, 1968b.

From the table it is clear that increasing linear carbon chain length enhances tannin mobility on both substrates.

The detailed explanation proposed by Russell *et al.*, (1967a, b, 1968a, b, c) to account for the effects described above is as follows: active sites on the substrate (cellulose or collagen) are considered to compete with environmental water and solvent molecules for polar binding sites on the tannin molecules. Any factor which increases water-tannin, solvent-tannin, water-cellulose or solvent-cellulose hydrogen bonding will commensurately reduce the potential for tannin-cellulose interactions. Such a factor would therefore promote tannin mobility. A similar mechanism is suggested to account for the affinity patterns shown on the collagen substrates. The effect of pendant hydrocarbon structure is described in terms of the shielding action of the apolar portion of the organic solvent molecule, which causes a reduction in the availability of water in its immediate vicinity. Thus the rate of exchange of environmental molecules at polar sites on the tannin and the substrate is diminished. The

overall effect represents an increase in the duration of residency of organic solvent molecules on tannin molecules and substrate as perturbant hydrocarbon chain length is increased. Hence there is a greater degree of solvation of tannin and substrate by the environmental atmosphere, and reduced probability that tannin-substrate hydrogen bonds will form. Thus at low concentrations of organic solvent the dominant effect is a reduction of the duration of polar interactions between tannin molecules and substrate due to competition for all polar sites by the perturbant molecules, this competitive ability being enhanced progressively by the shielding effect of increasing carbon chain length. When the concentration of organic perturbant in the environment becomes sufficiently high, however, tannin mobility decreases since the general lowering of dielectric constant becomes more significant than the vicinal effect in the immediate proximity of perturbant molecules. Under these circumstances all polar interactions are enhanced but the co-operative nature of the multi-point catechin-substrate attachment makes this the dominant associative tendency and mobility is sharply reduced. This theoretical approach is the direct corollary to that discussed in relation to the soluble collagen systems and is similarly subject to modification in terms of the influence of vicinal water structure on local dielectric constant.

The generality of effects of solvent composition with respect to tannin mobility on matrix collagen and on cellulose does not necessarily imply that the situation obtaining in systems containing soluble collagen can be interpreted in identical terms. The structure of a protein molecule in solution might reasonably be expected to be more sensitive to certain environmental influences such as hydrophobic stabilization processes (Tanford, 1962; Wetlaufer and Lovrien, 1963; von Hippel and Wong, 1965; Schnell, 1968) than would the gross, intact form. Nevertheless, in the present study,

comparison of the influence of solvent composition on catechin-cellulose affinity patterns with the effect on the stability and renaturation of soluble collagen proved very useful. The possible significance of the apparent similarities and differences evident in these systems is discussed in later chapters.

HYDROPHOBIC INTERACTIONS WITHIN THE TRIPLE HELIX

The concept of hydrophobic interaction as a factor contributing to the stabilization of the collagen triple helix is not infrequently invoked to explain the effects of certain lyotropic agents (Schnell, 1968; Harrap, 1969). The apparently well-established role for such interactions in relation to the globular proteins and model systems (Wetlaufer and Lovrien, 1963; Kiehs et al., 1966; Castellino and Barker, 1969; Gratzer and Beaven, 1969; Cecil and Louis, 1970; Timasheff, 1970) has led to the tentative assumption that similar processes might be significant with respect to collagen stability at the level of the monomeric macromolecule. Tanford (1970) states that, "It is now firmly established that the compact ordered structures of typical native proteins owe their existence to the fact that the hydrophobic side chains of proteins have an unfavourable free energy of interaction with water." While this approach appears to be valid in a number of cases, especially with respect to the globular proteins and probably also in relation to the polymerization of collagen monomeric units in the formation of the typical higher aggregation states, difficulties arise in extending this hypothesis to include the tropocollagen monomer itself. Examination of the fine structure of the collagen molecule makes it appear unlikely that apolar association could play a significant part. The Ramachandran (1967) model for the collagen triple helix shows that all side chains must be directed away from the central axis. Wherever an α-carbon atom carries a side

chain, that is, in all cases other than glycine, there is no possibility of the side chain being oriented towards the main axis; side chains must, in effect, be externally disposed around the "outside" of the tropocollagen macromolecule. Other collagen models, such as that proposed by Rich and Crick (1955), do not differ in this particular respect since β, γ and δ carbon atoms could not be proximally accommodated. The remaining possibility to be considered is that involving apolar association of contiguous non-polar side chains on the external surface of the monomer. It appears that any such contiguous interactions between suitably juxtaposed side chains would necessarily be of a limited nature. Nevertheless, the experimental evidence arising out of the present work suggests the existence of a role for such interactions as contributors to the conformational stability of the collagen molecule. This implies that it may not be essential for extensive chain segments of a predominantly apolar nature to come into very close proximity, as in the formation of tertiary structural elements in globular proteins or quaternary aggregation states in collagen, for hydrophobic effects to contribute significantly to stabilization of the triple helix.

It is evident from the literature that there is no consensus of opinion concerning the probability of hydrophobic interaction being a contributor to the stabilization of the triple helix. Harrington (1964), for example, makes the assumption that the only forces involved in the stabilization of the tropocollagen monomer in the typical triple helical conformation are peptide hydrogen bonds between the three chains, in addition to the stereochemical restrictions associated with the pyrrolidine-ring residues. In contrast, von Hippel (1967), in an assessment of Harrington's findings, contends that there is considerable evidence to suggest that collagen is stabilized by hydrophobic interactions and that attributing too exclusive a role to hydrogen bonding might be an oversimplification.

SYNTHETIC POLYPEPTIDES AND OTHER SYSTEMS

Studies on the collagen analogue $(Pro\text{-}Gly\text{-}Pro)_n$ are relevant to the present discussion. Engel (1967) has demonstrated the fundamental similarity of the $(Pro\text{-}Gly\text{-}Pro)_n$ and collagen structures with respect to temperature-induced conformational transitions. On the basis of Harrington's (1964) calculations for the two-hydrogen-bonded structure for collagen, Engel proceeded to compute the expected melting temperature of $(Pro\text{-}Gly\text{-}Pro)_n$ using the same entropy and enthalpy values but assuming only one hydrogen bond per tripeptide unit; only one hydrogen bond per tripeptide unit being possible since the nitrogen atom of the proline residue is tertiary. The value so obtained was 93° C which is in good agreement with the experimental estimate of 70°-100°C. These values are based on the assumption that hydrophobic interactions play little or no part in the stabilization of the monomeric triple helical structures in either case.

In contrast, it has been shown (von Hippel and Schleich, 1969) that perturbants such as urea and guanidinium chloride considerably increase the solubility of alkanes and non-polar amino acids, thus implying that interactions other than hydrogen bonding must be operative in this type of solubility process, which is regarded as being analogous to the destabilization of hydrophobically associated protein side chains.

The considerations outlined above suggest that although contacts between apolar side chains in the collagen molecule appear to be of a far more limited nature than those in globular proteins, the possibility that hydrophobic interactions might contribute significantly to the stabilization of the tropocollagen monomer cannot be dismissed. It is hoped that examination of the experimental findings to be detailed in the following chapters will allow a relative assessment to be made of the extent to which structure perturbation in the collagen molecule may be accounted for in terms of the foregoing hypotheses.

CHAPTER 2

METHODOLOGY

SPECIFIC ROTATION AND THE COLLAGEN↔GELATIN TRANSITION

The theoretical basis for the use of optical rotation as a parameter for monitoring the collagen↔gelatin transition has been discussed by Cohen (1955). The interpretation offered relates optical rotation and polypeptide chain conformation as follows. (1) In the denatured state chain conformation is completely randomized, the observed rotation being attributed to the additive contributions of the individual L-amino acid residues. (2) The very high specific rotation of native collagen, by comparison with the globular proteins, may be rationalized in terms of the large contribution by the unique collagen-fold structure which is superimposed on the contributions of the α-carbon atoms of the L-amino acid residues. Thus any change in the specific rotation is regarded as a direct measure of the amount of collagen-type helical structure existing in the main polypeptide chains. In the present study the specific rotation of a completely heat-denatured gelatin solution was taken as $[\alpha] = -460^{\circ}$ at $\lambda = 365\text{m}\mu$, and that of soluble native collagen as $[\alpha]^{15^{\circ}}_{365} = -1330^{\circ}$ (Drake et al., 1966). Calculations of specific rotation are based on the relation

$$[\alpha]^{t}_{365} = \frac{100}{dc}\alpha$$

where α = observed rotation

d = path length in decimetres

and c = concentration of protein in grams per decilitre.

The high content of the pyrrolidine-type residues, proline and hydroxyproline, in collagen is of particular importance with respect to the additivity of optical rotatory properties; under normal circumstances helical conformations have no preferred sense or direction of twist, providing that pyrrolidine-residue content is low. Hence, in globular proteins, it seems likely that areas of opposed helical sense could occur in the same polypeptide chain in the native state; the occasional

presence of pyrrolidine residues may in fact favour a reversal of the helical sense. In contrast, the unusually high pyrrolidine-residue content of collagen dictates stereochemical restrictions that preclude any sense reversals, thus accounting for the high optical rotatory power. The situation with respect to globular proteins is one in which the observed rotation is essentially a measure only of the extent to which one helical sense is in excess of the other. Since a peptide chain appears to fold more readily into a right-handed α-helix than a left-handed one (Haggis, 1964) it may be assumed that optical rotation readings represent the statistical dominance of the right-handed helical sense. As noted above, this situation does not exist in native collagen. In addition, the entire tropocollagen macromolecule may be regarded as being in the helical condition whereas globular proteins have regions of helix interspersed, to a greater or lesser degree, with regions of random coil.

Other aspects of the relationship between optical rotation and conformation of polypeptides and proteins have been comprehensively reviewed by Urnes and Doty (1961).

VISCOSITY

The viscosity of a solution is the manifestation of an essentially hydrodynamic phenomenon, changes in which are mediated by alterations in the overall size and shape of solute molecular domain, and hence provides a useful corroborative parameter for monitoring the collagen$\rightarrow$gelatin transition. The high viscosity of native collagen solutions reflects the asymmetry of the rodlike tropocollagen macromolecule, as distinct from the flexible denatured random coil which offers much less resistance to flow.

Viscosity measurements may be subjected to a number of theoretical treatments one of the more useful of which is the

calculation of the reduced viscosity. This is derived from the relation

$$\eta \text{ reduced} = \left(\frac{\eta - \eta_0}{\eta_0}\right)\frac{1}{c}$$

where η = viscosity of solution,

η_0 = viscosity of solvent,

c = concentration of solute in grams per decilitre.

Yang (1961) has reviewed the various viscometric relationships in current use and discussed in detail their application to different branches of protein chemistry.

THE USE OF FIXED-TIME REGAIN VALUES

A convenient means of comparing the effects of various perturbants on the renaturation process is on the basis of the optical rotation and viscosity regain values at fixed times after commencement of renaturation. Readings at 1 hour and 48 hours were used as indicators of recovery during the early and late stages of reaction, respectively. These were generally found to be in qualitative agreement with each other with respect to the overall order or ranking of perturbant effects. It had been expected that the 1 hour recovery values might also serve as an easily accessible measure of the kinetic situation at zero-time. However, in certain cases the relationship between 1 hour recovery values and initial reaction rates was found to be non-linear. In view of this finding, 1 hour recovery values were used as strictly qualitative indicators of the simple order of lyotropic effectiveness at finite time.

MATERIALS

Lyophilized, acid-soluble calf-skin collagen which had been prepared and checked for purity by amino acid analysis and ultracentrifugation according to the method described by Cooper and Davidson (1965) was used throughout.

Solvents and reagents were all commercial AR grade or Laboratory grade. In the studies of the effects of pH fluctuation the following buffer system was used:

component A' = 9.005 g/l glacial acetic acid
component B' = 14.722 g/l potassium acetate.

By combining suitable proportions of these two components a reasonably wide range of pH values could be selected.

In all other systems the buffer components were:

component A = 7.384 g/l glacial acetic acid
component B = 17.372 g/l potassium acetate.

Equal parts of components A and B produced a solution of pH 4.65 to 4.70 in the absence of added organic solvents.

In all cases the final ionic concentration of the combined components of the buffer systems was 0.15 molar.

Whatman No. 1 chromatography paper was used in all the chromatographic studies.

The (±) catechin used as chromatographic reference compound was supplied by Koch-Light Laboratories, Colnbrook, England. Catechin spots were visualized by spraying with a 14% solution of ammoniacal silver nitrate.

INSTRUMENTATION

Optical rotation measurements were taken by means of a Perkin-Elmer Model 141 readout polarimeter using the mercury lamp at a wavelength of 365 $m\mu$. The 10 cm polarimeter cells used were water-jacketed and the temperature controlled to $\pm$ 0.05°C using Colora Ultrathermostat water baths and Colora Tauchkühler TK64 immersion coolers. These were arranged in series so as to allow simultaneous operation of five polarimeter cells.

Viscosities were measured using Canon-Fenske flow viscometers, sizes 50 and 100, B.S. 188 and calculated according to the equation

$$V = Ct - \frac{B}{t}$$

where

V = kinematic viscosity in centistokes,
t = observed flow time in seconds,

and C and B are specific constants for each viscometer as supplied by the manufacturer (Gallenkamp & Co., London).

Viscometers were initially cleaned with chromic acid then rinsed with distilled water and dried with GR grade acetone. Following each experiment the viscometers were rinsed with tap water then filled with a $\pm$ 10% acetic acid solution and allowed to stand overnight. Viscometers were then rinsed with distilled water and dried with acetone.

Temperature control during viscometry was achieved using a Gallenkamp Model WF 615 viscometer bath linked to a Beckman Mobile Refrigeration Unit Model 133A, giving control to $\pm$ 0.05°C.

Tropocollagen solutions were clarified using an MSE 18 high speed centrifuge.

Optical rotation and viscosity data and the kinetic analyses were computed by means of a Hewlett-Packard Model 9100B programmable desk-top calculator.

METHODS

Collagen solutions were prepared by dissolving the estimated amount of freeze-dried acid-soluble collagen in the acetic acid component of the buffer by means of gentle stirring with a magnetic stirrer for $\pm$ 30 minutes, keeping the solution at $\pm$ 10°C to avoid heat denaturation. The potassium acetate solution was then added and the mixture allowed to stand overnight in the refrigerator at 4°C. The solution was centrifuged at 32,000 g for 1 hour at 5°C to remove any undissolved collagen. A concentration check was carried out by polarimetry using the value $[\alpha]_{365}^{15^\circ} = -1330^\circ$ as the basis for calculating the specific rotation of native tropocollagen (Drake et al., 1966) and the preparation was adjusted to the required concentration by the addition of buffer.

DENATURATION

Collagen $\rightarrow$ gelatin transitions were monitored by polarimetry using the "30-minute method" of von Hippel and Wong (1963a) in which systems are incubated for 30 minutes at each temperature prior to reading, thus allowing a pseudo-equilibrium state to be attained. Von Hippel and Wong (1963a) have shown that for true equilibrium to be reached the incubation periods at temperatures within the transition zone may have to be as long as 18 hours. However, the 30-minute procedure leads to an increase of only about 1°C in the apparent Tm and to melting curves which do not suffer any marked loss of symmetry by comparison with true equilibrium profiles. In the present work, melting temperatures

were used as a measure of the relative effects of various perturbants on the stability of the collagen triple helix, hence the small difference between Tm values obtained by the 30-minute method as compared with true equilibrium methods was not considered significant. In all cases Tm values were read at the transition midpoints.

RENATURATION

Both polarimetry and viscometry were used to follow the course of renaturation. Concentrated collagen/buffer solutions were denatured by heating at 45°C for 15 minutes, after which the systems were made up to final volume by the addition of predetermined quantities of organic solvent, buffer and distilled water at ambient temperature. The periods over which renaturation was followed, the temperatures chosen and the concentrations of collagen solutions are specified in conjunction with the descriptions of results or in legends to figures and tables.

EXPERIMENTAL ERROR

The error inherent in basing comparisons of lyotropic effectiveness on renaturation was determined using five replicate controls. Standard deviations were calculated for optical rotation data profiles only, since these were found to be more sensitive to experimental conditions than were the viscosity regain curves. The values obtained are shown below:

$[\alpha]_{365}^{15^\circ}$ at 1 hour

standard deviation = 10.04°

$[\alpha]_{365}^{15^\circ}$ at 48 hours

standard deviation = 6.14°

CHROMATOGRAPHY

A simple ascending technique was used. The air temperature in the chromatography room was maintained at $22^{o}C \pm 1^{o}C$. Chromatography paper was cut into strips measuring 18 by 2 cm. Solvent flow was in the machine direction in all cases. Pencil lines 2 cm and 12 cm from the lower ends of the strips served to mark the origins and 10 cm flow limits, respectively. A $\pm$ 5% solution of catechin in methanol was spotted at the origins, using a fine glass capillary, and allowed to air-dry for 2-3 minutes. Strips were then suspended in glass-stoppered reagent bottles with the lower ends of the strips clear of the solvent mixtures in the jars.

After equilibration for 20-30 minutes the strips were eased into lower positions in the jars, by raising and lowering the jar closures slightly, until the strips made contact with the solvent mixtures. In most cases development was allowed to proceed until the solvent fronts reached the pencil lines 10 cm above the origins, when the strips were removed and oven-dried at $\pm$ $100^{o}C$. When using high concentrations of very viscous solvents, however, the rates of ascent of the solvent fronts were extremely slow, hence strips were removed and dried before the 10 cm marks had been reached. In such cases the solvent fronts were marked by making a small cut in each paper strip upon removal from the jar; Rf values being calculated on the basis of the shorter ascent distances. When dry, the strips were sprayed with the ammoniacal silver nitrate solution to effect visualization of the catechin spots. Subsequently the strips were rinsed in two changes of distilled water followed by washing in running tap water for 20-30 minutes. The addition of a few crystals of sodium thiosulphate to the final rinse water helped to diminish strip discolouration.

MOLECULAR STRUCTURES OF ORGANIC SOLVENTS

Solvent	Structure
ethylene glycol	$HO-CH_2-CH_2-OH$
2-methoxy-ethanol	$HO-CH_2-CH_2-OCH_3$
2-ethoxy-ethanol	$HO-CH_2-CH_2-OC_2H_5$
2-butoxy-ethanol	$HO-CH_2-CH_2-OC_4H_9$
propane-1, 2-diol	$HO-CH_2-CH(OH)-CH_3$
propane-1, 3-diol	$HO-CH_2-CH_2-CH_2-OH$
butane-1, 4-diol	$HO-CH_2-CH_2-CH_2-CH_2-OH$
butane-1, 3-diol	$HO-CH_2-CH_2-CH(OH)-CH_3$
butane-2, 3-diol	$CH_3-CH(OH)-CH(OH)-CH_3$
pentane-1, 5-diol	$HO-CH_2-CH_2-CH_2-CH_2-CH_2-OH$
hexane-1, 6-diol	$HO-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-OH$
diethylene glycol (digol)	$HO-CH_2-CH_2-O-CH_2-CH_2-OH$
methyl digol	$HO-CH_2-CH_2-O-CH_2-CH_2-OCH_3$
ethyl digol	$HO-CH_2-CH_2-O-CH_2-CH_2-OC_2H_5$
glycerol	$HO-CH_2-CH(OH)-CH_2-OH$
methyl alcohol	CH_3-OH
n-propyl alcohol	$CH_3-CH_2-CH_2-OH$
n-butyl alcohol	$CH_3-CH_2-CH_2-CH_2-OH$
n-hexane	$CH_3-CH_2-CH_2-CH_2-CH_2-CH_3$

CHAPTER 3

COLLAGEN RENATURATION

THE MECHANISM OF RENATURATION

It is well established that the pyrrolidine rings present in the polypeptide chains of collagen are of singular importance in determining macromolecular conformational stability (Harrington, 1958; von Hippel and Harrington, 1959; Flory and Weaver, 1960; Piez, 1960; Harrington and McBride, 1966). The extent to which other factors contribute to the formation and stability of the poly-L-proline II type helix is the subject of considerable debate, however. On the basis of renaturation studies, which have shown the effects of neutral salts on mutarotation rates to be relatively independent of protein concentration, a three-stage model for the re-formation of the collagen structure from the random coil has been proposed (von Hippel and Harrington, 1959; Flory and Weaver, 1960; Harrington and von Hippel, 1961a; von Hippel and Wong, 1963b) which may be applicable to a wide range of linear polymer systems. A mechanism incorporating similar concepts has been invoked by Ross and Sturtevant (1962) to account for helix formation kinetics in the double-stranded helical complex of the synthetic polynucleotides, polyriboadenylic acid and polyribouridylic acid. The proposed process involves: (a) nucleation of the poly-L-proline II type helix in individual polypeptide chains in regions of high pyrrolidine ring content; (b) propagation of the helical arrangement through the length of the chain to include the regions of lower pyrrolidine ring content; (c) formation of the collagen triple helix through inter-chain hydrogen bonding. An important implication with respect to such a model is that perturbants that retard initial mutarotation rate must do so primarily by interfering with steps (a) or (b) since if (c) was necessary for the formation of the poly-L-proline II type helix a dependence on protein concentration would be expected.

Recent work has raised serious doubts concerning the three-stage mechanism described above. Beier and Engel (1966) and

Kühn et al. (1965) have shown that the extent to which the truly native collagen structure, which they term re-formed collagen, is regenerated during renaturation is highly temperature-dependent and at the higher renaturation temperatures mutarotation rate becomes markedly concentration-dependent. In addition, Engel (1962) and Beier and Engel (1966) have shown that very little renaturation to the authentic triple helical structure takes place when renaturation is carried out at low temperature (± 4° C), and the greater proportion of structures formed exhibit a low degree of asymmetry, in direct contrast to the highly asymmetrical shape of the native tropocollagen macromolecule. There is evidence to suggest that the small amount, about 5 to 10%, of real re-formation of the native state that does take place is due to the presence of a γ-component in which the three polypeptide chains are covalently linked (Altgelt et al., 1961). The α and β-components appear to assume non-native stabilization states that do not represent re-establishment of the triple helical arrangement.

Drake and Veis (1964) have proposed that individual polypeptide chains might double or triple back upon themselves, thereby serving to stabilize the poly-L-proline II type conformation, and accounting for the apparent concentration-independence of the renaturation process at low temperature. Thus it appears that the concentration-independence shown in renaturation studies carried out at low temperatures may be due to intrachain aggregation, and this process may be necessary for the formation of the poly-L-proline II type helix in collagen, under those circumstances where the potential for interchain interactions is minimized.

In the present work renaturation was carried out at 15°C in most cases and, as will be shown below, the process is independent of concentration only up to about 1 mg/ml. In contrast, at renaturation temperatures in the region of 4°C, it has been shown (von Hippel and

Harrington, 1959; Flory and Weaver, 1960; Harrington and von Hippel, 1961b) that the mutarotation process is independent of concentration up to 2-3 mg of protein per ml. This is in keeping with the findings of Beier and Engel (1966) and, as they propose, casts considerable doubt on the existence of a single-chain intermediate as required by the three-stage process. Thus it seems possible that inter-chain or inter-segment association may be essential to the stabilization of the poly-L-proline II type helix in collagen. The behaviour of the stable single polypeptide helix of the synthetic polymer poly-L-proline itself (Harrington and Sela, 1958; Steinberg et al., 1960; Kurtz and Harrington, 1966; Schleich and von Hippel, 1969; Engel and Schwarz, 1970; Mattice and Mandelkern, 1970) should not be directly equated with that of collagen since poly-L-proline does not form inter- or intra-chain hydrogen bonds (Engel, 1967). Therefore, although helical stability has been shown to be a function of pyrrolidine ring content in a number of collagens (von Hippel and Wong, 1963a; Harrington and Rao, 1967; Privalov, 1968), it appears tenuous to assume that the formation of poly-L-proline II type helices in collagen must necessarily be an independent process preceding inter-chain hydrogen bond formation, as required by the three-stage model.

RESULTS

CONCENTRATION DEPENDENCE

In view of the importance of concentration effects, with respect to the interpretation of experimental findings, an investigation of the influence of protein concentration on renaturation was undertaken before proceeding to systems containing added organic perturbants.

Figure 3.1 shows that renaturation at 15°C, as monitored by optical rotation recovery, is almost independent of concentration up to about 1 mg/ml. At higher concentrations, however, a definite effect becomes apparent as shown by the 1.66 and 3.33 mg/ml profiles. Examination of the 1 hour and 48 hour values emphasizes certain points (Table 3.1).

TABLE 3.1

Effect of varying protein concentration on collagen renaturation at 15°C: optical rotation recovery

Protein Concentration	$-[\alpha]_{365}^{15^\circ}$ at 1 Hour	$-[\alpha]_{365}^{15^\circ}$ at 48 Hours
0.208 mg/ml	925	1022
0.417 mg/ml	910	1038
0.833 mg/ml	890	1052
1.66 mg/ml	935	1112
3.33 mg/ml	953	1132

There appears to be a decrease, at the 1 hour level, in the extent of optical rotation recovery as protein concentration is increased from 0.208 mg/ml to 0.833 mg/ml. Further increases in concentration reverse the apparent trend, however. In contrast, at the 48 hour level, there is no trend reversal, the final values increasing in the same order as protein concentration. This set of systems serves to demonstrate that, at 15°C and concentrations above $\pm$ 1 mg/ml, the concentration-dependence of the mutarotation process becomes significant. In all subsequent work concentrations of below 1 mg/ml were used.

The pattern emerging from an examination of the concentration-dependence of viscosity regain is shown in Figure 3.2 and Table 3.2.

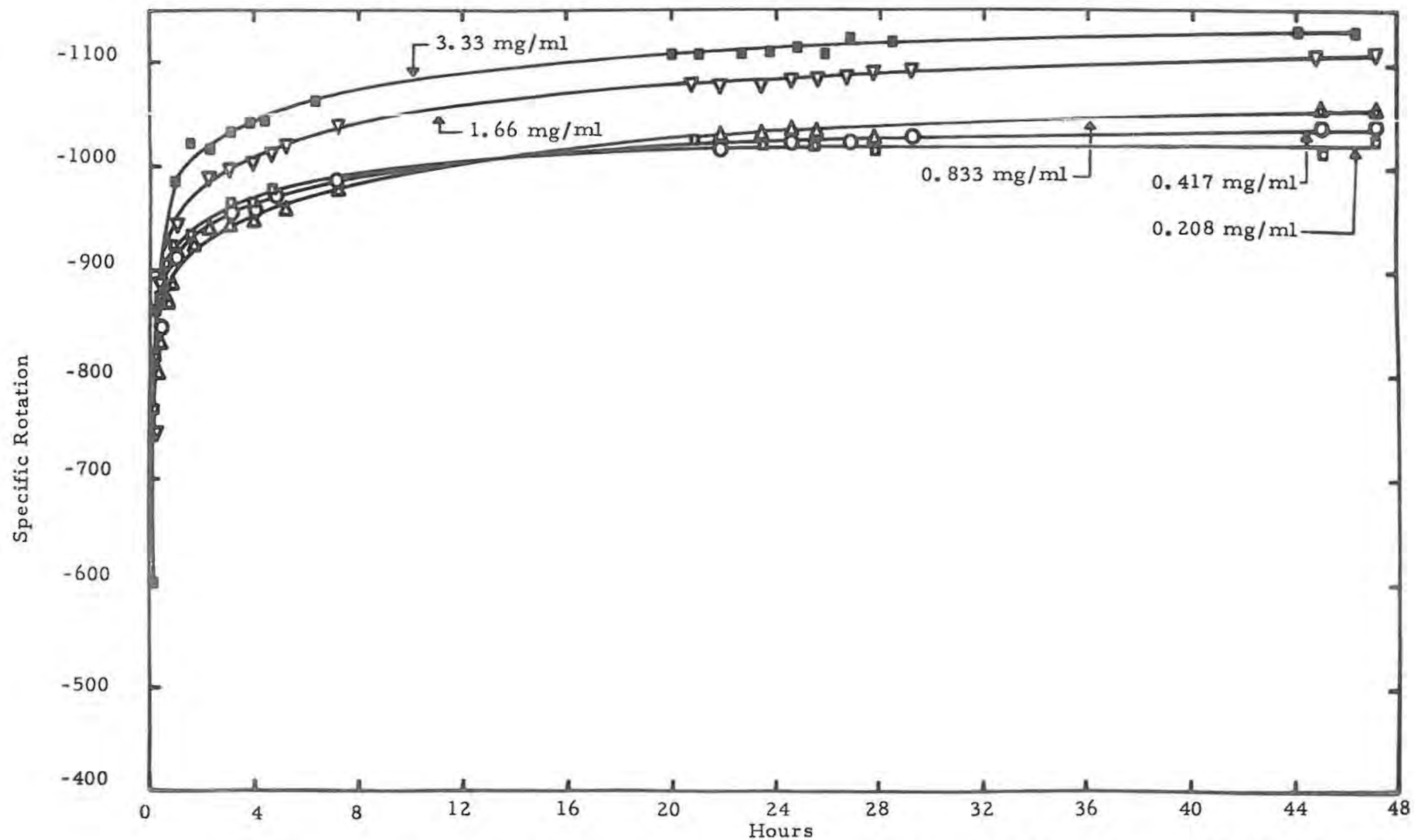

Figure 3.1. Effect of collagen concentration on optical rotation recovery of heat-denatured (45°C; 15 min) acid-soluble collagen in 0.15 M potassium acetate buffer. Renaturation temperature, 15°C.

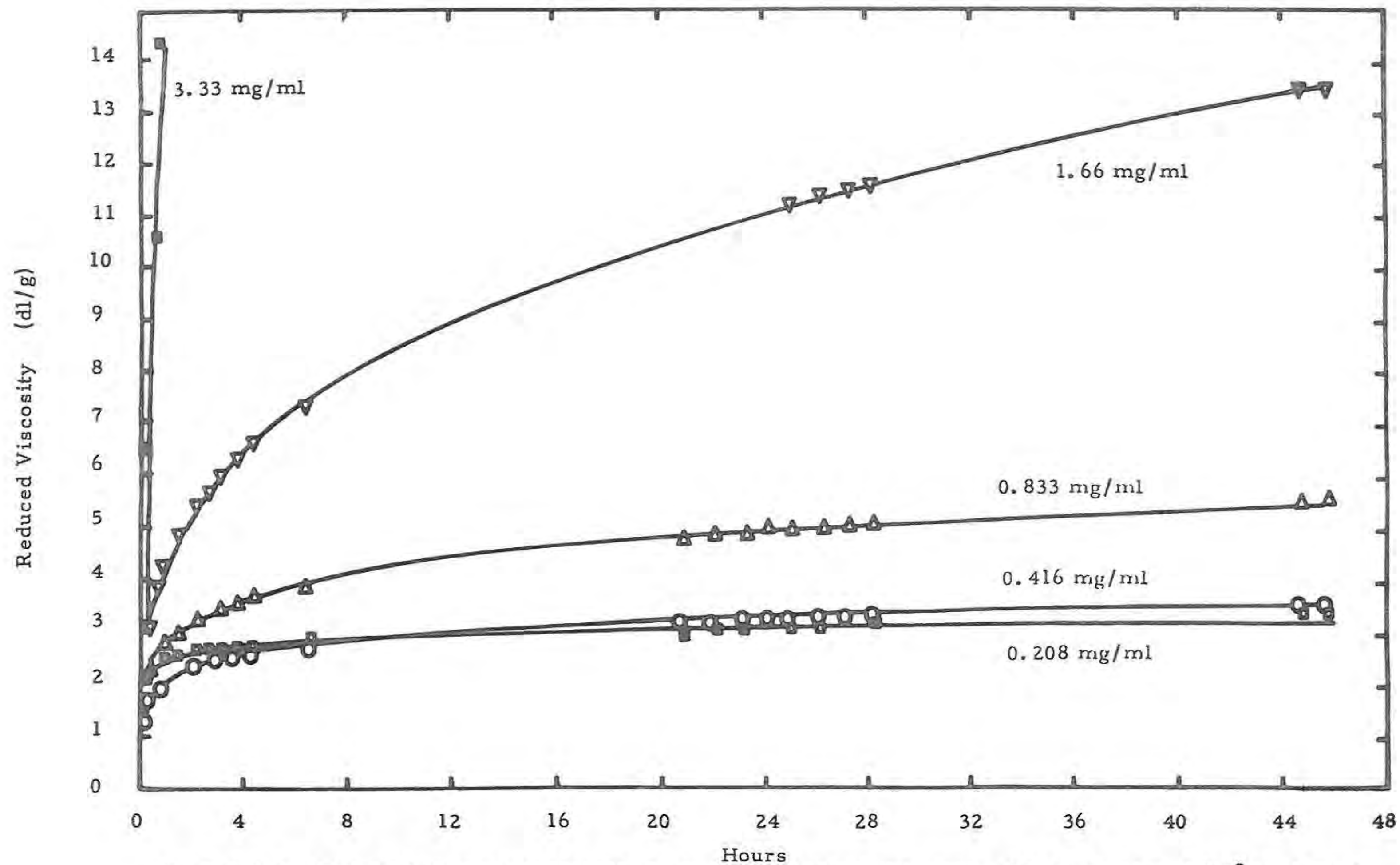

Figure 3.2. Effect of collagen concentration on reduced viscosity regain of heat-denatured (45°C; 15 min) acid-soluble collagen in 0.15 M potassium acetate buffer. Renaturation temperature, 15°C.

TABLE 3.2

Effect of varying protein concentration on collagen renaturation at $15^{o}C$: viscosity regain

Protein Concentration	η red at 1 Hour	η red at 48 Hours
0.208 mg/ml	2.37 dl/g	3.26 dl/g
0.416 mg/ml	1.95 "	3.54 "
0.833 mg/ml	2.70 "	5.28 "
1.66 mg/ml	3.97 "	13.29 "
3.33 mg/ml	$\pm$14.0 "	$\pm$260.0 "

There appears to be an anomaly with respect to the 1 hour regain values in the 0.208 and 0.416 mg/ml profiles, since the latter is lower than the former (Table 3.2). Subsequently, a crossover point appears at $\pm$ 9 hours (Figure 3.2), which is in support of the optical rotation patterns at about nine hours and low protein concentration (comparing Figures 3.1 and 3.2). Apart from this feature, it is clear that protein concentration has a decisive influence on viscosity regain at the 1 hour level, while the pseudo-equilibrium values (48 hours) show an even greater concentration-dependence. This high degree of concentration-dependence gives rise to a major experimental difficulty since viscometry results are not comparable unless obtained at identical protein concentrations.

pH DEPENDENCE

Table 3.3 shows that optical rotation values increase consistently as pH rises from 3.70 to 5.51. The 48 hour values do not precisely follow this trend since there is a slight decrease in specific rotation between pH 4.70 and 5.10. This is sufficiently small to be

due to inherent scatter, however, and the overall tendency is clearly towards increasing recovery as pH is raised.

TABLE 3.3

Effect of pH on collagen renaturation at 15°C

pH	$-[\alpha]_{365}^{15^\circ}$		η_{red} (dl/g)	
	1 hour	48 hours	1 hour	48 hours
3.70	823	1058	3.24	5.87
4.08	875	1076	3.20	5.95
4.70	940	1115	3.80	7.07
5.10	947	1108	3.59	7.00
5.51	960	1135	3.10	6.08

Collagen concentration = 0.83 mg/ml.

Reduced viscosity measurements do not show the same consistent pattern. From Table 3.3 it can be seen that at pH values between 4.70 and 5.10 there is not a great deal of variation at either 1 hour or 48 hours. Above and below these median values, however, there is a considerable fall in viscosity. This variation between trends suggests a differential effect of pH changes on separate aspects of renaturation. The formation of poly-L-proline II type helices appears to be relatively insensitive to pH adjustments, whereas the regeneration of the native, rodlike structures of re-formed collagen is considerably influenced by environmental pH; the former being revealed by optical rotation and the latter by viscosity measurements. It is suggested that in the latter case simple electrostatic (ion-pair) effects may be important in establishing suitable inter-chain registration and that raising or lowering pH tends to mask these interactions, thereby inhibiting the re-formation of the authentic

triple-stranded collagen fold. This suggestion is in direct contrast to the conclusion of Rauterberg and Kühn (1968) that electrostatic forces play no part in directing the alignment of the polypeptide chains.

The addition of organic solvents to the protein-buffer systems caused a slight pH increase in most cases. In general, variation did not exceed half a pH unit, ranging from $\pm$ 4.7 in the pure buffer to a maximum of $\pm$ 5.2 at high perturbant concentrations. Thus the overall effect on mutarotation introduced by pH fluctuations was not considered to be significant in relation to the inherent error of the method. As shown above, viscosity regain shows more substantial variation in response to pH adjustments than does mutarotation. Nevertheless, in the primary area of interest (pH 4.7 to $\pm$ 5.2) there seems to be little disturbance of regain potential.

RENATURATION IN THE PRESENCE OF PERTURBANTS

The main body of the renaturation work consisted of an investigation of the effects of the presence of certain diols and related compounds on the course of recovery. These solvents proved to be particularly useful due to the complete water-miscibility of a number of homologues and derivatives. Typical data profiles for systems renaturing in the presence of varying concentrations of perturbant are shown in Figures 3.3 and 3.4. Profiles of this type were constructed for all the perturbants studied in the renaturation work. As is evident from the figures, the production of a smooth profile necessitates the recording of a large number of data points over an extended period of time. In the discussion that follows the most significant aspects of the renaturation profiles are collated in tabular form; the individual profiles are not shown.

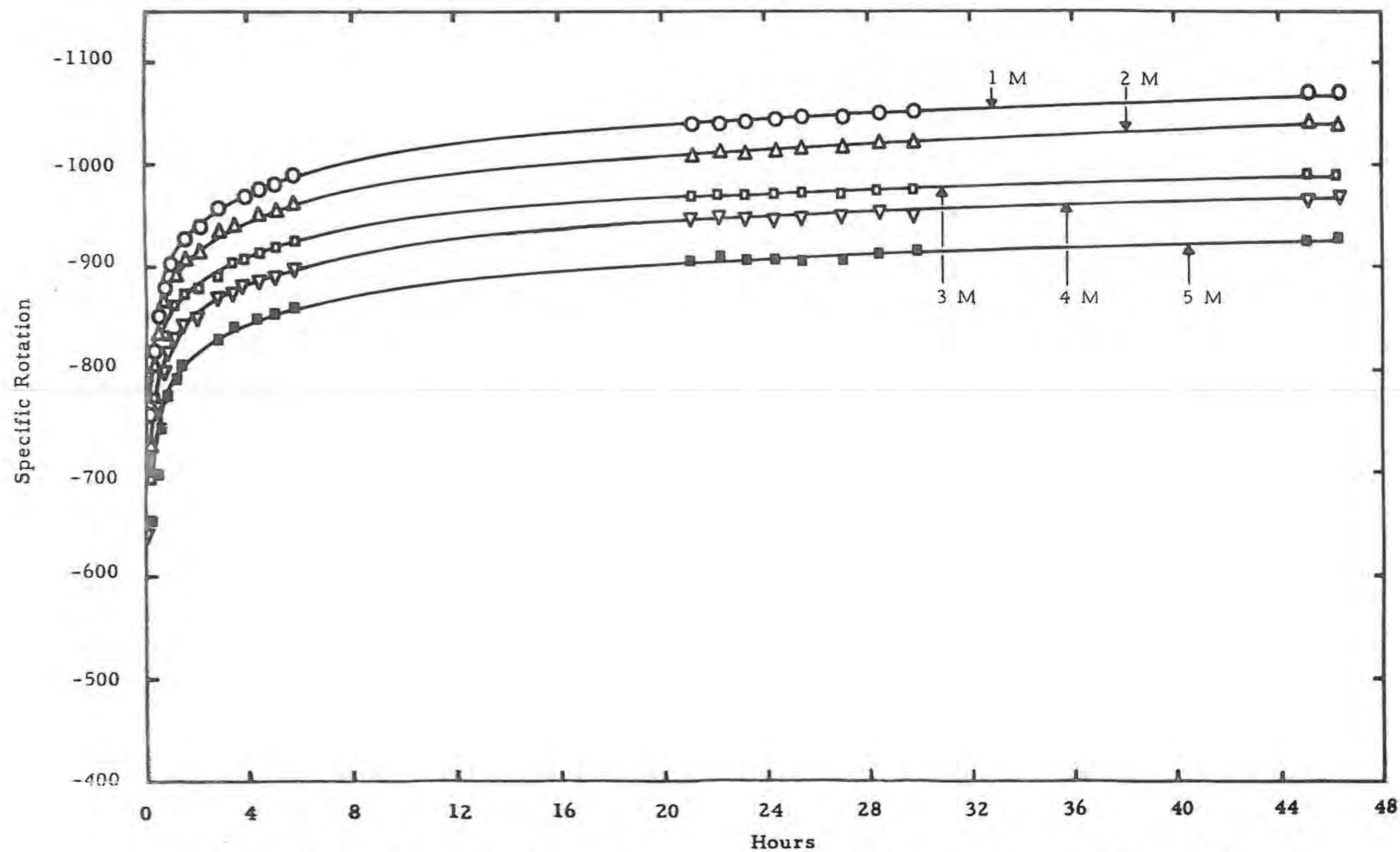

Figure 3.3. Effect of propane-1, 2-diol concentration on optical rotation recovery of heat-denatured (45°C; 15 min) acid-soluble collagen (0.84 mg/ml in 0.15 M potassium acetate buffer). Renaturation temperature, 15°C.

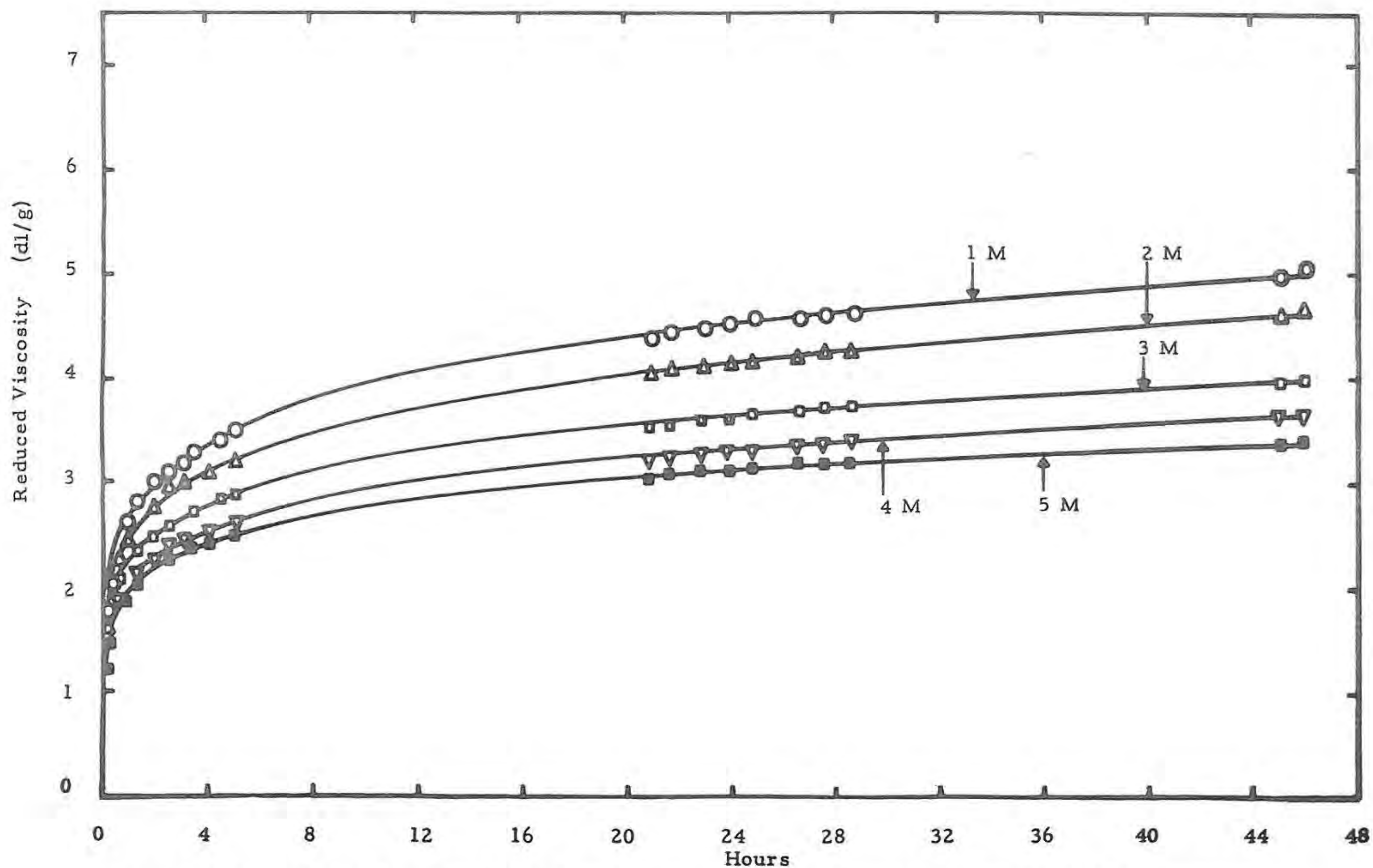

Figure 3. 4. Effect of propane-1, 2-diol concentration on reduced viscosity regain of heat-denatured (45°C; 15 min) acid-soluble collagen (0. 84 mg/ml in 0. 15 M potassium acetate buffer). Renaturation temperature, 15°C.

DIOLS AND GLYCEROL: The effects of a series of related diols and glycerol are shown in Tables 3.4 and 3.5. Ethylene glycol exhibits low molar effectiveness with respect to retardation of mutarotation. There appears to be a slight enhancement of renaturation at a concentration of 1.5 molar. The reduced viscosity measurements do not confirm this suggested enhancement at 1.5 M, the latter parameter indicating slightly increased renaturation at 0.5 M but reduction of regain at 1.5 M, however, such variations are too small to be regarded as significant. In most of the later work optical rotation was found to be the most satisfactory parameter from which to assess the lyotropic effectiveness of the perturbants studied, viscosity readings serving largely to support the rotation findings. Hence, in those cases where the viscosity data do not usefully supplement the information obtainable from the specific rotation readings, the former are omitted.

The replacement of a hydrogen atom on one of the methylene groups of ethylene glycol by a methyl group, to yield propane-1,2-diol, produces a marked increase in lyotropic effectiveness, which becomes apparent at concentrations of 3 molar and above. The isomer, propane-1,3-diol, does not retard renaturation to the same extent, however. Thus the sterically more accessible methyl group of the 1,2-isomer appears to exert a significant influence on activity. The difference in effect of isomers is less clearly demonstrated by the viscosity measurements, becoming evident only at higher molarities.

Introduction of a third hydroxyl group to give glycerol reduces apparent lyotropic activity almost to the level of ethylene glycol, which has the same polar atom / carbon atom ratio. The significance of this ratio in relation to perturbant effects is discussed in detail in Chapter 7.

TABLE 3.4

Effect of diols and glycerol on optical rotation recovery of collagen renaturing at 15°C

Solvent	Molarity	$-[\alpha]_{365}^{15^\circ}$	
		1 hour	48 hours
Control	-	875	1051
Ethylene glycol	0.5	894	1043
	1.5	916	1058
	3.0	901	1033
	5.0	887	1015
Propane-1,2-diol	1.0	895	1068
	2.0	882	1040
	3.0	857	990
	4.0	823	968
	5.0	777	927
Propane-1,3-diol	1.0	892	1056
	2.0	900	1050
	3.0	879	1000
	4.0	855	990
	5.0	830	975
Glycerol	1.0	921	1088
	2.0	911	1052
	3.0	896	1012
	4.0	887	1009
	5.0	854	977

Collagen concentration = 0.84 mg/ml.

TABLE 3.5

Effect of diols on viscosity regain of collagen renaturing at 15°C

Solvent	Molarity	η red (dl/g)	
		1 hour	48 hours
Control	-	2.79	5.50
Ethylene glycol	0.5	2.85	5.51
	1.5	2.76	5.10
	3.0	2.52	4.38
	5.0	2.50	4.08
Propane-1,2-diol	1.0	2.62	5.02
	2.0	2.49	4.66
	3.0	2.25	4.00
	4.0	2.04	3.68
	5.0	1.93	3.42
Propane-1,3-diol	1.0	2.55	4.82
	2.0	2.54	4.58
	3.0	2.48	4.14
	4.0	2.38	4.04
	5.0	2.36	3.58

Collagen concentration = 0.84 mg/ml.

The relative lyotropic effectiveness, with respect to retardation of rotation recovery, of the four perturbants discussed above is shown in Figure 3.5.

The next higher homologue in the series ethylene glycol, propane-1,3-diol is butane-1,4-diol; the relative effects of these are shown in Figure 3.6. At low molarities separation of trend lines is not sufficient to show the activity sequence. Above 4 molar, however, there is a clear gradation of lyotropic power corresponding to increasing carbon chain length and decreasing polar atom to carbon atom ratio.

An interesting comparison may be made between diethylene glycol and butane-1,4-diol, from which the former differs by the presence of an oxygen atom in the centre of the chain. Tables 3.6 and 3.7 show that diethylene glycol has a smaller effect on optical rotation recovery than does butane-1,4-diol whereas it shows a greater tendency to retard viscosity regain than does butane-1,4-diol. This inconsistency might be due to the symmetrical ether structure of diethylene glycol. None of the other perturbants examined exhibit this degree of symmetry; some do contain ether linkages but the carbon atoms are not symmetrically disposed about a central oxygen as in diethylene glycol. It seems possible that the symmetrical diethylene glycol molecules could exert a laminating effect on neighbouring molecules, thus causing a reduction in shear which results in a decrease in apparent viscosity.

MONOALKYL DERIVATIVES OF ETHYLENE GLYCOL: The effects on renaturation are shown in Tables 3.8 and 3.9. A much higher order of effectiveness is evident than with the parent diols themselves, despite the fact that the alkyl derivatives have half the number of potential donor hydrogen-bonding groups. The relative activities are summarized in Figure 3.7 which shows renaturation to be increasingly retarded as hydrocarbon content of perturbant molecules increases.

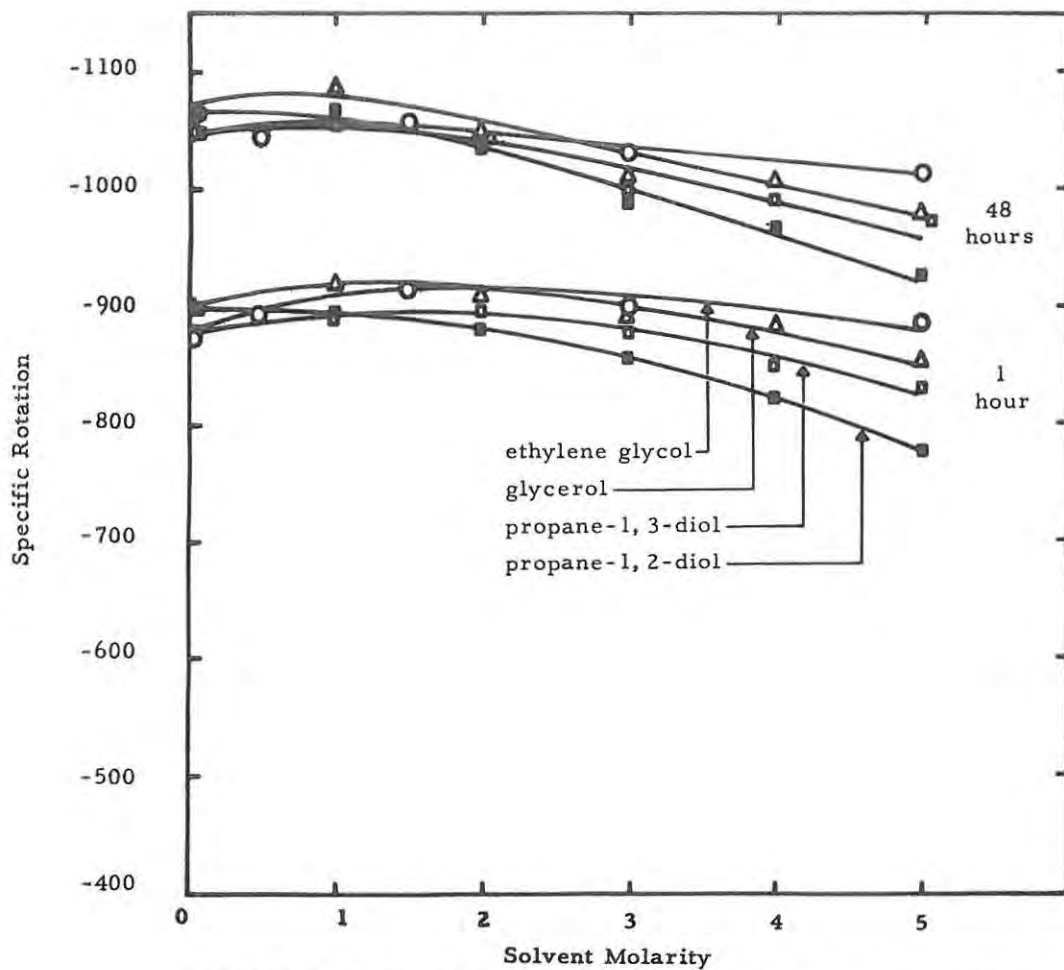

Figure 3. 5. Comparison of effects of ethylene glycol, glycerol and isomeric propanediols on 1 hour and 48 hour optical rotation recovery values of heat-denatured (45°C; 15 min) acid-soluble collagen in 0. 15 M potassium acetate buffer. Renaturation temperature, 15°C.

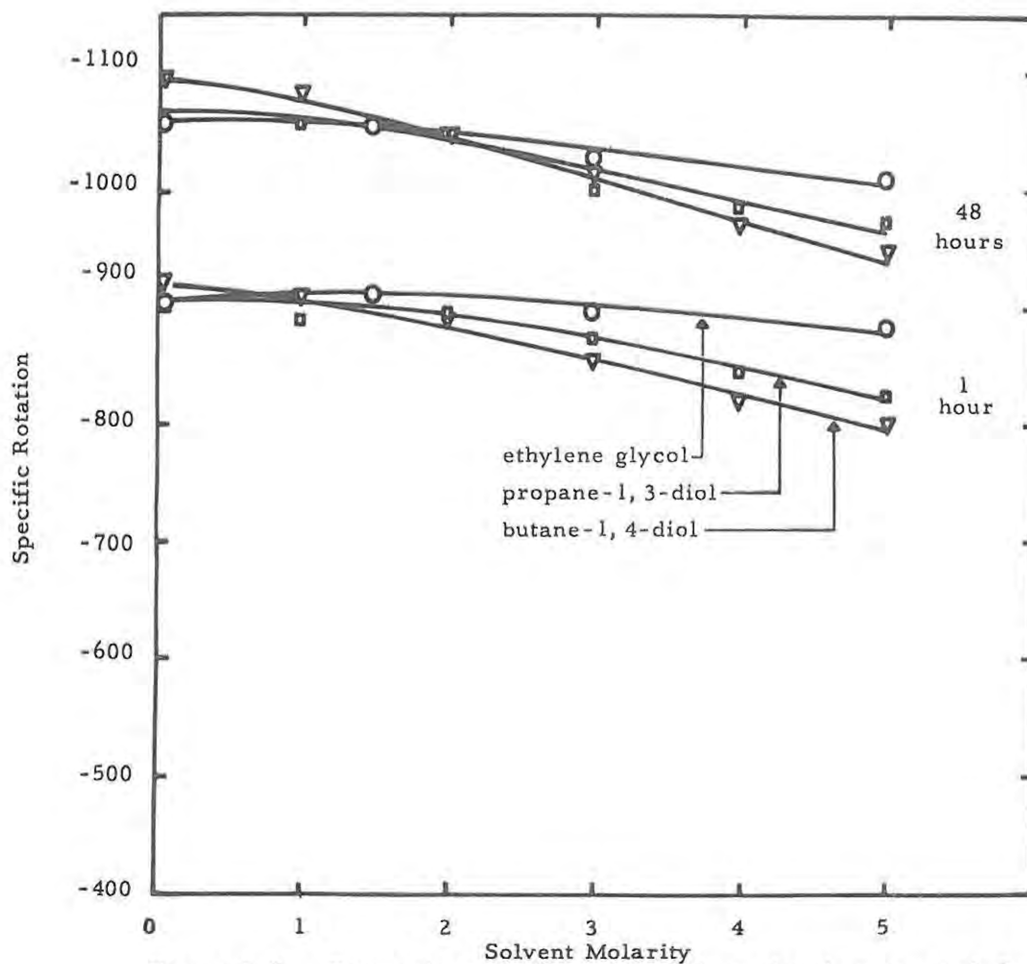

Figure 3. 6. Comparison of effects of ethylene glycol, propane-1, 3-diol and butane-1, 4-diol on 1 hour and 48 hour optical rotation recovery values of heat-denatured (45°C; 15 min) acid-soluble collagen in 0.15 M potassium acetate buffer. Renaturation temperature, 15°C.

TABLE 3.6

Effect of butane-1,4-diol and diethylene glycol on optical rotation recovery of collagen renaturing at 15°C

Solvent	Molarity	$-[\alpha]_{365}^{15^\circ}$	
		1 hour	48 hours
Control	-	938	1112
Butane-1,4-diol	1.0	913	1086
	2.0	893	1048
	3.0	858	1019
	4.0	823	974
	5.0	804	952
Diethylene glycol	1.0	950	1102
	2.0	940	1074
	3.0	900	1024
	4.0	868	990

Collagen concentration = 0.86 mg/ml.

TABLE 3.7

Effect of butane-1,4-diol and diethylene glycol on viscosity regain of collagen renaturing at 15°C

Solvent	Molarity	η_{red} (dl/g)	
		1 hour	48 hours
Control	-	3.08	6.15
Butane-1,4-diol	1.0	3.22	6.06
	2.0	3.16	5.81
	3.0	2.58	4.52
	4.0	2.35	4.08
	5.0	2.20	3.52
Diethylene glycol	1.0	3.10	5.73
	2.0	2.78	4.99
	3.0	2.57	4.40
	4.0	2.08	3.49

Collagen concentration = 0.86 mg/ml.

TABLE 3.8

Effect of monoalkyl derivatives of ethylene glycol on optical rotation recovery of collagen renaturing at 15°C

Solvent	Molarity	$-[\alpha]_{365}^{15^\circ}$	
		1 hour	48 hours
Control	-	930	1105
2-methoxy-ethanol	1.0	908	1062
	2.0	902	1012
	3.0	880	1002
	4.0	843	963
	5.0	800	907
2-ethoxy-ethanol	1.0	875	1037
	2.0	847	958
	3.0	794	917
	4.0	718	853
	5.0	650	764
2-butoxy-ethanol	1.0	787	1031

Collagen concentrations: with 2-methoxy-ethanol and 2-ethoxy-ethanol, 0.84 mg/ml; with 2-butoxy-ethanol, 0.86 mg/ml.

TABLE 3.9

Effect of monoalkyl derivatives of ethylene glycol on viscosity regain of collagen renaturing at 15°C

Solvent	Molarity	η red (dl/g)	
		1 hour	48 hours
Control	-	2.82	5.31
2-methoxy-ethanol	1.0	2.77	5.06
	2.0	2.63	4.46
	3.0	2.20	3.37
	4.0	1.62	2.32
	5.0	1.13	1.75
2-ethoxy-ethanol	1.0	2.60	4.99
	2.0	2.22	3.61
	3.0	1.35	2.01
	4.0	0.94	1.42
2-butoxy-ethanol (control)	0.0	3.26	6.40
	1.0	2.69	5.94

Collagen concentrations: with 2-methoxy-ethanol and 2-ethoxy-ethanol, 0.84 mg/ml: with 2-butoxy-ethanol, 0.86 mg/ml; the 2-butoxy-ethanol readings were displaced upwards owing to the higher protein concentration used, therefore a separate control was required.

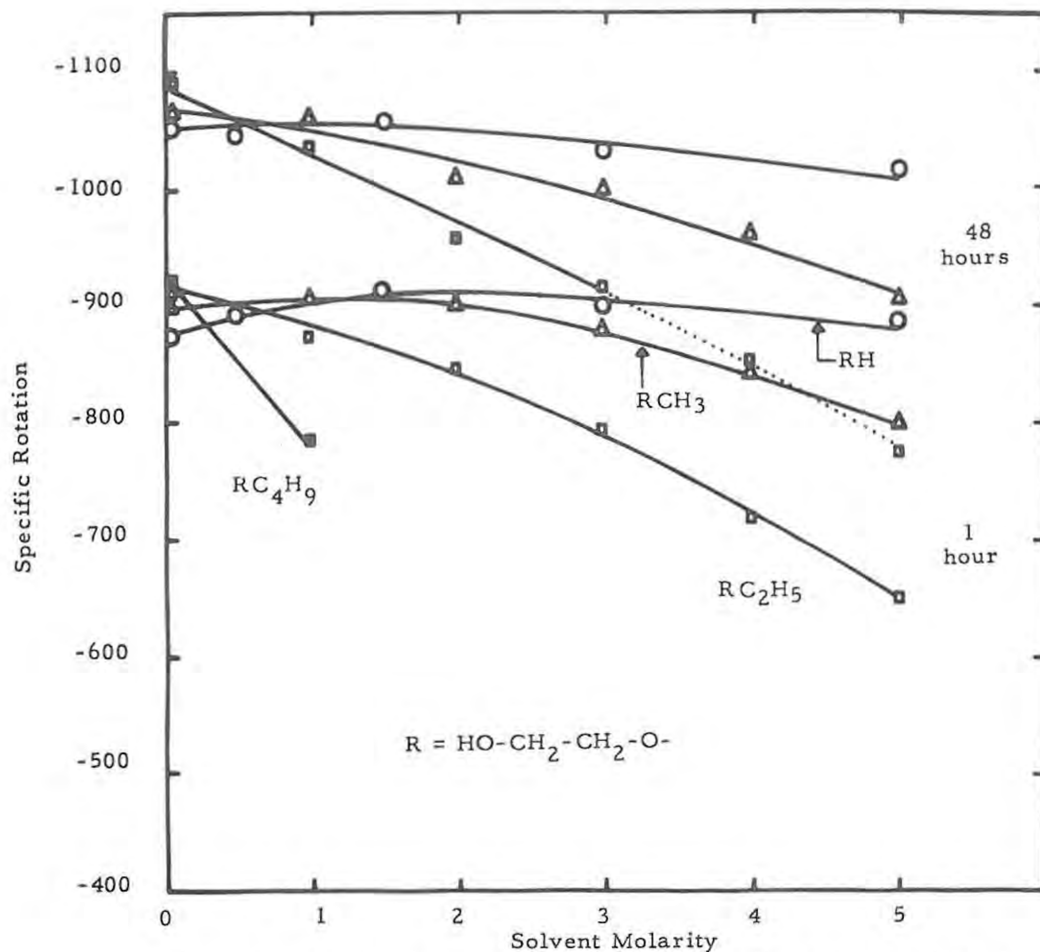

Figure 3.7. Effect of monoalkyl substitution of ethylene glycol on 1 hour and 48 hour optical rotation recovery values of heat-denatured (45°C; 15 min) acid-soluble collagen in 0.15 M potassium acetate buffer. Renaturation temperature, 15°C.

DISCUSSION

In general, the results outlined above indicate increased retardation of the renaturation process as hydrocarbon content of solvent molecules is increased. A certain degree of stereoselectivity is also apparent as evidenced by the greater lyotropic activity of propane-1, 2-diol as compared with its 1, 3-isomer.

An interesting comparison may be made between butane-1, 4-diol and 2-ethoxy-ethanol (Figure 3. 8) which show a marked difference in lyotropic effectiveness. The diol retards mutarotation to a considerably smaller extent than does 2-ethoxy-ethanol, despite the presence of an additional potential donor hydrogen-bonding function and a longer unbroken hydrocarbon chain in the former. This may be interpreted in terms of the increased probability of hydrophobic interaction between sterically accessible segments of perturbant hydrocarbon chain and apolar residues of the protein molecule. Thus, the terminal ethyl group of 2-ethoxy-ethanol may confer a higher degree of lyotropic effectiveness on the molecule than does the centrally situated ethylene chain of the diol. An explanation in terms of a shielding mechanism, by which the apolar segment enhances the hydrogen-bonding potential of perturbant polar groups, also appears tenable. An attempt to define the mechanism of perturbant action more precisely will be made in Chapter 7, in which the most useful of the results tabulated in this and the following chapters will be discussed in detail.

In certain cases (e. g. diethylene glycol, Table 3. 6) there appeared to be an enhancement of 1 hour optical rotation recovery at low concentration, by comparison with the control value. The possible significance of this phenomenon is dealt with in the kinetic analyses in Chapters 6 and 7.

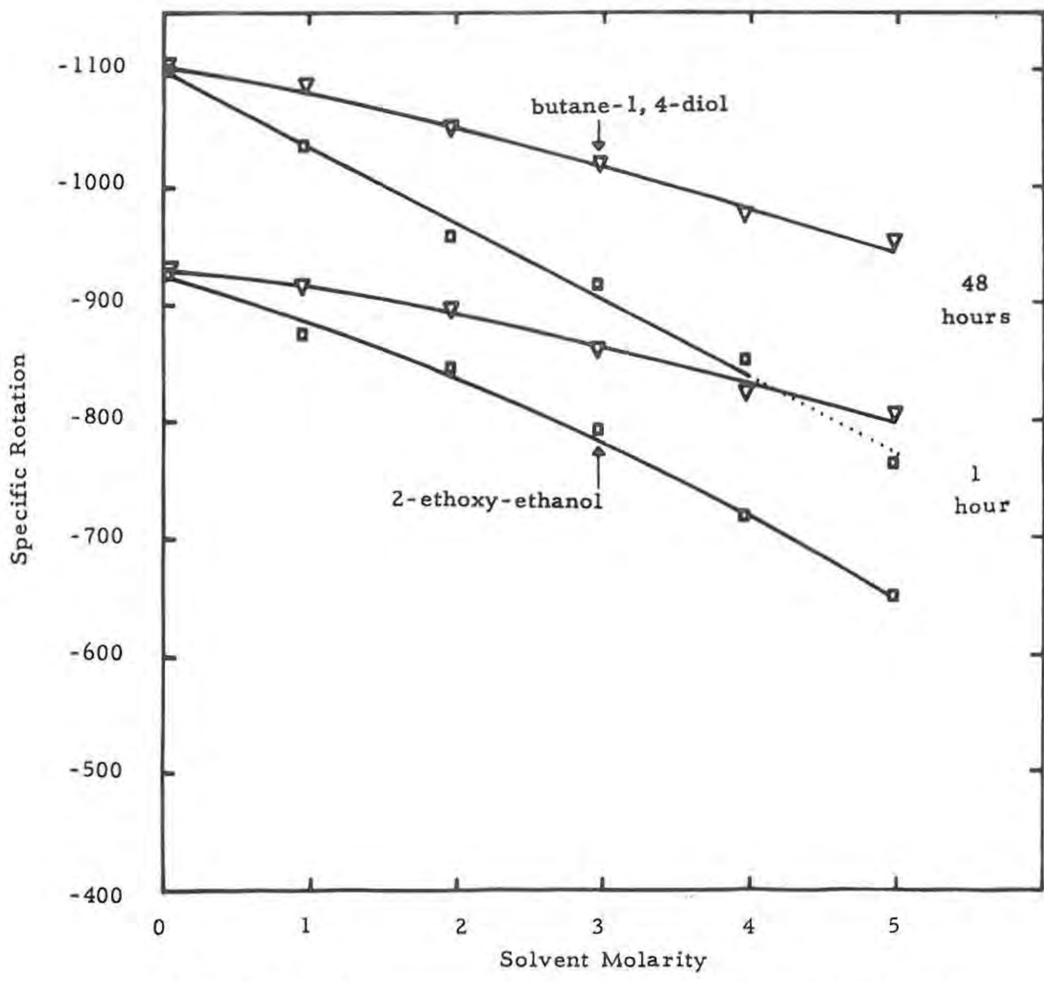

Figure 3.8. Comparison between effects of butane-1, 4-diol and 2-ethoxy-ethanol on 1 hour and 48 hour optical rotation recovery values of heat-denatured (45°C; 15 min) acid-soluble collagen in 0.15 M potassium acetate buffer. Renaturation temperature, 15°C.

CHAPTER 4

MELTING CURVES

Information obtained in respect of the thermally induced collagen → gelatin transition was in many cases less equivocal and more readily interpreted than the renaturation data. Typically divergent melting curves are shown in Figures 4. 1 and 4. 2. The former shows that the transition temperature, Tm, is progressively increased as perturbant concentration rises, in the case of propane-1,3-diol, whereas the latter shows the opposite to hold for butane-2, 3-diol. The effects of a number of related solvents were investigated by this method. In some cases the highest perturbant concentrations were inaccessible, due to precipitation or gelation of the protein; generally, however, trends were very well defined.

RESULTS

The effects of the monoalkyl derivatives of ethylene glycol on Tm are shown in Table 4. 1. Precipitation of the protein occurred at concentrations above 1 molar. It is clear, however, that alkyl substitution of the parent compound results in progressive lowering of Tm as the size of the hydrocarbon substituent is increased. Notably, the unsubstituted diol and the lowest derivative, 2-methoxy-ethanol, produce Tm values that are above that of the control, whereas the higher homologues lower Tm below the control value. Thus there appears to be a direct relationship between perturbant hydrocarbon content and the effect on Tm, despite the removal of a potential donor hydrogen-bonding function upon substitution of the parent diol. The phenomenon of increased perturbational power of solvents which have fewer potential hydrogen-bonding sites may be significant with respect to the possible nature of macromolecular stabilization processes and is discussed further in Chapter 7.

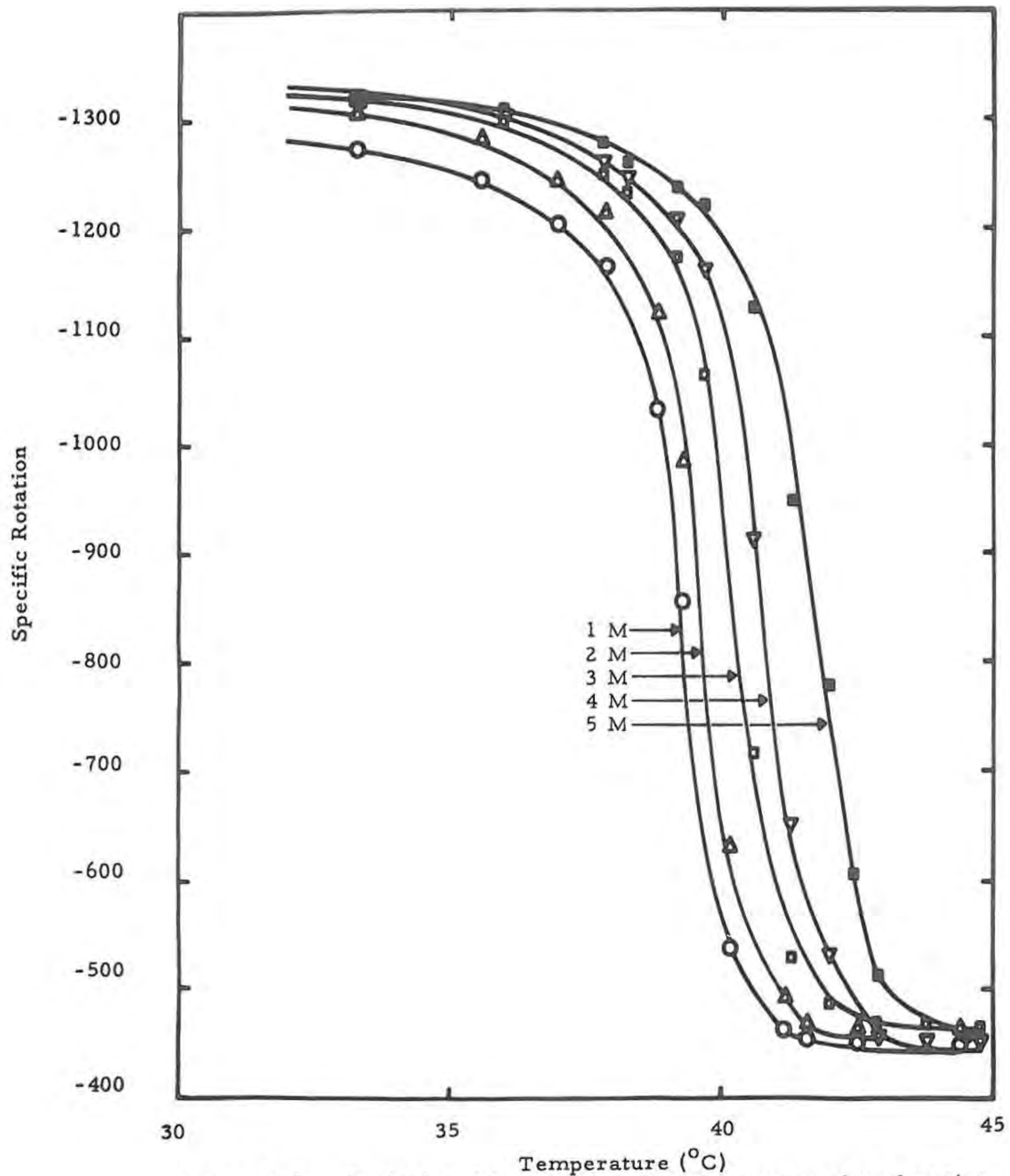

Figure 4.1. Typical melting profiles showing progressive elevation of Tm of acid-soluble collagen with increasing concentration of propane-1, 3-diol (buffer, 0.15 M potassium acetate).

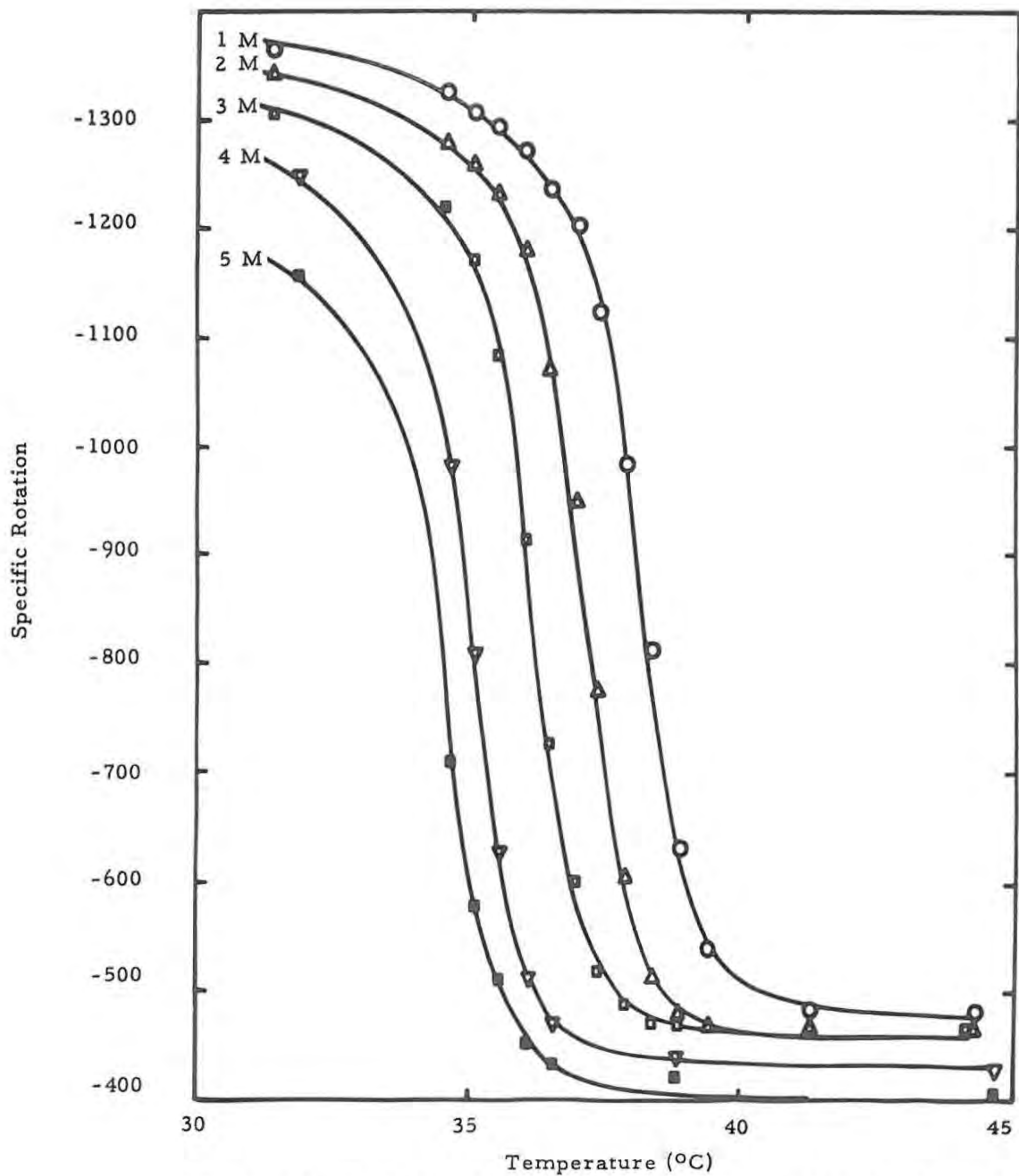

Figure 4.2. Typical melting profiles showing progressive reduction of Tm of acid-soluble collagen with increasing concentration of butane-2,3-diol (buffer, 0.15 M potassium acetate).

TABLE 4.1

Effects of monoalkyl derivatives of ethylene glycol on Tm of collagen

Solvent	Molarity	Tm°C
Ethylene glycol	1	39.25
2-methoxy-ethanol	1	38.95
Control	-	38.90
2-ethoxy-ethanol	1	38.05
2-butoxy-ethanol	1	35.30

Collagen concentration = 0.86 mg/ml.

Unlike the monoalkyl derivatives, ethylene glycol itself did not cause precipitation difficulties, and its influence on Tm could be followed to high concentration. As shown in Table 4.2, a steady increase in Tm occurs as solvent molarity is increased. Table 4.2 also shows the effects of glycerol, which has an even greater stabilizing influence on the native protein than does ethylene glycol. Both these perturbants have the same polar atom / carbon atom ratios and all the methylene groups carry polar substituents, rendering hydrophobic interactions unlikely. The difference in activity appears to be a consequence of the differing molar volumes. Since these perturbants stabilize the native state of the protein it is implied that they are, on a volume basis, weaker perturbants than water itself. Thus, the reduction in environmental polar atom concentration appears to be the dominant influence, resulting in a decrease in the extent to which the co-operative hydrogen-bonded structure of the protein is competitively perturbed by interaction with the environment.

TABLE 4.2

Effects of ethylene glycol and glycerol on Tm of collagen

Solvent	Molarity	Tm^{o} C
Control	-	38.90
Ethylene glycol	1	39.25
	2	39.65
	3	39.96
	4	40.18
	5	40.64
	6	40.97
	7	41.17
	8	41.62
	9	41.84
	10	42.21
Glycerol	1	39.86
	2	40.59
	3	41.51
	4	42.38
	5	43.26
	6	44.47
	7	45.56
	8	46.75
	9	48.22
	10	49.45

Collagen concentrations: with ethylene glycol, 0.86 mg/ml; with glycerol from 1 to 9 M, 0.43 mg/ml; 10 M, 0.25 mg/ml.

THE ISOMERIC PROPANEDIOLS

The isomeric propanediols differ considerably in perturbational activity, as shown in Table 4. 3. Propane-1, 3-diol stabilized the native protein structure whereas propane-1, 2-diol lowered Tm systematically up to a concentration of ± 7 molar, after which there was a reversal of trend.

The effects of the propanediols, glycerol and ethylene glycol are compared graphically in Figure 4. 3.

ETHYLENE GLYCOL HOMOLOGUES

The effects of the members of the homologous series extending from butane-1, 4-diol to hexane-1, 6-diol are shown in Table 4. 4. Increasing concentrations of butane-1, 4-diol produced a low-concentration minimum at $\pm$ 2 molar, after which there was a steady increase in Tm. Pentane-1, 5-diol produced even more marked destabilization, followed by a trend reversal at 3 to 4 molar. Hexane-1, 6-diol caused very sharp reduction of Tm at the accessible molarities.

The effects of all the members of the homologous diol series, ethylene glycol to hexane-1, 6-diol, are compared graphically in Figure 4. 4 which shows the general tendency towards destabilization of the protein at lower concentrations as hydrocarbon content of perturbant molecules increases. Trend reversals are apparent, especially with butane-1, 4-diol and pentane-1, 5-diol. Although high molarities were not accessible in the case of hexane-1, 6-diol, owing to the formation of a gel-like precipitate, there is a definite suggestion of a potential trend reversal.

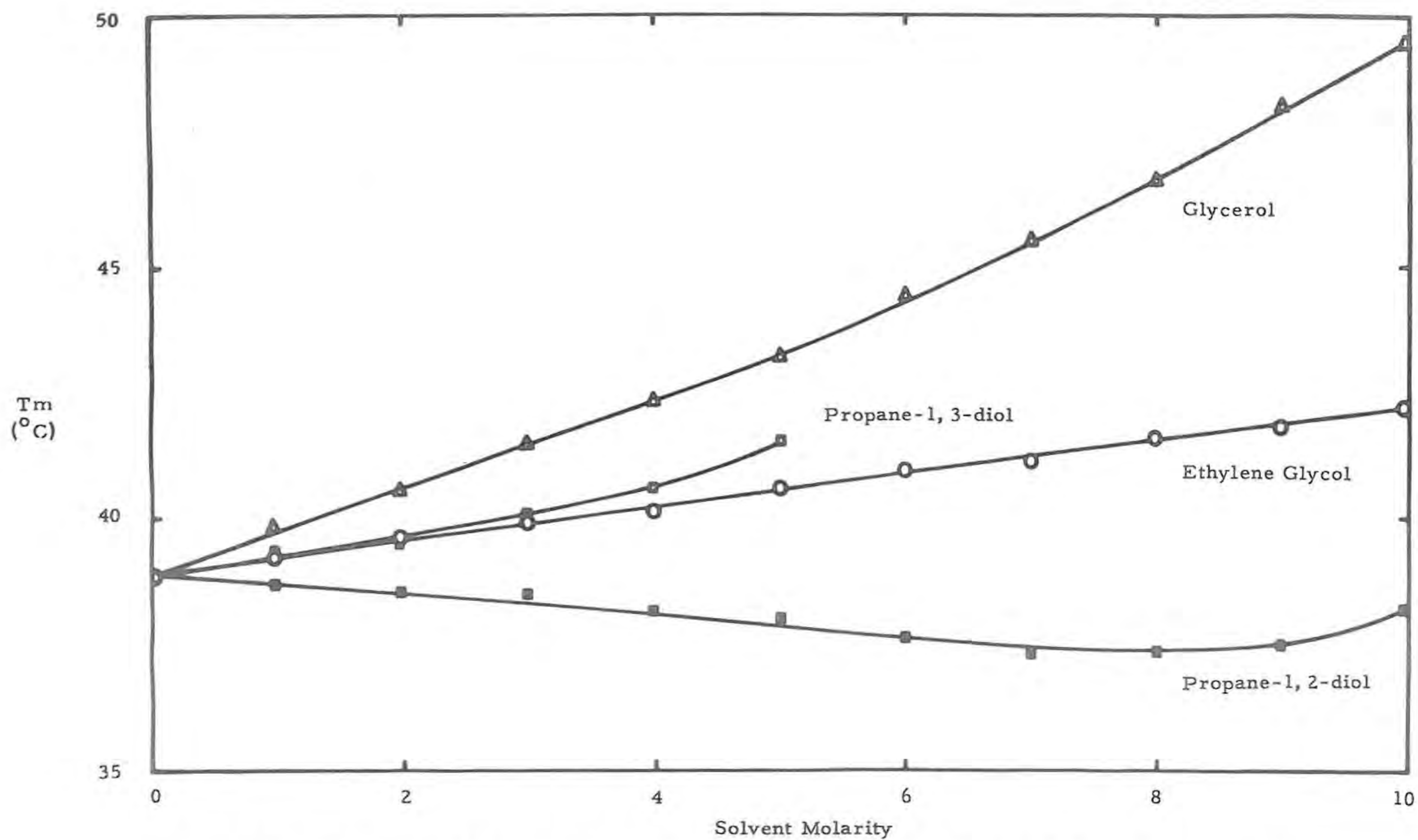

Figure 4.3. Comparison of effects of isomeric propanediols, ethylene glycol and glycerol on Tm of acid-soluble collagen in 0.15 M potassium acetate buffer.

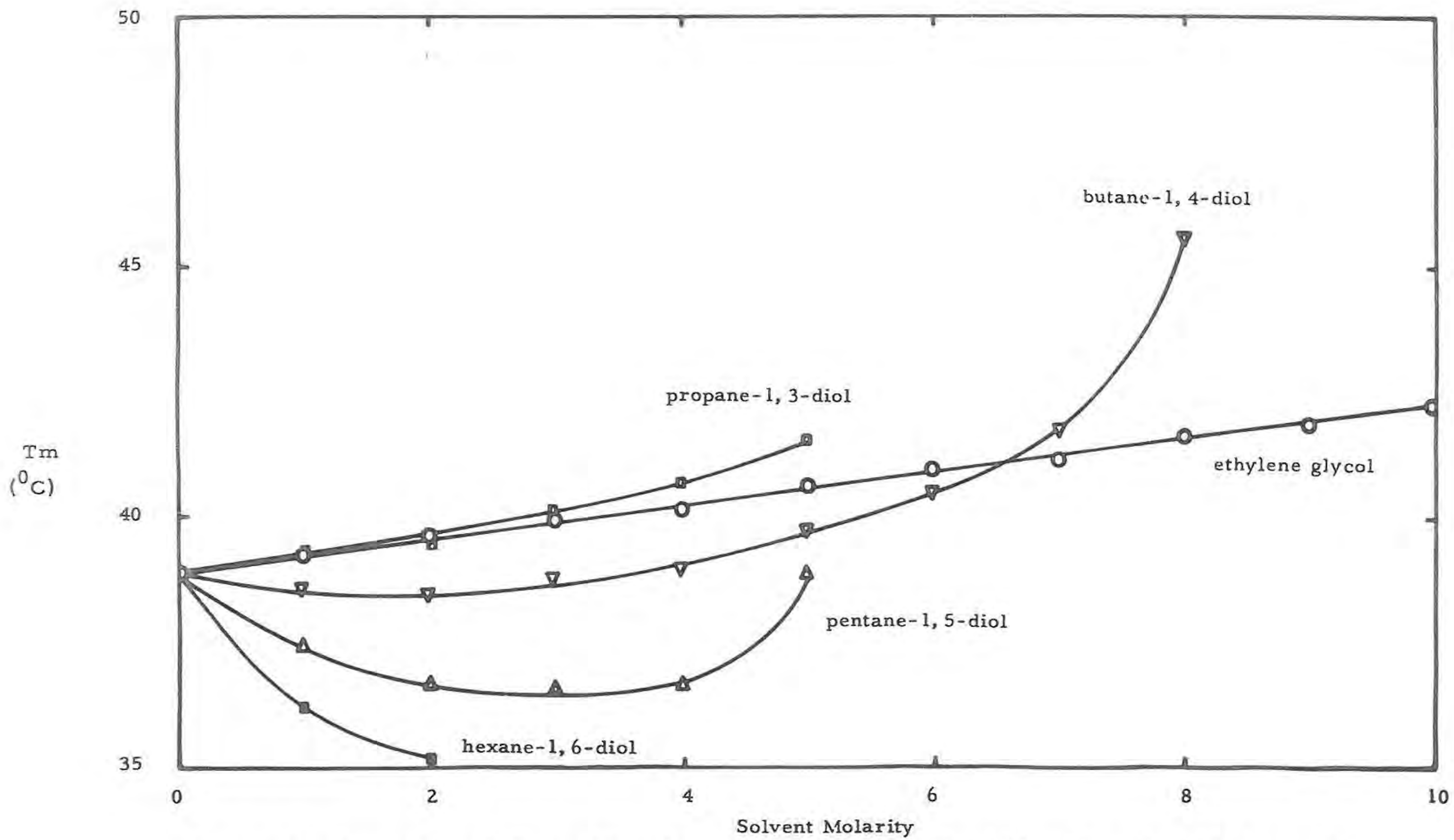

Figure 4.4. Comparison of effects of homologous diols on Tm of acid-soluble collagen in 0.15 M potassium acetate buffer.

TABLE 4.3

Effects of isomeric propanediols on Tm of collagen

Solvent	Molarity	Tm°C
Control	-	38.85
Propane-1,3-diol	1	39.28
	2	39.51
	3	40.12
	4	40.65
	5	41.55
Propane-1,2-diol	1	38.71
	2	38.56
	3	38.52
	4	38.19
	5	38.04
	6	37.62
	7	37.31
	8	37.36
	9	37.48
	10	38.21

Collagen concentrations: with propane-1,3-diol, 0.86 mg/ml; with propane-1,2-diol from 1 to 9 M, 0.43 mg/ml; 10 M, 0.25 mg/ml.

TABLE 4.4

Effects of higher ethylene glycol homologues on Tm of collagen

Solvent	Molarity	Tm°C
Control	-	38.90
Butane-1,4-diol	1	38.57
	2	38.46
	3	38.76
	4	38.99
	5	39.73
	6	40.50
	7	41.76
	8	45.59
Pentane-1,5-diol	1	37.42
	2	36.62
	3	36.59
	4	36.66
	5	±39.10
Hexane-1,6-diol	1	36.22
	2	35.20

Collagen concentrations: with butane-1,4-diol from 1 to 6 M, 0.43 mg/ml; 7 and 8 M, 0.25 mg/ml; with pentane-1,5-diol and hexane-1,6-diol, 0.43 mg/ml.

TABLE 4.5

Effects of butanediol isomers and diethylene glycol on Tm of collagen

Solvent	Molarity	Tm°C
Control	-	38.90
Butane-1,3-diol	1	38.56
	2	38.32
	3	38.25
	4	38.21
	5	38.56
Butane-2,3-diol	1	38.05
	2	37.07
	3	36.12
	4	35.04
	5	34.30
	6	34.71
Diethylene glycol	1	39.81
	2	40.39
	3	41.15
	4	41.88
	5	42.35
	6	43.19
	7	44.22

Collagen concentrations: with 5 and 6 M butane-2,3-diol, 0.25 mg/ml; all others, 0.43 mg/ml.

BUTANEDIOL ISOMERS AND DIETHYLENE GLYCOL

The effects of the isomers related to butane-1, 4-diol and of diethylene glycol are shown in Table 4. 5. Butane-1, 3-diol causes destabilization up to a concentration of 4 molar, after which Tm starts to increase. Butane-2, 3-diol destabilizes up to 5 molar, following which there is a trend reversal. Diethylene glycol differs from butane-1, 4-diol only by virtue of the centrally situated ether oxygen atom of the former. Although this provides an additional potential polar interaction site, diethylene glycol stabilizes the protein to a considerably greater extent than does butane-1, 4-diol, as shown by comparing Tables 4. 4 and 4. 5. The presence of the additional polar atom appears to be the source of this difference in activity.

The relative activities of the isomeric butanediols and diethylene glycol are compared graphically in Figure 4. 5.

DISCUSSION

The Tm studies emphasise the effect of the direction from which the collagen⟷gelatin transition is approached on the apparent activity of organic perturbants. The highly co-operative system of non-covalent forces, that stabilize the native triple helical structure, appears to be much less sensitive to perturbant action than is a renaturing system. The re-formation of the triple helix and of non-native renaturation products seems to be subject to inhibition by solvents that do not destabilize the native structures, and may in fact stabilize them. A detailed discussion of the possible significance of this observation with respect to the kinetic situation is undertaken in Chapter 6.

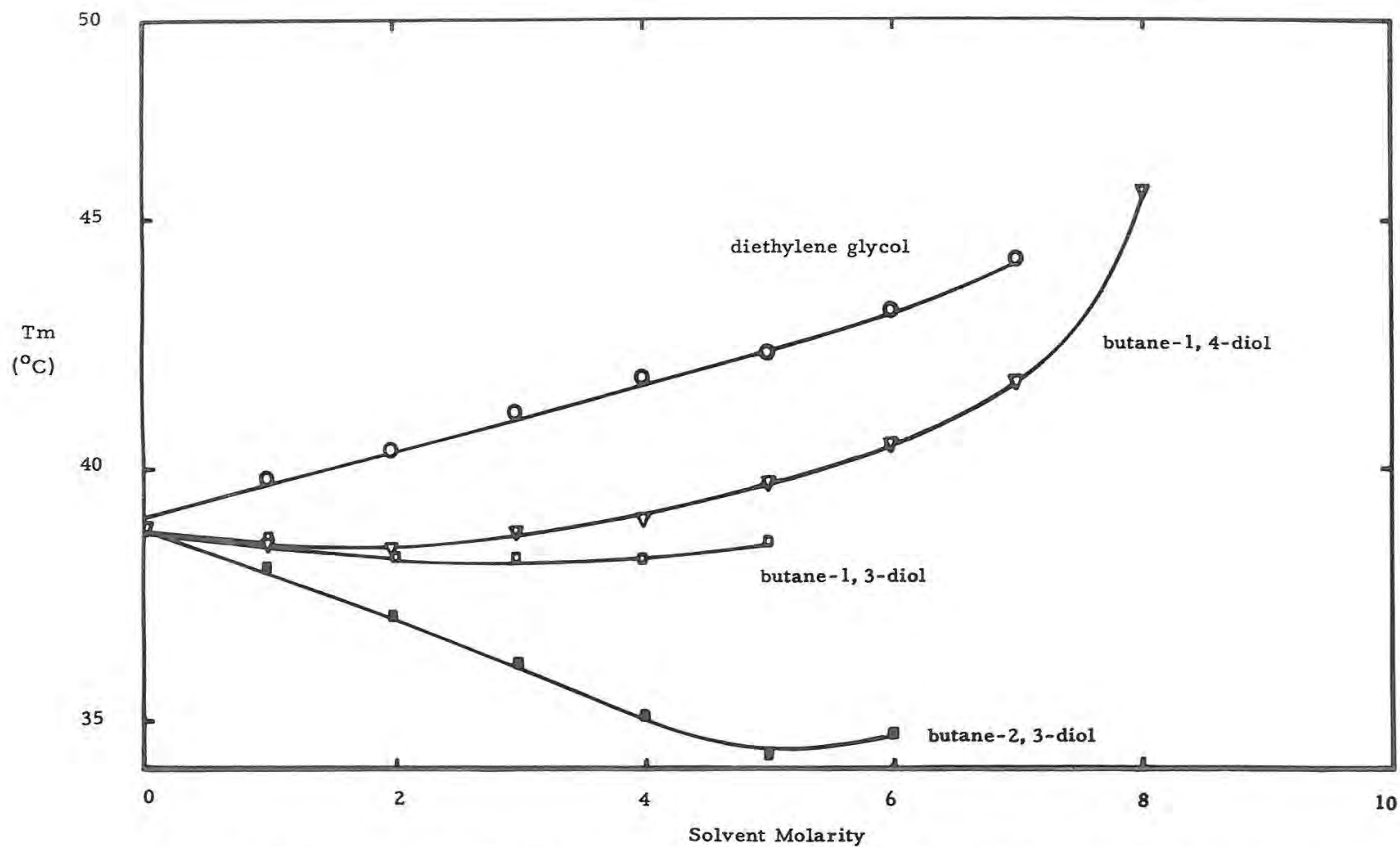

Figure 4. 5. Comparison of effects of isomeric butanediols and diethylene glycol on Tm of acid-soluble collagen in 0. 15 M potassium acetate buffer.

CHAPTER 5

PAPER CHROMATOGRAPHY

Chromatographic procedures using polyacrylamide and dextran gels to provide evidence for direct interaction between denaturing agents and amide groups have recently been investigated (Egan, 1968; Saunders and Pecsok, 1968; St. Pierre and Jencks, 1969). Studies of this nature support the concept of direct interaction between lyotropic agents and peptide or amide groups in proteins and certain model systems (Mandelkern and Stewart, 1964; Nagy and Jencks, 1965; Robinson and Jencks, 1965a, b; Russell and Cooper, 1970). It seems possible that the solvent-substrate interactions involved are of an even more general nature than is apparent from the studies cited above. The work to be described in this chapter was undertaken in an attempt to demonstrate such generality of effects for substrates as diverse as cellulose and soluble collagen. The apparent similarities and differences between the mechanisms of solvent-substrate interaction in the two systems will be discussed in Chapter 7.

All the solvents which produced interesting results in the soluble collagen systems were made use of in the paper chromatographic work. Catechin mobility patterns showed systematic variations which may be interpreted in terms of perturbant polar atom content, steric accessibility of functional groups, and changes in the dielectric properties of the medium.

RESULTS

ETHYLENE GLYCOL AND MONOALKYL DERIVATIVES

The first series of homologues examined was that consisting of ethylene glycol and its monoalkyl ethers. Figure 5.1 shows the original form of a typical mobility profile. The Rf values obtained from such profiles using ethylene glycol and its monoalkyl

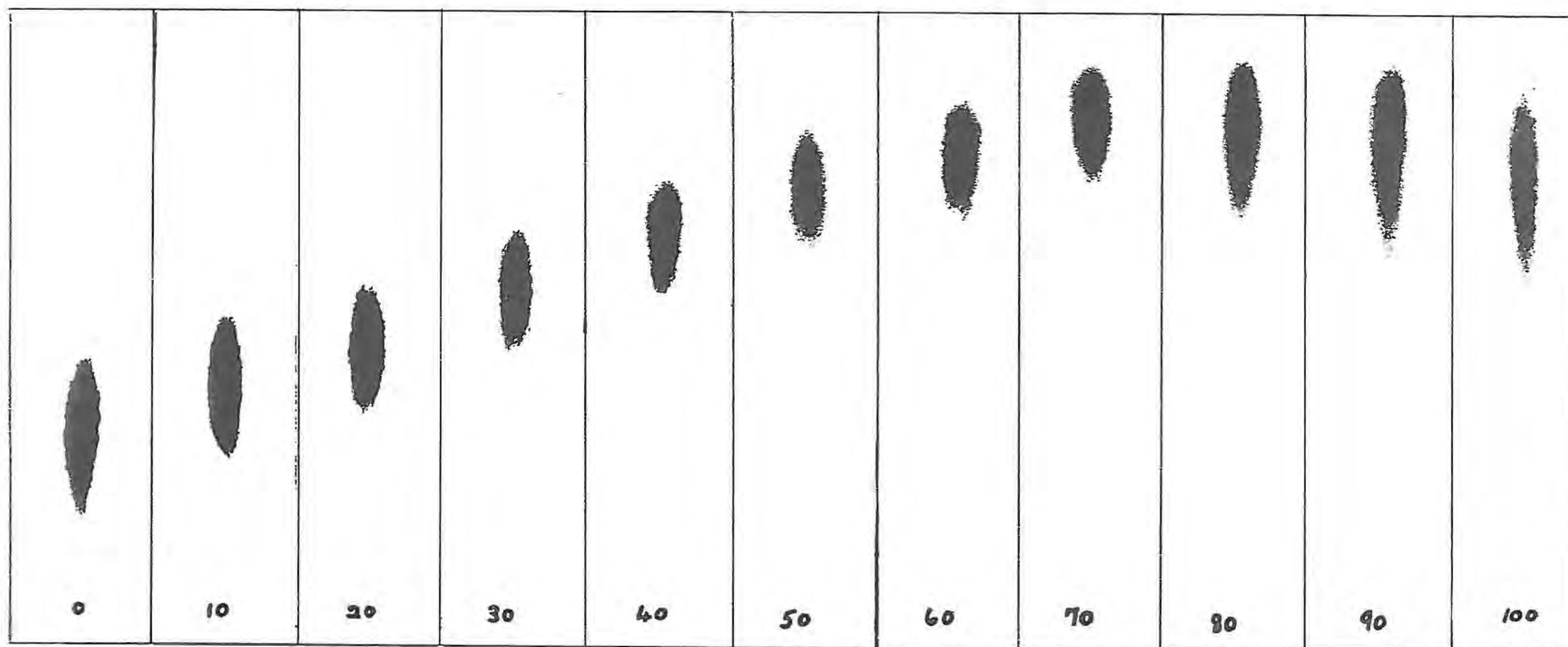

Figure 5. 1. Example of original chromatographic profile produced by catechin on paper strips as methanol/water ratio is changed. The methanol concentration (v/v) is inscribed at the base of each strip. Temperature, 21. 5°C.

derivatives are shown in Figure 5.2 plotted as functions of molarity of organic component in water. From the figure it can be seen that ethylene glycol causes a progressive increase in catechin mobility as concentration of organic component is increased, no maximum being observed at intermediate concentrations. In contrast, the alkyl derivatives produce mobility maxima at intermediate concentrations, this effect becoming more marked with increasing length of the alkyl substituent. Simultaneously, there is a sharper decline in mobility at the high-concentration extreme as perturbant hydrocarbon content rises. The peculiar double peak of the 2-butoxy-ethanol profile was confirmed in replicate experiments.

PROPANEDIOL ISOMERS

In Figure 5.3 the effects of ethylene glycol and the propanediol isomers are compared. The additonal methyl group of propane-1,2-diol and methylene group of propane-1,3-diol shift the mobility maxima to lower concentrations, and cause a sharper decline in mobility after the maxima have been passed, than does ethylene glycol itself. The 1,2-isomer appears to promote mobility to a lesser extent than does propane-1,3-diol.

THE ISOMERIC BUTANEDIOLS AND DIETHYLENE GLYCOL

The effects of the isomeric butanediols on catechin mobility are shown in Figure 5.4. These isomers all have very similar influences at low to intermediate concentrations. This is in direct contrast to their effects on Tm, as shown in Figure 4.5, where considerable activity differences are apparent, especially with butane-1,3-diol and butane-2,3-diol.

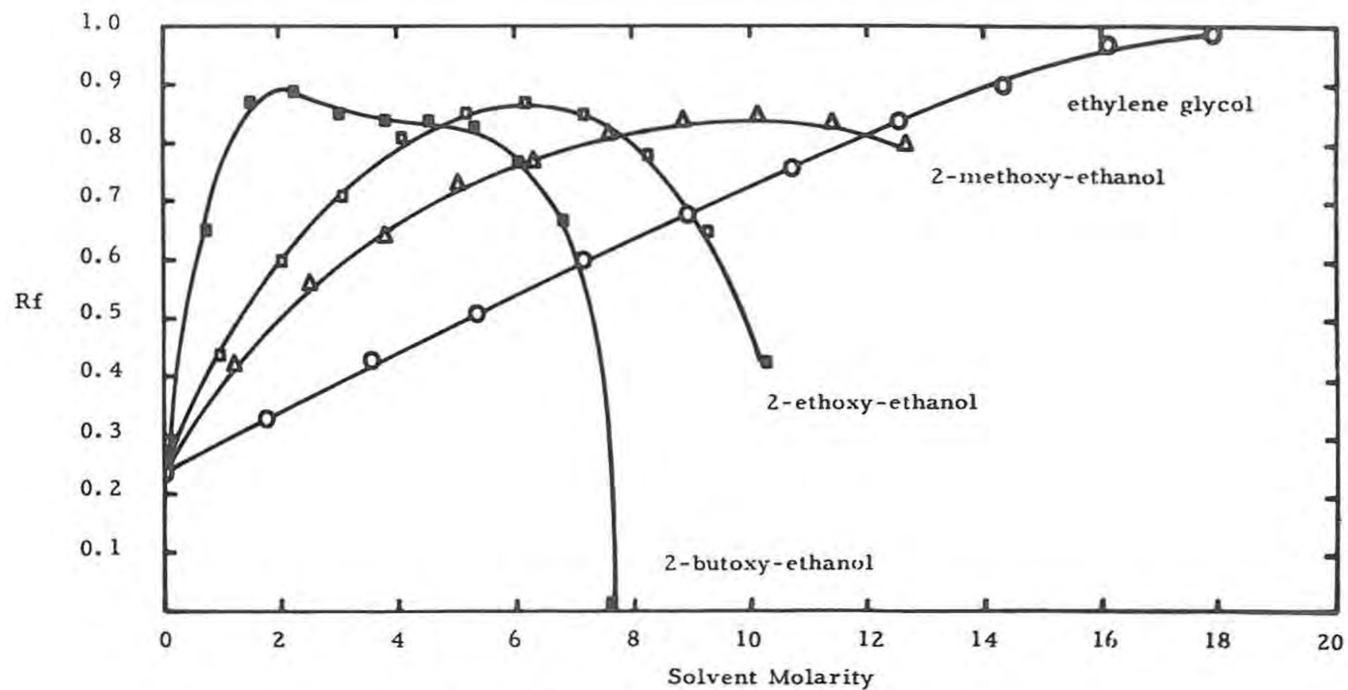

Figure 5.2. Effect of monoalkyl substitution of ethylene glycol on chromatographic mobility of catechin on cellulose in solvent/water systems. Temperature, 21.5°C.

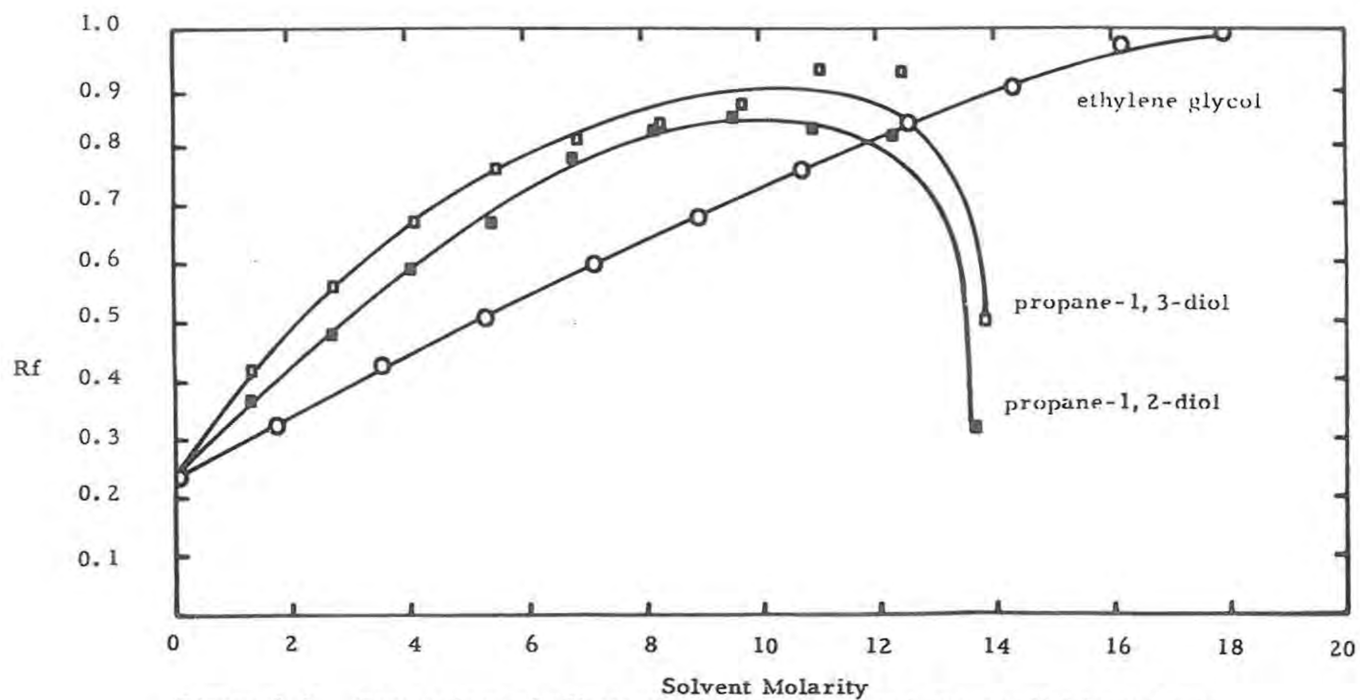

Figure 5.3. Comparison of effects of ethylene glycol and propanediol isomers on chromatographic mobility of catechin on cellulose in solvent/water systems. Temperature, 21.5°C.

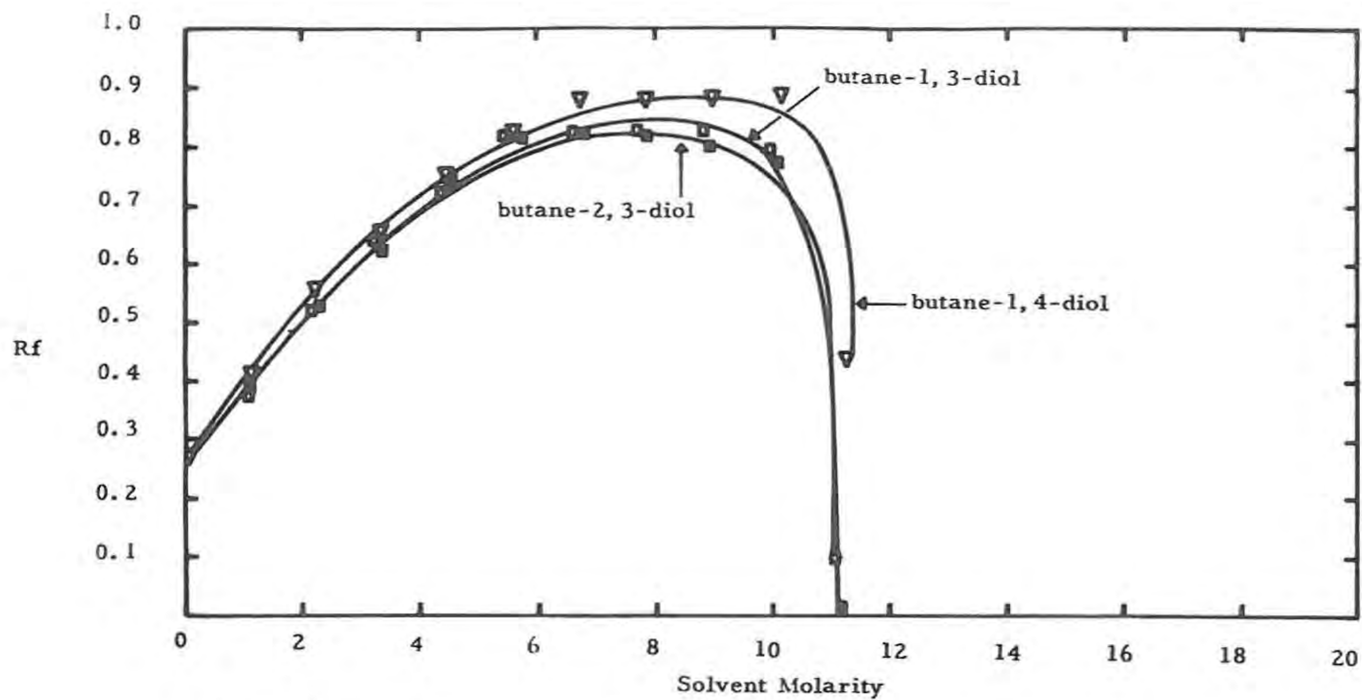

Figure 5.4. Comparison of effects of isomeric butanediols on chromatographic mobility of catechin on cellulose in solvent/water systems. Temperature, 21.5°C.

Figure 5.5 shows that diethylene glycol promotes catechin mobility to a greater extent than does butane-1,4-diol. In addition there is no mobility decline at high concentrations of diethylene glycol. The significance of these observations is considered in detail in Chapter 7.

ETHYLENE GLYCOL HOMOLOGUES

Mobility patterns using ethylene glycol and its higher homologues, propane-1,3-diol, butane-1,4-diol, pentane-1,5-diol and hexane-1,6-diol, are shown in Figure 5.6. The following relationships are evident at low to intermediate concentrations of organic solvent:

(1) Enhancement of catechin mobility:
hexane-1,6-diol > pentane-1,5-diol > butane-1,4-diol > propane-1,3-diol > ethylene glycol

(2) Perturbant polar atom to carbon atom ratio:
hexane-1,6-diol < pentane-1,5-diol < butane-1,4-diol < propane-1,3-diol < ethylene glycol

Thus it appears that low polar atom content of perturbant molecules favours mobility enhancement at low to intermediate concentrations. This implies that some factor other than direct polar interaction between organic solvent molecules and the substrates may be involved in masking polar interactions between catechin and cellulose.

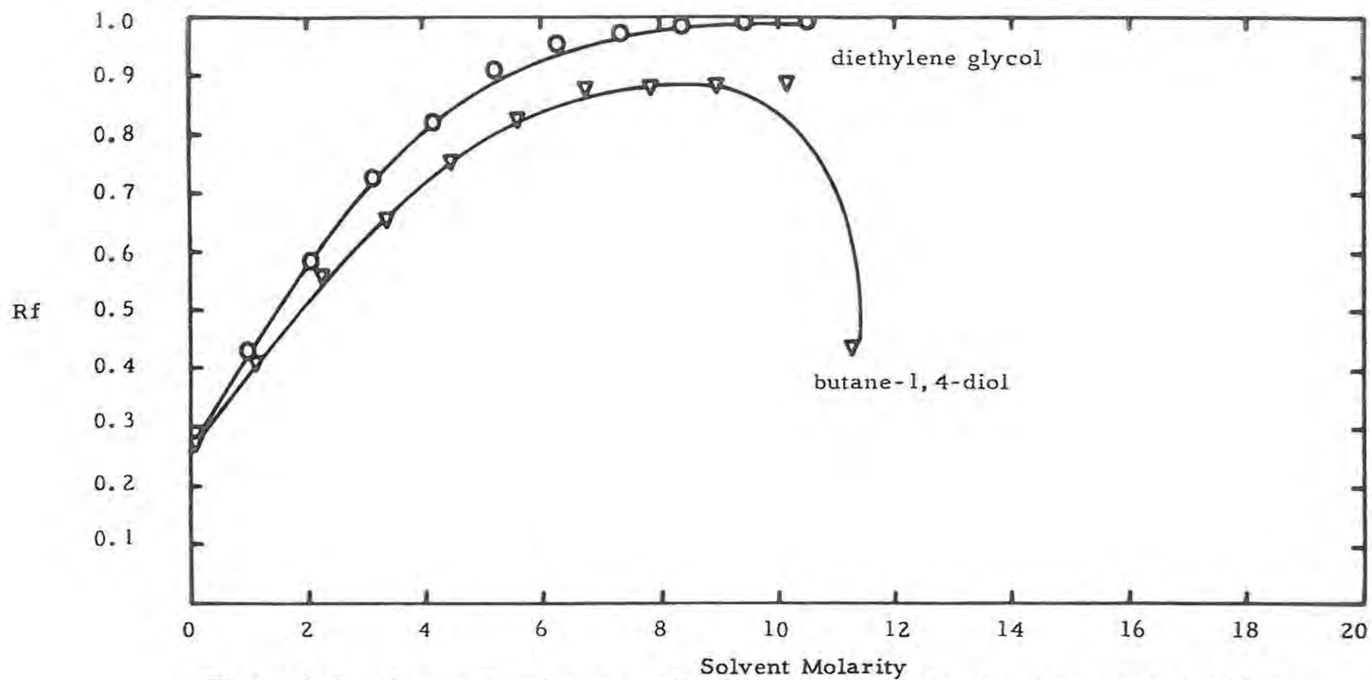

Figure 5.5. Comparison between effects of diethylene glycol and butane-1, 4-diol on chromatographic mobility of catechin on cellulose in solvent/water systems. Temperature, 21.5°C.

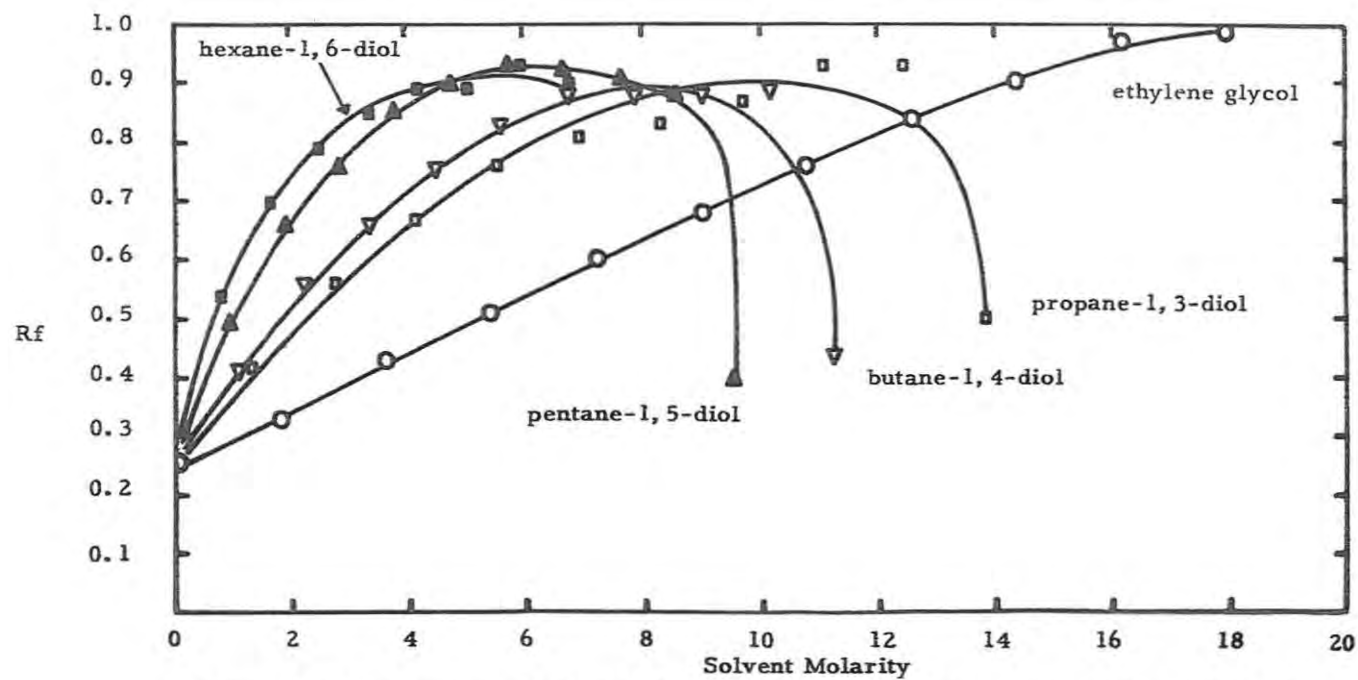

Figure 5.6. Comparison of effects of homologous diols on chromatographic mobility of catechin on cellulose in solvent/water systems. Temperature, 21.5°C.

POLAR ATOM/CARBON ATOM RATIOS

Figure 5.7 shows the distinct similarity, with respect to curve shape, of the profiles produced by solvents having the same polar atom/carbon atom ratios. Methanol, ethylene glycol and glycerol all show the long shallow climb to a maximum Rf at high molarities, followed by curve flattening or a slight decrease in Rf as the limiting condition is approached. In contrast, Figure 5.8 demonstrates that alteration of the polar atom/carbon atom ratio gives rise to curves that are considerably different in shape. As can be seen from the figure, the effect of adding polar groups in the series n-propanol, propane-1,2-diol, glycerol, is to reduce catechin mobility at low solvent proportions while increasing mobility at high solvent proportions.

THREE - COMPONENT SOLVENT SYSTEMS

The final experiment in the series was an attempt to detect non-specific effects arising purely out of changes in the properties of the bulk medium and which could not be attributed to direct polar interactions between the organic components of the solvent system and the substrates. Chromatographic mobility of catechin on cellulose was examined in three-component systems consisting of a non-polar organic solvent, n-hexane, added to constant ratio n-propanol/water mixtures. The results are tabled below. Solubility limitations did not permit the use of higher concentrations of n-hexane. There was nevertheless a detectable enhancement of mobility with increasing hexane concentration.

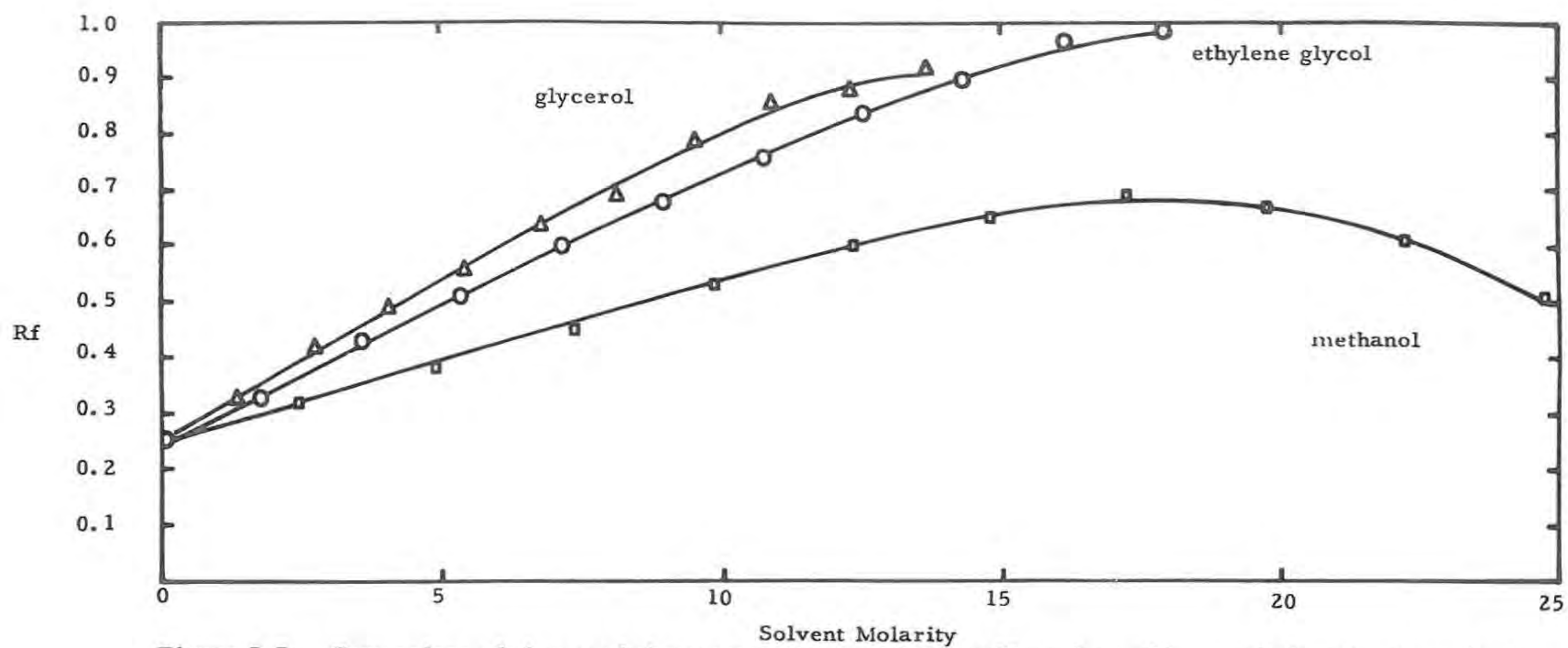

Figure 5.7. Comparison of shapes of chromatographic mobility profiles of catechin on cellulose in solvent/water systems when solvents have identical polar atom/carbon atom ratios. Temperature, 21.5°C.

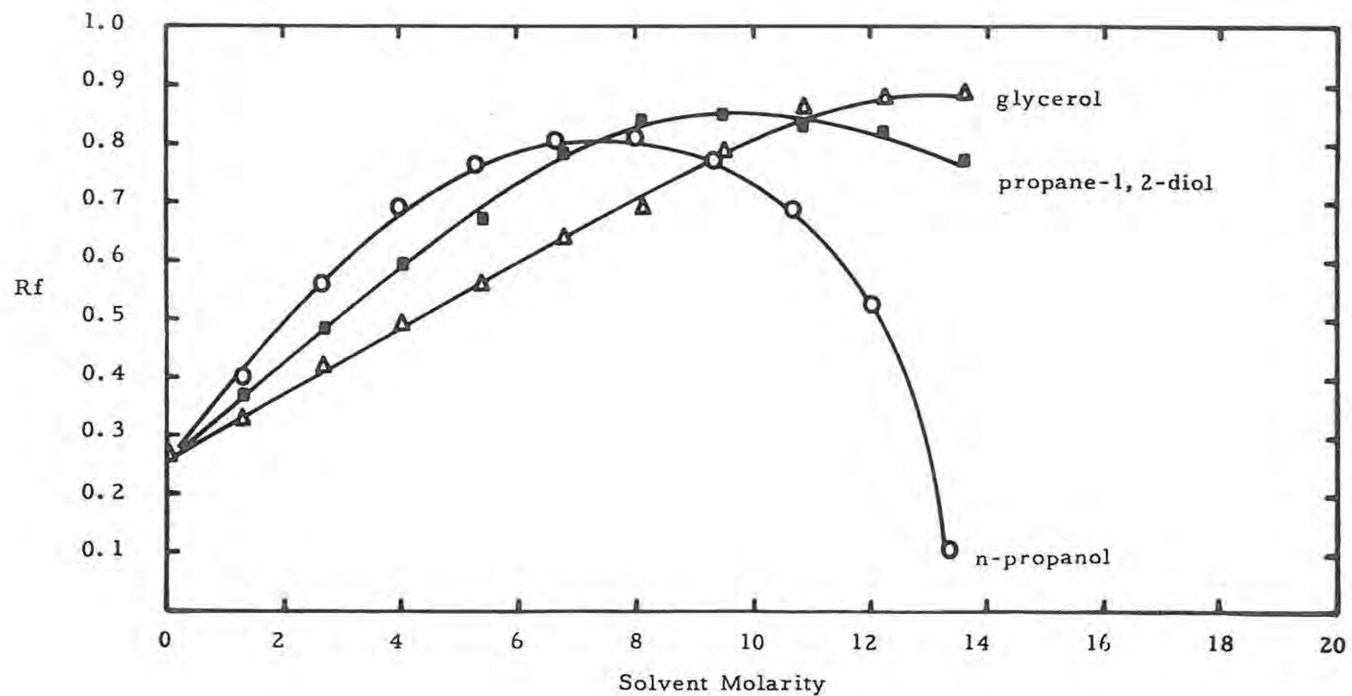

Figure 5.8. Comparison of shapes of chromatographic mobility profiles of **catechin** on cellulose in solvent/water systems when solvents have different polar **atom/carbon** **ratios.** Temperature, 21.5°C.

TABLE 5.1

The addition of hexane to a propanol/water mixture: effect on Rf of catechin on cellulose

n-Propanol:water ratio(v/v)	Hexane(volumes %)	Rf
0.6667	0.000	0.750
0.6667	2.439	0.758
0.6667	4.762	0.761

Temperature, 21.0°C

DISCUSSION

The overall pattern emerging from the results obtained using the chromatographic systems is summarized below.

(1) Increasing the concentration of the organic component of a simple organic solvent/water system invariably resulted in an enhancement of catechin mobility at low proportions of organic solvent.

(2) Low polar atom to carbon atom ratios of the organic components gave rise to higher mobilities at low proportions of organic solvent, while reducing mobilities at high organic solvent concentrations.

(3) Organic solvents such as methanol, ethylene glycol and glycerol, which have high polar atom/carbon atom ratios, do not produce maxima at low concentration nor do they cause any sharp decline in mobility as concentration approaches the pure organic solvent extreme. This is most

noticeable with ethylene glycol and glycerol and also in the case of diethylene glycol, suggesting that direct polar interaction between perturbant and substrates is the dominant process in these cases.

There appear to be two possible mechanisms in terms of which to account for the observed effects. The first involves the specific hydrophobic shielding concept described in Chapter 1. An alternative approach may be formulated based on the assumption that general, not specific, influences contribute significantly to solvent effects. The latter argument requires that an increase in the hydrocarbon content of the solvent system should enhance catechin mobility even where no direct hydrophobic shielding mechanism can be operative. The results shown in Table 5.1 tend to support the suggested non-specificity of solvent action since (1) hexane itself is unlikely to contribute to catechin solvation and (2) the addition of hexane must render the environment more hydrophobic, and hence in terms of direct-shielding mediated propanol-substrate interactions, would be expected to result in a mobility decrease. Thus the appearance of a slight mobility increase, upon addition of a small quantity of hexane, raises the possibility that changes in the general hydrophobicity of the medium may exert a significant effect on a molecular species other than the organic components of the system. A detailed discussion of this suggestion will be undertaken in Chapter 7.

CHAPTER 6

INITIAL RATES OF REACTION DURING COLLAGEN RENATURATION

A number of studies (Flory and Weaver, 1960; Harrington and von Hippel, 1961a; von Hippel and Wong, 1962, 1963a; von Hippel, 1967) have shown that a modified expression based on the Arrhenius equation adequately describes the effect of temperature on mutarotation rates. In the absence of an added perturbant the equation (Flory and Weaver, 1960) is

$$K = \text{Const exp} \times \left[\frac{-A}{kT(Tm^* - T)}\right]$$

where

K	=	reversion rate constant
T	=	renaturation temperature
Tm*	=	denaturation temperature
A	=	apparent activation energy
k	=	Boltzmann constant

Following on from the above, it has been shown (Mandelkern and Stewart, 1964) that the same reverson mechanism may be assumed to apply in the presence of a lyotropic agent which lowers Tm; renaturation temperature and protein concentration being fixed in this case. The approach has been further extended by Russell and Cooper (1969) to show that, with perturbants that lower Tm, there is a linear relationship between 1 hour optical rotation values and the logarithm of zero-time reversion rates; 1 hour [α] values therefore serving as a convenient measure of initial rates.

The existence of a linear relationship between 1 hour rotation values and the log of the initial rate has been substantiated in the present work, in those cases where mutarotation decreased significantly and systematically with increasing perturbant concentration, namely, with propane-1,2-diol, butane-1,4-diol, and 2-ethoxy-ethanol (Tables 3.4, 3.6 and 3.8, respectively). In certain other cases however, where recovery decrease was not large and systematically related to perturbant concentration, as with ethylene glycol (Table 3.4),

it was considered necessary to evaluate the reliability of 1 hour rotation values as relative measures of initial recovery rates.

ORDER OF THE REACTION

From an examination of van't Hoff plots of log $(d[\alpha]/dt)$ against log $([\alpha]_\infty - [\alpha]t)$, derived from the equation

$$\frac{d[\alpha]}{dt} = k([\alpha]_\infty - [\alpha]_t)^n ,$$

Harrington and von Hippel (1961a) have shown the initial stages of renaturation to be apparent second order ($n = 2.2 \pm 0.15$), irrespective of temperature or protein concentration. The use of a second order equation in the present work provided a convenient means of determining initial rate values. The most suitable form (Gardiner, 1969) was found to be

$$\frac{1}{[A]} = \frac{1}{[A]_o} - \nu_A k'(T)t$$

where

$[A]_o$ = concn of A at start of reaction
$[A]$ = concn of A at variable upper limit
T = absolute temperature
ν_A is negative and equals -1 or -2
t = time
$k'(T)$ = the rate constant, k; T is included to indicate the temperature dependence of the rate constant.

Letting $k = -\nu_A k'$

$$\frac{1}{[A]} = \frac{1}{[A]_o} + kt \qquad \text{.....} (1)$$

Introducing the terms

y_o = observed value of $[\alpha]$ at zero time = -460° (i.e. the specific rotation of fully denatured collagen)

y = $([\alpha]$ at time $t) - y_o$

y_∞ = assumed maximum (i.e. equilibrium) value for $[\alpha]$

y' = $y_\infty - y$

and since y' is directly related to $[A]$

and y_∞ is directly related to $[A]_o$

equation (1) becomes: $$\frac{1}{y_\infty - y} = \frac{1}{y_\infty} + kt$$

$$\therefore \quad y_\infty - y = \frac{y_\infty}{kty_\infty + 1}$$

$$\therefore \quad y = y_\infty \left[1 - \frac{1}{kty_\infty + 1} \right] \quad \ldots\ldots (2)$$

$$\therefore \quad y = \frac{y_\infty^2 \cdot kt}{y_\infty \cdot kt + 1}$$

$$\therefore \quad \frac{1}{y} = \frac{y_\infty \cdot kt + 1}{y_\infty^2 \cdot kt}$$

i.e. $$\frac{1}{y} = \frac{1}{y_\infty} + \frac{1}{y_\infty^2 \cdot k} \cdot \frac{1}{t} \quad \ldots\ldots (3)$$

This is of the form $y = mx + c$, thus a plot of $\frac{1}{y}$ vs. $\frac{1}{t}$

will have slope = $\frac{1}{y_\infty^2 \cdot k}$

and intercept = $\frac{1}{y_\infty}$

In order to test the accuracy with which a second order rate equation of this form could be fitted to experimental data profiles, a comparison between experimental and theoretical curves was made using a Hewlett-Packard Model 9100B programmable desk-top calculator, employing a programme written specifically for this purpose. Basing calculations on the data points covering the first three hours of renaturation, the theoretical curves obtained from the rate equation were compared with the experimental values as shown in Figure 6.1. The experimental and theoretical profiles are almost superimposable both in the absence of perturbant and in the presence of a relatively high concentration (4 molar) of one of the more highly lyotropic perturbants investigated, namely, 2-ethoxy-ethanol. The correlation coefficient relating the experimental data and theoretical curves in both cases shown in Figure 6.1 is 0.99 indicating a very good fit. The register between experimental data and theoretical profiles was checked in similar manner for all the perturbants examined and found to be satisfactory in every case, correlation coefficients of the order of 0.99 being the rule.

THE RELATIONSHIP BETWEEN [α] AT 1 HOUR AND LOG INITIAL RATE

Having established the utility of the second order kinetic equation in relation to renaturing systems, in the presence and absence of added organic perturbants, the equation was further modified as follows:

(from equation (2))
$$y = y_{\infty}\left[1 - \frac{1}{kty_{\infty} + 1}\right]$$

$$= y_{\infty} - \frac{y_{\infty}}{(kty_{\infty} + 1)}$$

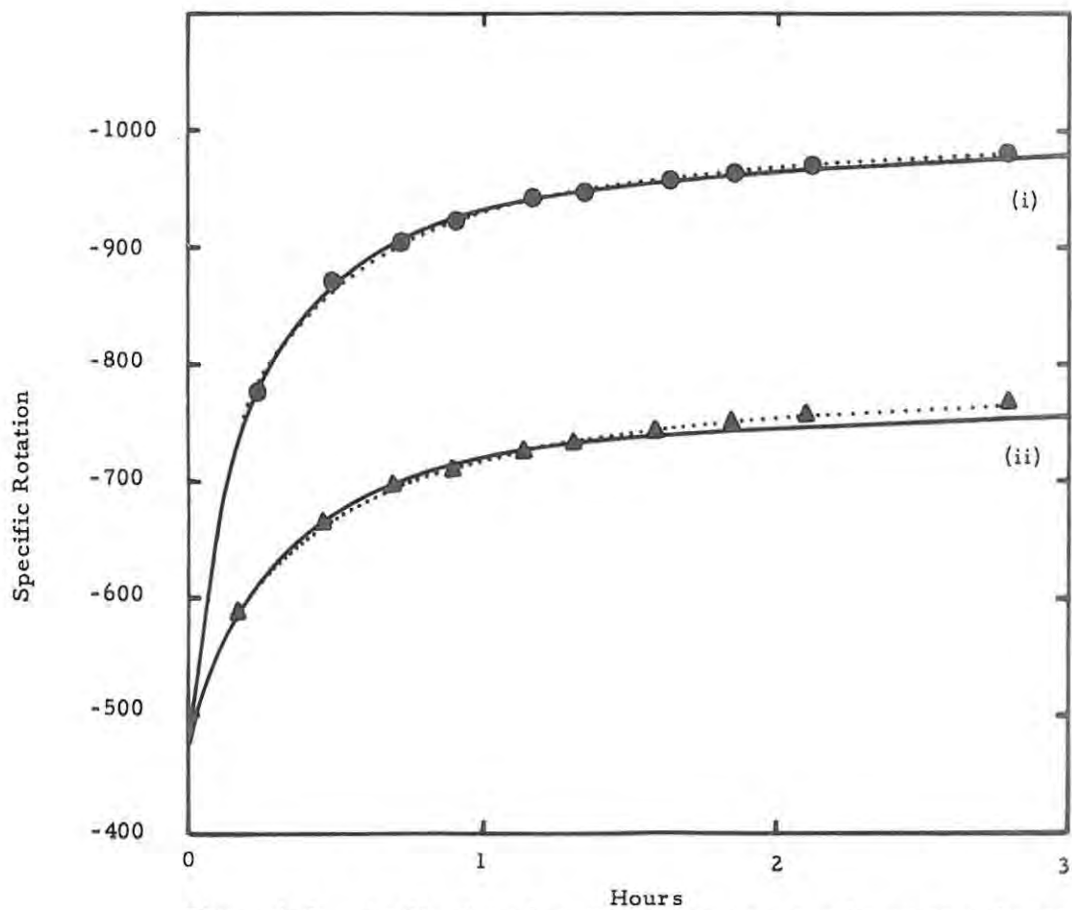

Figure 6.1. Application of second order rate equation to experimental data for renaturation of acid-soluble collagen at 15°C. Solid curves show theoretical second order recovery profiles predicting the course of the first three hours of renaturation (i) in the absence of perturbant, (ii) in the presence of 4 M 2-ethoxy-ethanol. Dotted lines show experimental profiles; ●, without perturbant; ▲, with 4 M 2-ethoxy-ethanol.

Differentiation of this equation with respect to time yields a differential of the form

$$d\left(\frac{u}{v}\right) = \frac{v.du - u.dv}{v^2}$$

and hence at t=zero the rate equation reduces to

$$\left(\frac{dy}{dt}\right)_o = y_\infty^2 \ . k$$

An assessment of the utility of 1 hour [α] values, as a measure of initial mutarotation rates, was then made by plotting log $y_\infty^2 \ . \ k$ as a function of 1 hour [α] values. Figure 6.2 illustrates the use of the method, the values for log initial rate vs. [α] at 1 hour being plotted for various concentrations of 2-ethoxy-ethanol in a system renaturing at 15°C. A linear relationship between the two parameters is evident, the correlation coefficient being 0.99. This serves as the model case where there is a significant and systematic decrease in mutarotation rate as perturbant concentration is increased.

Application of the above method of analysis to other perturbant systems, however, shows that there are cases where the direct relationship between [α] at 1 hour and log initial rate is not apparent. This is illustrated by Figure 6.3 which shows that with diethylene glycol, in a system renaturing at 15°C, the two parameters exhibit no linear register, the correlation coefficient being 0.30. All the perturbants used in the renaturation studies were subjected to similar analyses and a number of cases were found where the correlation between [α] at 1 hour and log of initial rate was poor. Furthermore, it was noticed that there was an apparent relationship between the effects of perturbants on Tm and the register between [α] at 1 hour and log initial rate, as shown in Table 6.1.

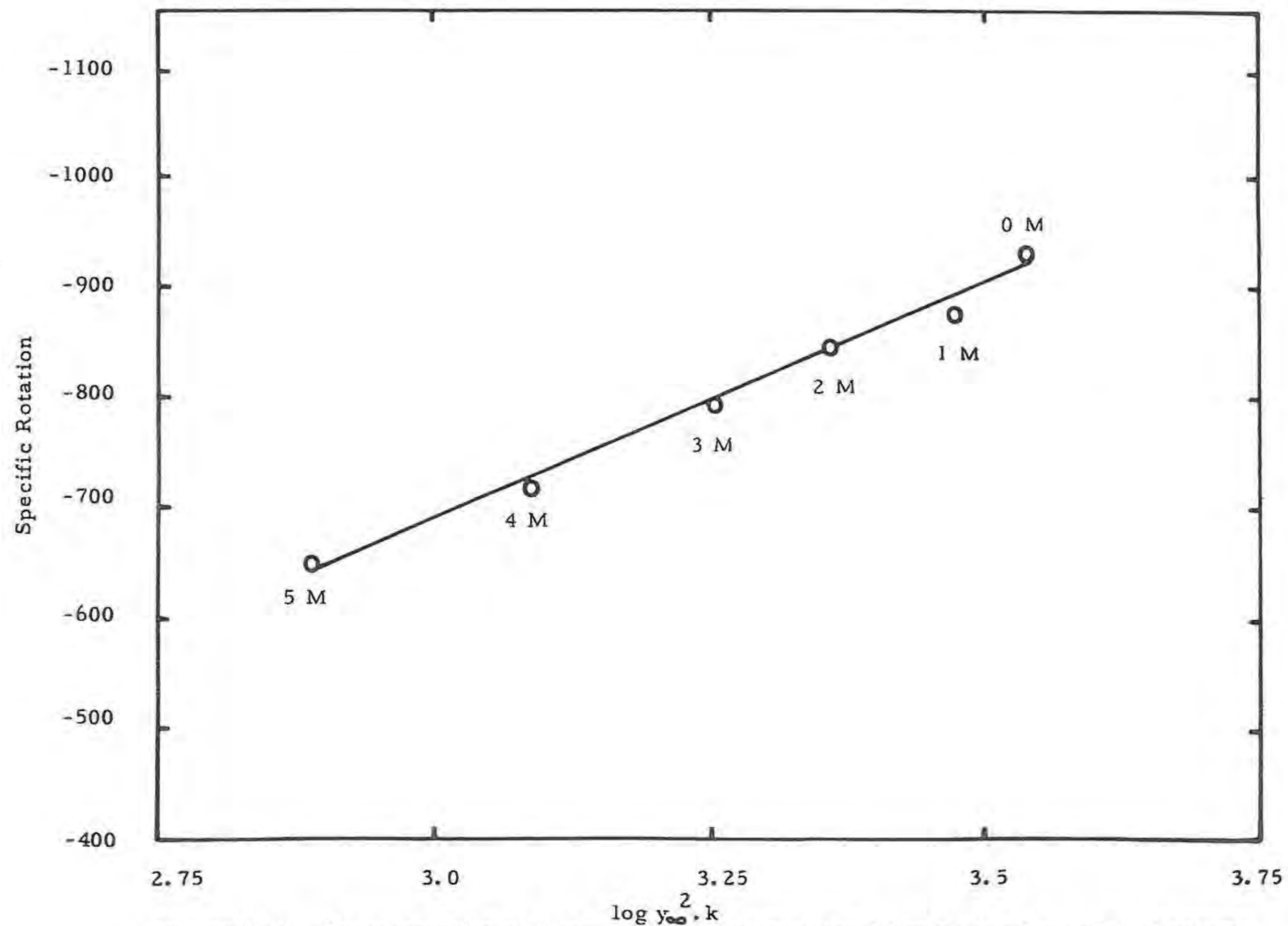

Figure 6.2. Demonstration of linear relationship between 1 hour optical rotation recovery value and log initial mutarotation rate of heat-denatured (45°C; 15 min) acid-soluble collagen (0.84 mg/ml in 0.15 M potassium acetate buffer) in the presence of various concentrations of 2-ethoxy-ethanol. Renaturation temperature, 15°C.

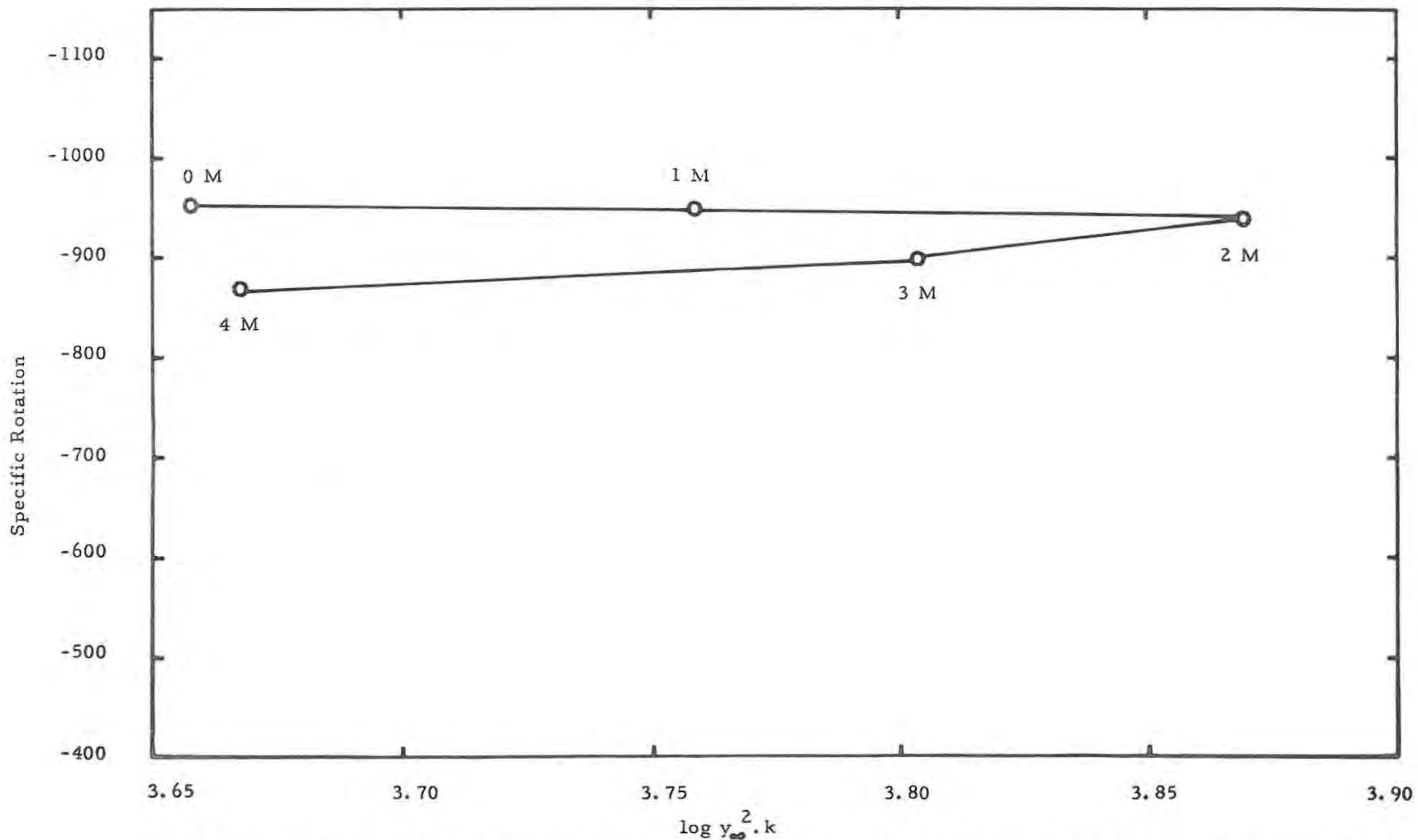

Figure 6.3. Demonstration of absence of linear relationship between 1 hour optical rotation recovery value and log initial mutarotation rate of heat-denatured (45°C; 15 min) acid-soluble collagen (0.86 mg/ml in 0.15 M potassium acetate buffer) in the presence of various concentrations of diethylene glycol. Renaturation temperature, 15°C.

TABLE 6.1

Relationship between correlation coefficient for [α] at 1 hour vs. log initial rate and the effect on Tm of collagen

Solvent	Corr. coeff. for [α] at 1 hour and log y_{∞}^{2} . k at 15°C	Effect on Tm at 1 molar	Description
glycerol	0.88	+ 0.96	stabilizer
diethylene glycol	0.30	+ 0.91	"
propane-1,3-diol	0.74	+ 0.38	"
ethylene glycol	0.60	+ 0.35	"
2-methoxy-ethanol	0.77	+ 0.05	"
propane-1,2-diol	0.97	- 0.19	destabilizer
butane-1,4-diol	0.99	- 0.33	"
2-ethoxy-ethanol	0.99	- 0.85	"

Perturbants are referred to as stabilizers or destabilizers according to whether they raise or lower the Tm of native soluble collagen with respect to the control value.

Table 6.1 shows that stabilizers have low correlation coefficients with respect to the [α] 1 hour: log y_{∞}^{2}. k relation, for renaturation at 15°C, whereas destabilizers show high correlation coefficients. It appears that [α] at 1 hour is a simple function of initial mutarotation rate only with perturbants which destabilize the native collagen molecule. Thus, in the case of the stabilizers, although the [α] values at 1 hour may be regarded as valid indicators of the simple order or ranking of perturbant effectiveness at finite time, they do not serve as reliable functions of the reaction rate at zero time for renaturations carried out at 15°C. It is noted that the use of 1 hour [α] values elsewhere in the current work, as a convenient measure of the relative lyotropic

power of the various perturbants at fixed time, is unaffected by the implications of the present discussion.

THE EFFECT OF TEMPERATURE

Since mutarotation rate depends markedly on the temperature at which renaturation takes place (Flory and Weaver, 1960; Harrington and von Hippel, 1961b), it was considered to be of interest to ascertain the effect, on the above relationships, of slowing the overall reaction rates by raising the renaturation temperature. This would simultaneously allow more precise monitoring of the early stages of the process. A stabilizing and a destabilizing perturbant were examined for their effects on renaturation kinetics at 20°C. The perturbants used were 2-ethoxy-ethanol, a powerful destabilizer, and diethylene glycol, a highly effective stabilizer.

When renaturation is monitored at 15°C, $[\alpha]$ at 1 hour, in the absence of perturbant, is approximately -900°. A comparable reaction stage for a control renaturing at 20°C is $[\alpha]$ at 3 hours, since $[\alpha]$ is then in the region of -900°. Thus the correlation between $[\alpha]$ at 3 hours and log initial rate for renaturations at 20°C is regarded as the most useful equivalent of the $[\alpha]$ 1 hour: log initial rate relation for renaturations at 15°C. Values for $[\alpha]$ at 3 hours at varying concentrations of the two perturbants used are shown in Table 6.2. The effect of increased renaturation temperature, on the correlation between fixed-time $[\alpha]$ values and initial rates, is shown in Table 6.3.

TABLE 6.2

Effects of 2-ethoxy-ethanol and diethylene glycol on optical rotation recovery of collagen renaturing at 20°C

Solvent	Molarity	$-[\alpha]_{365}^{20^{o}}$ at 3 hours
control	-	908
2-ethoxy-ethanol	1	879
	2	813
	3	746
	4	671
	5	607
diethylene glycol	1	917
	2	876
	3	852
	4	834
	5	780

Collagen concentration = 0.86 mg/ml

TABLE 6.3

Comparison of correlation coefficients relating fixed-time$[\alpha]$ values and log of initial rates for collagen renaturation in the presence of 2-ethoxy-ethanol and diethylene glycol at different temperatures

Solvent	Corr. coeff. between $[\alpha]$ at 1 hour and log $y_{\infty}^{2}.k$, at 15°C	Corr. coeff. between $[\alpha]$ at 3 hours and log $y_{\infty}^{2}.k$, at 20° C
2-ethoxy-ethanol	0.99	0.99
diethylene glycol	0.30	0.95

From Table 6. 3 it appears that renaturation temperature is not a decisive factor affecting the correlation between fixed-time [α] values and initial rates in the case of 2-ethoxy-ethanol. In contrast, systems containing the stabilizer, diethylene glycol, show a much improved correlation between the fixed-time [α] values and the initial rate parameter when the reaction is slowed by renaturing at the higher temperature.

THE RELATIONSHIP BETWEEN PERTURBANT CONCENTRATION AND REACTION RATE

A linear relationship between perturbant concentration and log zero-time reversion rate has been demonstrated for neutral salts and for urea by von Hippel and Wong (1962, 1963a). A similar linear relationship between perturbant concentration and recovery rates has been found in studies using polar organic solvents of the lower homologous alcohol series (Russell and Cooper, 1969) and various amides and alkyl-substituted amides (Russell and Cooper, 1970). In the present study, however, there have arisen a number of examples of an apparently non-linear relationship between zero-time reversion rates and perturbant concentration. These are shown in Figures 6. 4, 6. 5, 6. 6, 6. 7, 6. 8, 6. 9, 6. 10. Commencing with the powerful stabilizer, diethylene glycol, it appears from Figure 6. 4 that there is a marked acceleration of log initial mutarotation rate as perturbant concentration is increased, reaching a maximum at about 2 molar, after which a phase of progressive rate decrease becomes evident. The less powerful stabilizer, 2-methoxy-ethanol (Figure 6. 5), gives rise to a similar pattern of effects; however the acceleration of zero-time reversion rate at low perturbant concentrations is not as marked as with diethylene glycol.

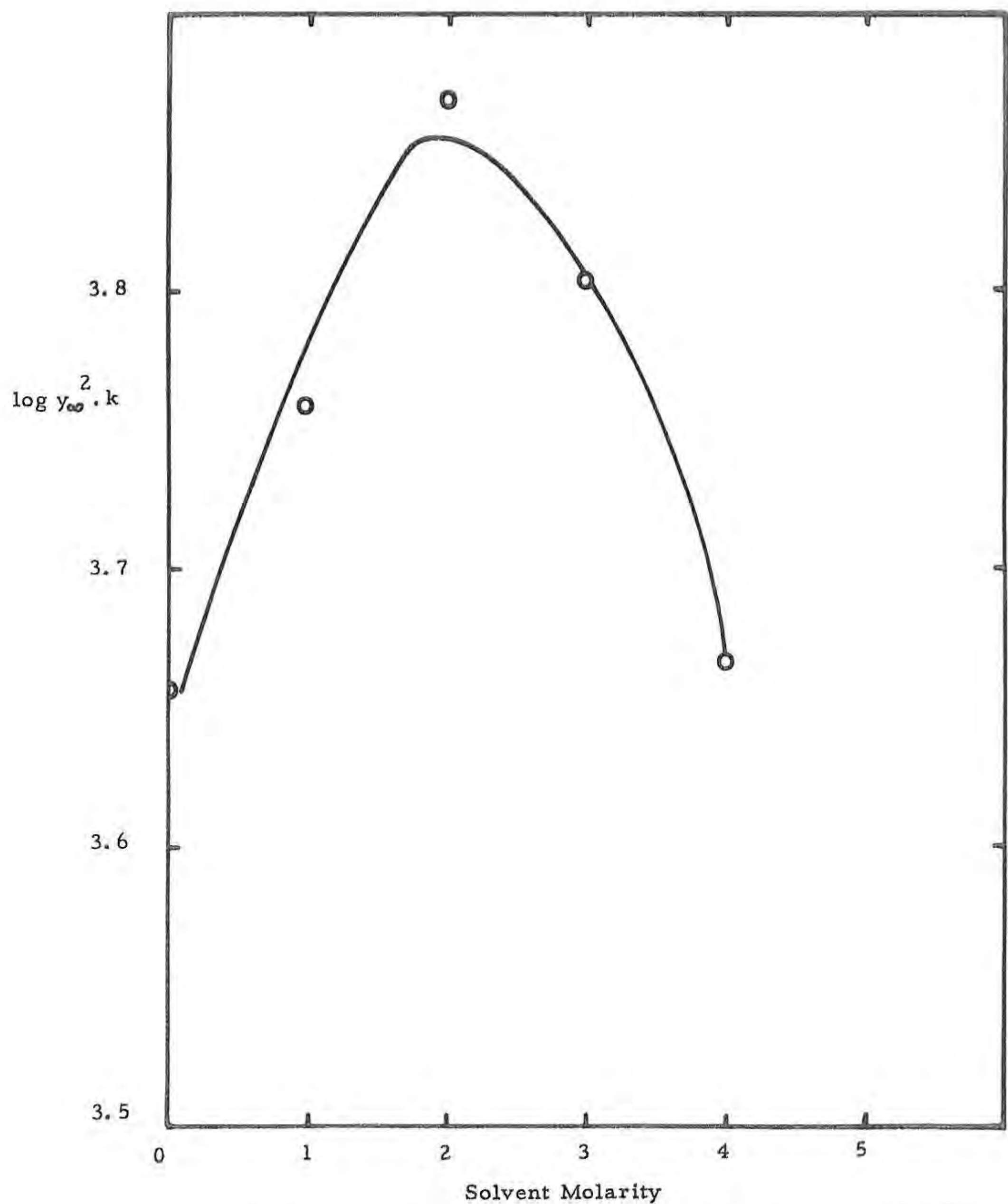

Figure 6.4. Effect of diethylene glycol concentration on log initial mutarotation rate of heat-denatured (45°C; 15 min) acid-soluble collagen (0.86 mg/ml in 0.15 M potassium acetate buffer). Renaturation temperature, 15°C.

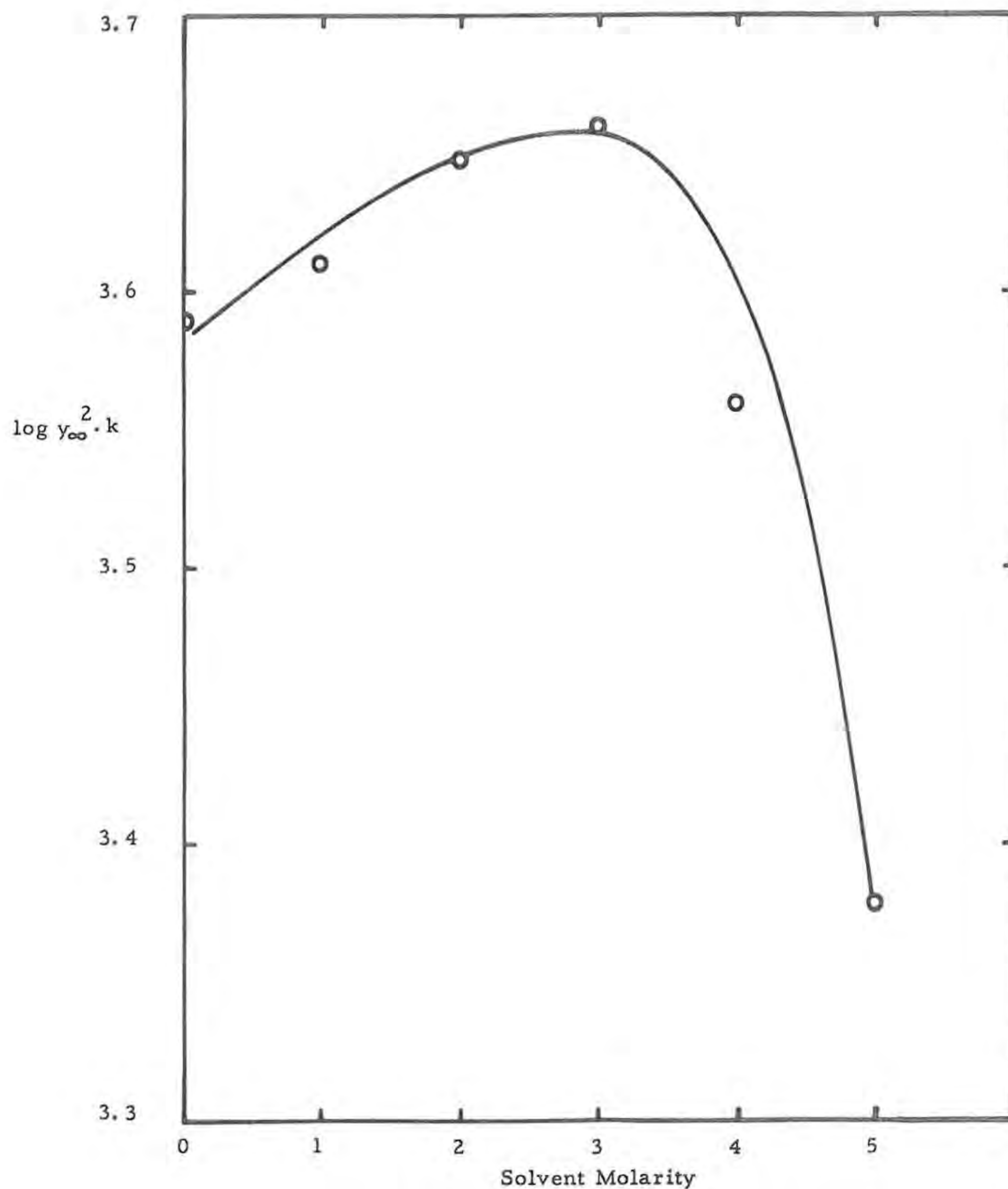

Figure 6.5. Effect of 2-methoxy-ethanol concentration on log initial mutarotation rate of heat-denatured (45°C; 15 min) acid-soluble collagen (0.84 mg/ml in 0.15 M potassium acetate buffer). Renaturation temperature, 15°C.

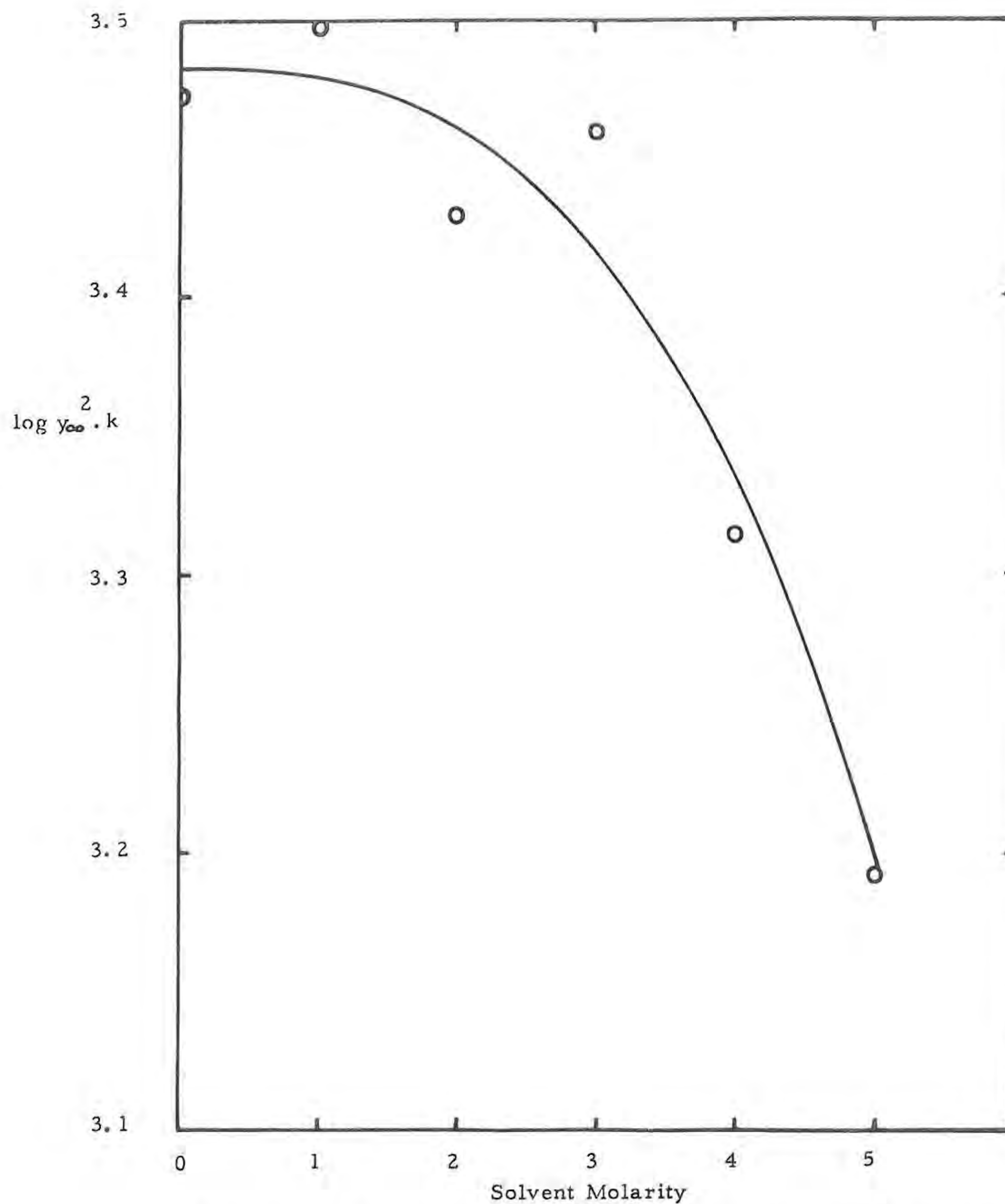

Figure 6.6. Effect of propane-1, 2-diol concentration on log initial mutarotation rate of heat-denatured (45°C; 15 min) acid-soluble collagen (0.84 mg/ml in 0.15 M potassium acetate buffer). Renaturation temperature, 15°C.

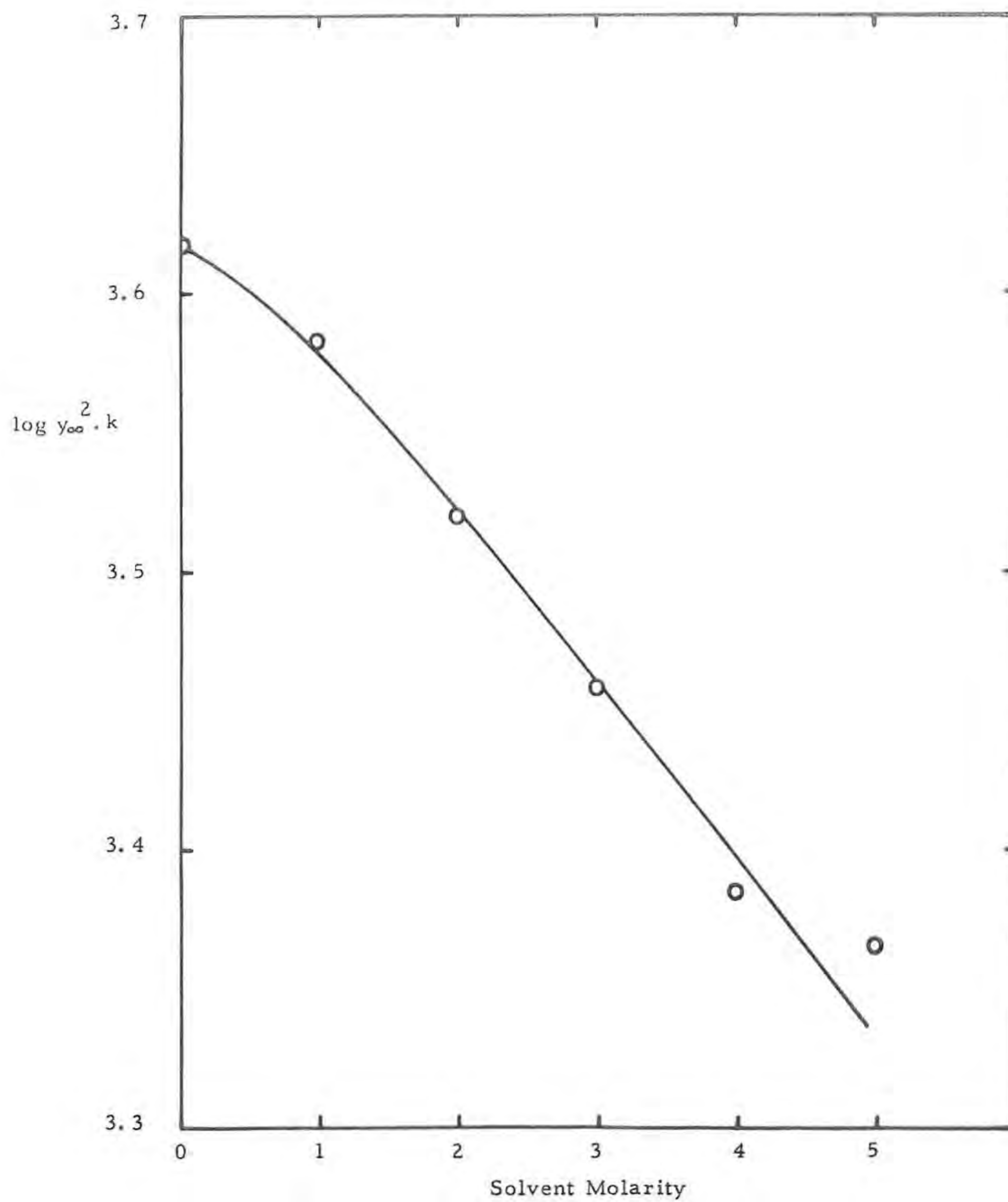

Figure 6.7. Effect of butane-1, 4-diol concentration on log initial mutarotation rate of heat-denatured (45°C; 15 min) acid-soluble collagen (0.86 mg/ml in 0.15 M potassium acetate buffer). Renaturation temperature, 15°C.

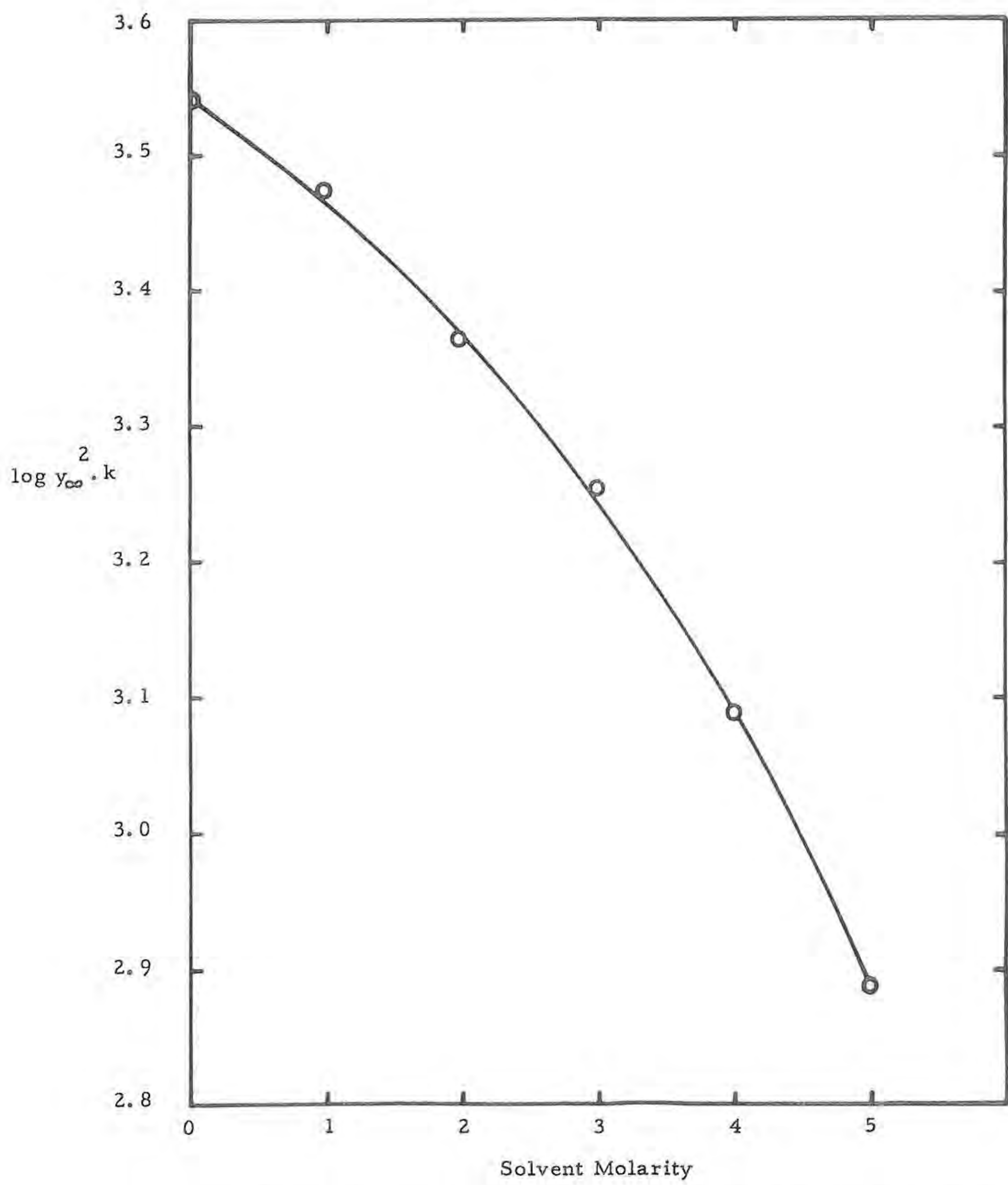

Figure 6.8. Effect of 2-ethoxy-ethanol concentration on log initial mutarotation rate of heat-denatured (45°C; 15 min) acid-soluble collagen (0.84 mg/ml in 0.15 M potassium acetate buffer). Renaturation temperature, 15°C.

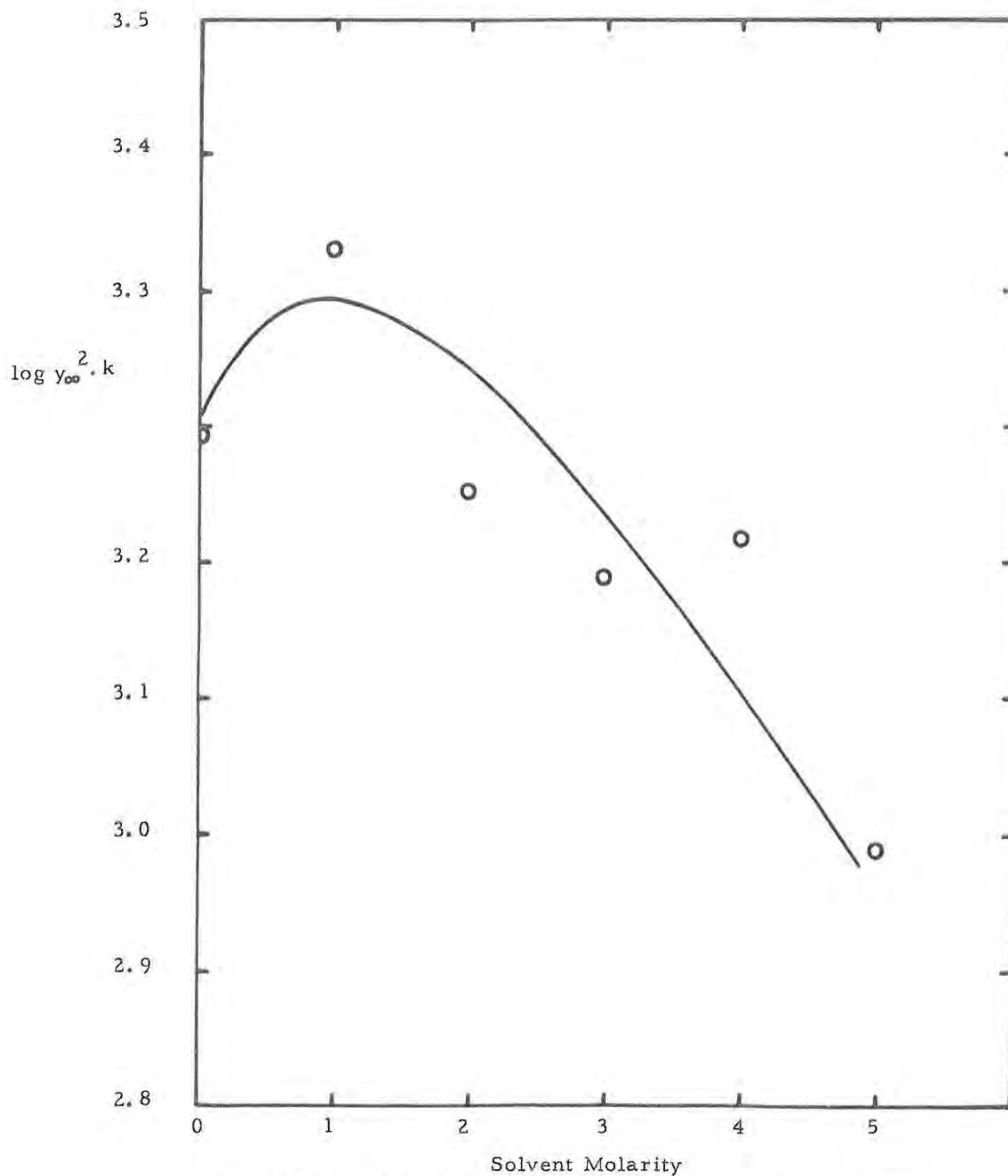

Figure 6.9. Effect of diethylene glycol concentration on log initial mutarotation rate of heat-denatured (45°C; 15 min) acid-soluble collagen (0.86 mg/ml in 0.15 M potassium acetate buffer). Renaturation temperature, 20°C.

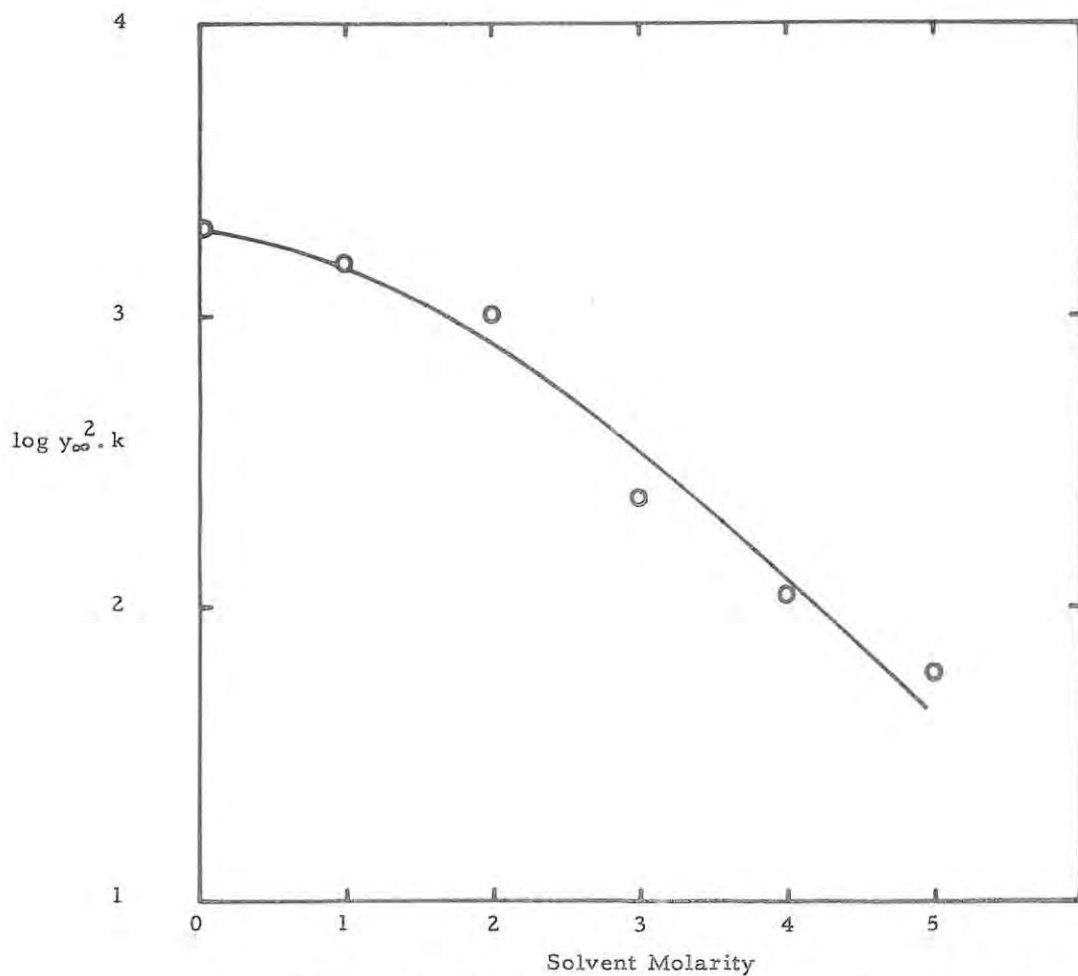

Figure 6.10. Effect of 2-ethoxy-ethanol concentration on log initial mutarotation rate of heat-denatured (45°C; 15 min) acid-soluble collagen (0.86 mg/ml in 0.15 M potassium acetate buffer). Renaturation temperature, 20°C.

On proceeding to the weakest of the destabilizers examined, namely propane-1, 2-diol (Figure 6. 6), it is seen that the tendency towards initial acceleration of mutarotation at low solvent concentrations has disappeared, although there remains a period of induction up to about 2 molar, after which mutarotation rate decelerates rapidly. This trend in the direction of increasing linearity is maintained with the more effective destabilizers, butane-1, 4-diol and 2-ethoxy-ethanol, as shown in Figures 6. 7 and 6. 8, although slight steepening of the curves as perturbant concentration increases is still suggested.

The trends shown in the systems renaturing at 15°C were confirmed by an examination of the 20°C renaturation profiles on the same basis. Diethylene glycol serves as the prototype for perturbants showing a strong tendency to stabilize the native state of the protein, and 2-ethoxy-ethanol as an example of a powerful destabilizer. From Figure 6. 9 it appears that with diethylene glycol the initial acceleration of mutarotation rate at low perturbant concentration, followed by a trend reversal after which rate retardation rapidly increases, is again manifested. This serves to confirm the findings for this perturbant at 15°C (Figure 6. 4). Similarly, the results obtained at 20°C using the destabilizer, 2-ethoxy-ethanol, substantiate the apparent 15°C trends, as may be seen by comparing Figures 6. 10 and 6. 8. The curves are closely similar in shape and both show slight curvature, suggesting increasing lyotropic activity in the system at higher perturbant concentrations.

The non-linearity of the log zero-time reversion rate vs. concentration plots has important implications for the mechanism of perturbant action. It appears that direct interaction between perturbant molecules and protein might not be the only factor affecting the course of events in the renaturing systems. Certain

other competing influences, in the case of stabilizers, or complementary effects, in the case of destabilizers, must be invoked to explain the complexities of the curves. A theoretical interpretation of the observed trends is attempted in the following chapter in the section dealing with renaturation kinetics.

CHAPTER 7

THEORETICAL INTERPRETATION OF SOLVENT-SUBSTRATE INTERACTIONS IN HYDROCARBON-WATER SYSTEMS

PART I

GENERAL CONSIDERATIONS

The basic assumption underlying the experimental approach to the present study has been that an examination of solvent-substrate interactions using substrates as diverse as soluble collagen, catechin and cellulose might be of value in establishing the relative contributions of various non-covalent forces to the solvation process. Should a mechanism emerge which can be applied with equal facility to the diverse substrates examined, the existence of a common, non-specific solvation process would be implied which would be largely independent of entropy-mediated effects such as hydrophobic interactions. Conversely, if the experimental findings are regarded as indicative of fundamental differences between the nature of the interactions in the chromatographic and proteinaceous systems, it becomes necessary to account for these differences in terms of processes other than exclusively polar interaction mechanisms.

The current work has revealed interesting relationships between the chromatographic systems and the collagen studies. Paper chromatography has contributed significantly to the construction of an interpretative framework upon which to base an attempted explanation of the experimental evidence. A model for the mechanism of solvent action in the paper chromatographic systems will be discussed in detail below, before proceeding to consider the applicability of such an interpretation to the stabilization and perturbation of the structure of soluble collagen.

CATECHIN-CELLULOSE INTERACTIONS

The presence of hydroxyl groups in the cellulose polymer is considered to be the main factor governing the chemical reactivity of paper surfaces (Knight, 1962). Cellulose is capable of entering into strong polar association with environmental molecules of a sufficiently

electronegative nature. Consden, Gordon and Martin (1944), however, have contended that the paper serves merely as a support for the aqueous stationary phase and does not actively participate in polar interactions with the constituent molecules of the mobile phase or with the sample itself. In these terms the stationary phase is regarded as a relatively immobile film of water molecules hydrogen-bonded to the cellulose matrix. A major objection to this approach arises out of the use of water-miscible solvents to effect separations (Arden et al., 1948; Hanes and Isherwood, 1949; Bentley and Whitehead, 1949, 1950). This would not be possible if separations were due to a simple partitioning process. The explanation offered by Hanes and Isherwood (1949) postulates the existence of a water-cellulose complex, partitioning between this complex and the mobile phase being the effective mechanism. It seems unrealistic to ignore the possibility of direct polar interactions between cellulose and environmental molecules other than water, however, and the fact that many dyes and other substances are known to adsorb is of considerable relevance in this connection (Lederer and Lederer, 1957).

On the basis of the above arguments, and since the polyhydroxy catechin molecule presents a relatively large number of polar groups to the external environment, it is concluded that direct catechin-cellulose polar interactions do occur, and that any interference with these interactions will affect the chromatographic mobility of catechin on a cellulose substrate.

In all the organic solvent/water systems examined, an increase in the hydrocarbon content of the systems caused an initial increase in catechin mobility. This increase was gradual and maintained, with those solvents having high polar atom to carbon atom ratios, whereas solvents having low polar atom/carbon atom ratios produced sharp maxima at low solvent/water ratios, followed by a marked reduction

in mobility at high concentrations of organic solvent. Any mechanistic interpretation of these results must account adequately not only for the progressive enhancement of catechin mobility at low solvent proportions, but also for the progressive mobility decline at high solvent concentration as the polar atom content of perturbant molecules increases. Thus the model must reconcile the apparent weakening of catechin-cellulose polar interactions, due to the presence of lower concentrations of hydrocarbon, with the considerable enhancement of these interactions when the hydrocarbon content of the system is high.

It is proposed that, with solvents having low polar atom to carbon atom ratios, the masking of catechin-cellulose polar interactions might not be due solely to hydrogen bond formation between organic solvent molecules and catechin and cellulose polar groups. Instead it is suggested that in such systems the water molecules are, on a mass-action basis, the most active hydrogen-bonding species present, and that the introduction of hydrocarbon into the environment renders water better able to solvate catechin and cellulose polar groups. In molecular terms this model may be described as follows: the presence of hydrocarbon molecules in the solvent environment reduces the affinity of water molecules for that environment since it contains a lower concentration of polar atoms than does pure water. In contrast, the affinity of water molecules for polar groups on catechin and cellulose is not diminished by reduction of the polar atom content of the liquid environment. The resultant effect is a decreased tendency for water molecules to exchange with the solvent mixture, and hence the period of residency of water molecules at polar sites on catechin and cellulose is prolonged. Thus, the extent to which polar groups of catechin and cellulose are solvated by water is increased, and the consequent reduction in the potential for catechin-cellulose hydrogen bonding

enhances chromatographic mobility. Although hydrogen bond formation between organic solvent polar groups and catechin and cellulose must also contribute to the solvation process, it is suggested that this interaction alone could not account for the observed pattern of effects. Mobility enhancement is more readily explained by incorporating the concept of enhanced water activity due to increasing hydrocarbon content of the system. Masking of catechin-cellulose polar interactions by perturbant molecules would be augmented by the reduced tendency of water molecules at polar surfaces to exchange with the liquid environment as the hydrocarbon content of that environment is increased.

The above suggestion is supported by the findings using the three-component hexane/propanol/water system (Table 5.1). As already mentioned (Chapter 5), the effect of the addition of hexane cannot be adequately accounted for in terms of direct-shielding mediated propanol-substrate interactions, since such a mechanism requires that the introduction of hexane molecules into the environment should result in decreased catechin mobility. The observed mobility increase is readily explained, however, if it is regarded as an effect of the presence of hexane on the activity of the third component of the solvent system, namely water, in accordance with the mechanism outlined above.

In contrast to the effects at low solvent concentrations, the presence of high concentrations of solvents having high hydrocarbon contents reduces chromatographic mobility to a value below the Rf maximum, which occurs at lower solvent/water ratios. The overall situation with respect to solvents with low polar atom to carbon atom ratios, arbitrarily taken as those in which this ratio is 0.5 or less, is summarized in Table 7.1.

TABLE 7.1

Influence of solvents with low polar atom/carbon atom ratios on mobility of catechin on cellulose

Solvent	polar atoms / carbon atoms	Rf max.	Conc. at which Rf max. occurs (volumes %)	Rf at 90% v/v
2-ethoxy-ethanol	0.50	0.87	60	0.65
butane-1, 4-diol	0.50	0.89	90	0.86
butane-1, 3-diol	0.50	0.83	70	0.80
butane-2, 3-diol	0.50	0.83	60	0.78
pentane-1, 5-diol	0.40	0.93	60	0.83
hexane-1, 6-diol	0.33	0.93	70	---
2-butoxy-ethanol	0.33	0.89	30	0.60

Table 7.1 shows that in each case the Rf at very high solvent concentration (90%) is lower than the maximum Rf. This supports the suggestion that water is an active contributor to mobility enhancement. Thus, when water concentration falls below a critical minimum, the reduced concentration of this highly active molecular species renders the solvent mixture unable to sustain a high degree of solvation of catechin and cellulose polar groups, hence catechin mobility decreases.

A different situation obtains where polar atom/carbon atom ratios of organic solvents are higher:

Table 7.2 shows that solvents with polar atom/carbon atom ratios of 0.67 produce a small decline in catechin mobility when the concentration of the organic component exceeds the optimum value. In contrast, solvents having polar atom/carbon atom ratios of 0.75 or more, show the opposite tendency. Diethylene glycol, ethylene glycol and glycerol produce maximum mobility at the high

concentration extreme (100 volumes %). By comparing these results (Table 7.2) with those shown in Table 7.1 it can be seen that there is a tendency for Rf maxima to occur at progressively higher concentrations of organic component as polar atom/carbon atom ratios increase. In the latter cases the polar atom concentration in the liquid environment is relatively high, even in the absence of water, hence it seems probable that the masking of catechin-cellulose polar interactions may be due, to an increasing extent, to direct solvation of catechin and cellulose polar groups by organic solvent molecules.

TABLE 7.2

Influence of solvents with high polar atom/carbon atom ratios on mobility of catechin on cellulose

Solvent	polar atoms / carbon atoms	Rf max.	Conc. at which Rf max. occurs (volumes%)	Rf at 90% v/v
2-methoxy-ethanol	0.67	0.85	80	0.84
propane-1, 2-diol	0.67	0.85	70	0.82
propane-1, 3-diol	0.67	0.90	80	0.88
diethylene glycol	0.75	0.99	100	0.99
ethylene glycol	1.00	0.99	100	0.97
glycerol	1.00	0.92	100	0.89

It is regarded as significant that solvents with high polar atom ratios are less effective mobility enhancers at low concentrations than are solvents having low polar atom ratios. This remains valid for comparisons made both on a volumes % and a molar basis, as shown in Table 7.3, from which certain interesting relationships are apparent. Methanol, ethylene glycol and glycerol enhance mobility only slightly at low concentration, whereas solvents with lower polar atom ratios cause

TABLE 7.3

Influence of solvent polar atom/carbon atom ratios on mobility of catechin on cellulose at low solvent concentrations

Solvent	polar atoms / carbon atoms	Rf at 10%v/v	Rf at 1M
methanol	1.00	0.32	0.28
ethylene glycol	1.00	0.33	0.29
glycerol	1.00	0.34	0.32
diethylene glycol	0.75	0.43	0.43
propane-1, 2-diol	0.67	0.37	0.34
propane-1, 3-diol	0.67	0.42	0.37
2-methoxy-ethanol	0.67	0.42	0.39
2-ethoxy-ethanol	0.50	0.44	0.44
butane-1, 4-diol	0.50	0.41	0.39
butane-1, 3-diol	0.50	0.39	0.38
butane-2, 3-diol	0.50	0.39	0.38
pentane-1, 5-diol	0.40	0.50	0.51
hexane-1, 6-diol	0.33	0.54	0.58
2-butoxy-ethanol	0.33	0.64	0.70

progressively larger mobility increments. Solvents having the lowest polar atom ratios (hexane-1, 6-diol and 2-butoxy-ethanol) cause the greatest mobility enhancement at low concentration but, as previously mentioned, are associated with lower Rf values at high concentrations.

With respect to the possible influence of the presence of organic solvent molecules on the polar bonding activity of water, it appears that the introduction of solvent molecules having high polar atom ratios into the environment does not greatly enhance water-catechin and water-cellulose polar interactions. This may be explained on the following basis. The addition, to the liquid environment, of molecules which have high polar atom ratios does not sufficiently reduce the environmental polar atom content. Consequently, the tendency for water molecules, hydrogen bonded to catechin and cellulose, to exchange with the solvent atmosphere is not diminished to an extent that significantly increases the period of residency of water molecules at polar sites on the substrates. This is in contrast to the situation when organic solvents with low polar atom ratios are introduced into the medium. In such cases the environmental exchange affinity of water is decreased, leading to prolongation of water-catechin and water-cellulose interactions, with a consequently sharp mobility increase at low proportions of the organic component.

ACCESSIBILITY OF POLAR GROUPS

The effects, at high concentration, of the isomeric butanediols and isomeric propanediols on the chromatographic mobility of catechin on cellulose are of considerable interest. A difference in steric accessibility of solvent polar groups is suggested, depending

on whether such groups are centrally or terminally situated. Table 7.4 shows that, at high solvent concentrations, isomers in which hydroxyl groups are terminally situated promote catechin mobility to a greater extent than do their counterparts which have the polar groups nearer to their geometric centres.

TABLE 7.4

Effect of the positions of solvent polar groups on the mobility of catechin on cellulose at high solvent concentrations

Solvent	Rf at 90%v/v	Rf at 100%v/v
propane-1, 3-diol]	0.93	± 0.50
propane-1, 2-diol]	0.82	± 0.32
butane-1, 4-diol]	0.86	± 0.44
butane-1, 3-diol]	0.80	± 0.10
butane-2, 3-diol]	0.78	± 0.01

Although there is considerable error in estimating the Rf values when using the pure organic solvent, owing to streaking of catechin spots, the order of effects is nevertheless quite clear.

It seems likely that terminally situated polar groups are sterically more accessible than those nearer the geometric midpoints of unbranched carbon chains. Consequently, organic solvent molecules with terminal polar groups might have statistically higher polar interaction potentials than would their isomers. Thus butane-2, 3-diol, for example, might less effectively solvate catechin and cellulose polar

groups than would butane-1, 3-diol, which would, in turn, be less effective than butane-1, 4-diol. At low solvent proportions, however, the isomeric butanediols produce only very slight mobility differences (Figure 5.4). Nevertheless, in support of the suggested effects of polar group accessibility, it is noteworthy that propane-1, 3-diol promotes catechin mobility to a considerably greater extent than does propane-1, 2-diol, even at low concentrations (Figure 5.3). Since the propanediols have a higher polar atom/carbon atom ratio than do the butanediols, it is to be expected that the effect of isomerism should be relatively greater in the former case. In other words, changed hydroxyl group position represents a more significant alteration to molecular structure with the compound of lower molecular weight. It is suggested that a mechanistic interpretation of this nature may account for the apparently greater polar interaction potentials displayed, at various concentrations, by those butanediol and propanediol isomers in which the polar groups are nearer the carbon chain extremities.

A necessary corollary follows from the above molecular explanation of the apparent differences in polar interaction potentials of isomers, since reduced steric accessibility of polar groups implies greater accessibility of the carbon chain itself. This inference is significant with respect to the influence of isomerism on the effects of organic solvents on the denaturation and renaturation of soluble collagen, and is discussed in detail in the following section.

COLLAGEN-SOLVENT INTERACTIONS

In recent years much evidence has been produced in support of the concept of apolar interaction as a major contributor to the maintenance of the solution structure of a number of different proteins. Nevertheless, the generally complex nature of the systems studied presents great difficulty with respect to the formulation of an unequivocal interpretation of the experimental findings. Many cases arise in which a given set of results may be explained in terms of different basic assumptions, the validity of which is not open to empirical verification. In this connection it is noteworthy that, even with respect to the globular proteins, recent findings appear to cast doubt on the widely accepted theory that hydrophobic interactions contribute significantly to conformational stability (Brandts et al., 1970). Similarly, in the present study the general tendencies, as revealed by the experimental findings discussed in Chapters 3 and 4, can be interpreted in a number of ways. The arguments that follow represent an attempt to account for the observed effects in terms of a limited number of theoretical approaches.

RENATURATION

Polar atom to carbon atom ratios and steric considerations were found to be closely related to perturbant effectiveness. This resembles the findings arising from the paper chromatography studies. There were a number of notable differences, however, the possible significance of which will be discussed below.

From Table 7.5 it is clear that increasing hydrocarbon content of perturbant molecules results in an apparent increase in effectiveness of ethylene glycol derivatives, as monitored by retardation of optical rotation recovery. Similarly, Table 7.6 shows

the order of effects in all comparable systems to be related to the polar atom/carbon ratios.

TABLE 7.5

Influence of solvent polar atom/carbon atom ratios on collagen renaturation at 15°C, as monitored by 1 hour optical rotation regain values

Solvent	polar atoms / carbon atoms	$-[\alpha]^{15°}_{365}$ at 1 hour and 1 molar
ethylene glycol	1.00	922
2-methoxy-ethanol	0.67	908
2-ethoxy-ethanol	0.50	875
2-butoxy-ethanol	0.33	787

Nevertheless, the problem of interpretation remains unsolved. Results could be rationalized in terms of (1) the shielding influence of the apolar chain on the hydroxyl group, (2) direct apolar interactions between perturbant hydrocarbon chains and apolar groups on the protein itself, or (3) the influence of the presence of hydrocarbon in the environment on the hydrogen-bonding capacity of water molecules.

A more definitive interpretation can be placed upon the differing effects of the isomeric propanediols, shown in Table 7.7:

propane-1,3-diol retards recovery to a smaller extent than does its isomer. This is regarded as significant in view of the finding that, in the paper chromatographic systems, propane-1,3-diol was a more effective mobility promoter than was propane-1,2-diol (Tables 7.2, 7.3 and 7.4). The chromatographic results were interpreted as implying that the 1,3-diol is capable of forming hydrogen bonds more effectively than its 1,2-isomer, due to the greater steric accessibility of the

TABLE 7.6

Influence of solvent polar atom/carbon atom ratios on collagen renaturation at 15°C, as monitored by 1 hour optical rotation regain values

Solvent	polar atoms / carbon atoms	Molarity	$-[\alpha]_{365}^{15}$ at 1 hour
* ethylene glycol	1.00	5.0	887
* glycerol	1.00	5.0	854
propane-1, 3-diol	0.67	5.0	830
butane-1, 4-diol	0.50	5.0	804
glycerol	1.00	4.0	887
diethylene glycol	0.75	4.0	868
diethylene glycol	0.75	0.8	951
** methyldigol	0.60	0.8	945
** ethyldigol	0.50	0.8	931

* The apparently greater effectiveness of glycerol as compared to ethylene glycol may be explained in terms of their differing molar volumes, since glycerol has a molecular weight approximately 50% greater than ethylene glycol, while their densities are similar.

** The effects of alkyl derivatives of diethylene glycol have not been discussed elsewhere in the present work, since precipitation difficulties greatly restrict their usefulness. They are shown in the above comparison, however, because they tend to confirm the presence of an apparent retardation of renaturation upon alkyl substitution of the parent compound.

terminally situated hydroxyl groups. It seems reasonable to expect, therefore, that the 1, 3-diol should be capable of greater retardation of renaturation than its isomer, provided that direct polar interaction between perturbant and collagen is the dominant process. The opposite situation prevails, however, since the 1, 2-isomer retards renaturation to a much greater extent than does the 1, 3-diol (Table 7.7). It appears that an additional mechanism must be operative in the protein-containing systems and is, at this particular concentration, the most significant of the non-covalent interactions involved. The fact that the 1, 2-diol contains a sterically more accessible methyl group than does its isomer, suggests that hydrophobic interactions between the accessible methyl groups and apolar side chains of the protein may be implicated.

TABLE 7.7

Effects of the isomeric propanediols on collagen renaturation at 15°C, as monitored by 1 hour optical rotation regain values

Solvent	polar atoms / carbon atoms	$-[\alpha]_{365}^{15^\circ}$ at 1 hour and 5 molar
propane-1, 3-diol	0.67	830
propane-1, 2-diol	0.67	777

DENATURATION

Investigation of the influence of the various organic solvents on Tm has, in many cases, provided the most readily interpretable data concerning the possible mechanisms involved in perturbational activity. The generally most convenient concentration at which to compare

melting temperatures, and relate them to polar atom ratios, was found to be 2 molar. The monoalkyl ethers of ethylene glycol were exceptional, however, since they caused precipitation of the protein at concentrations above 1 molar.

TABLE 7.8

Influence of solvent polar atom/carbon atom ratios of ethylene glycol and derivatives on Tm of collagen

Solvent	polar atoms / carbon atoms	Tm at 1 molar (° C)
ethylene glycol	1.00	39.25
2-methoxy-ethanol	0.67	38.95
control	---	38.90
2-ethoxy-ethanol	0.50	38.05
2-butoxy-ethanol	0.33	35.30

Table 7.8 shows that ethylene glycol raises Tm to a value appreciably above that of the control, even at low concentration, whereas the methyl ether raises Tm only slightly. In contrast, the higher alkyl derivatives cause a progressively greater reduction of Tm. The fact that ethylene glycol has one more potential donor hydrogen atom than do the derivatives, is apparently of lesser significance than the presence of additional hydrocarbon in the molecules of the latter. A simple relationship between perturbant polar atom/carbon atom ratios and Tm is evident. This in itself, however, does not give any indication of the direct cause of destabilization with increasing hydrocarbon content.

TABLE 7.9

Influence of solvent polar atom/carbon atom ratios of unsubstituted polyols on Tm of collagen

Solvent	polar atoms / carbon atoms	Tm at 2 molar (°C)
glycerol	1.00	40.59
ethylene glycol	1.00	39.65
diethylene glycol	0.75	40.39
propane-1, 3-diol	0.67	39.51
control	---	38.90
propane-1, 2-diol	0.67	38.56
butane-1, 4-diol	0.50	38.46
butane-1, 3-diol	0.50	38.32
butane-2, 3-diol	0.50	37.07
pentane-1, 5-diol	0.40	36.62
hexane-1, 6-diol	0.33	35.20

Table 7.9 shows the close relationship between polar atom content of the unsubstituted parent polyols and their influence on Tm. Glycerol, ethylene glycol and diethylene glycol, which have high polar atom ratios, cause a considerable elevation of Tm above that of the control. The fact that diethylene glycol raises Tm to a greater extent than does ethylene glycol, despite the lower polar atom/carbon atom ratio of the former, is probably due to the larger (± 70%) molar volume of diethylene glycol. It has been argued, with respect to catechin-cellulose interactions, that high polar atom/carbon atom ratios are commensurate with a high degree of direct solvation of the substrates by organic solvent molecules; this becoming most evident at high solvent concentrations. The reverse is seen to apply to the influence

of these solvents on the Tm of collagen, since it is clear that they caused stabilization of the native structure (Table 7. 9). This effect was maintained, up to the highest accessible molarities, as shown in Table 7. 10.

TABLE 7. 10

Effects of high concentrations of solvents with high polar atom/ carbon atom ratios on the Tm of collagen

Solvent	polar atoms / carbon atoms	Molarity	Tm (°C)
glycerol	1. 00	10	49. 45
ethylene glycol	1. 00	10	42. 21
diethylene glycol	0. 75	7	44. 22
propane-1, 3-diol	0. 67	5	41. 55

It appears that whereas direct solvation by organic solvents, with high polar atom ratios, successfully disrupts catechin-cellulose hydrogen bonds, this type of direct solvation is unable to perturb the highly co-operative triple helical structure of native collagen. This leads to the additional implication that interactions other than hydrogen bond formation may be involved in the stabilization of the triple helix. Under these circumstances, the main effect of reducing the polar atom concentration in the liquid environment would be an enhancement of the highly co-operative system of polar interactions in the protein. The non-interference with apolar stabilization mechanisms by solvents which have high polar atom/carbon atom ratios, such as glycerol, ethylene glycol, diethylene glycol and propane-1, 3-diol, could be accounted for in terms of (1) the steric inaccessibility of the hydrocarbon portions of these molecules, and (2) the reduction of the polar atom concentration in the liquid environment being insufficient to nullify the tendency of apolar groups

of the protein to associate with one another by hydrophobic interaction. The reduction in environmental polar atom concentration would, however, be sufficient to enhance electrostatic stabilization of the highly co-operative structure.

The question of steric accessibility is regarded as being of major importance. Returning to Table 7.9, the relevance of steric considerations, to the effects of solvent on Tm, is apparent from the marked difference in the action of propane-1, 3-diol and its isomer, propane-1, 2-diol. It appears that the greater steric accessibility of the terminal methyl group of the 1, 2-isomer may be responsible for destabilising the collagen structure. A similar order of effects is apparent with the isomeric butanediols, where increasing exposure of the carbon chain results in increasing destabilization of the protein. A more detailed assessment of the significance of these results is possible when the effects of the isomers on the protein systems are compared with their influence on the chromatographic mobility patterns, as shown in Table 7.11.

TABLE 7.11

Comparison of effects of butanediol and propanediol isomers on Tm of collagen and mobility of catechin on cellulose

Solvent	$\frac{\text{polar atoms}}{\text{carbon atoms}}$	effect on Tm at 2 M (°C)	Rf at 90% v/v
propane-1, 3-diol	0.67	+0.61	0.88
propane-1, 2-diol	0.67	-0.34	0.82
butane-1, 4-diol	0.50	-0.44	0.86
butane-1, 3-diol	0.50	-0.58	0.80
butane-2, 3-diol	0.50	-1.83	0.78

Positive Tm values represent those higher than the control, and the negative values those lower than the control.

It has been argued that the effects of high concentrations of organic solvents on chromatographic mobility must be due to direct masking of catechin-cellulose polar interactions, since the concentration of water is so reduced as to largely eliminate its role as a solvating species. Mobility patterns at a concentration of 90 volumes percent should, therefore, give an indication of the comparative hydrogen bonding capacities of isomers. Since it appears that propane-1, 3-diol more effectively masks catechin-cellulose interactions than does propane-1, 2-diol, it is assumed that the terminal disposition of the hydroxyl groups of the 1, 3-isomer allows for a statistically greater potential for the formation of hydrogen bonds between the solvent and the catechin-cellulose system. (This suggestion has already been discussed in detail.) Consequently, it is regarded as significant that propane-1, 3-diol raises Tm whereas propane-1, 2-diol lowers it. This result is in direct contrast to what would be expected if direct hydrogen bond formation between solvent and protein was the dominant interaction mechanism, since this would require that the 1, 3-diol be the more effective destabilizer. Similarly, the isomeric butanediols show the reverse order of effects in the protein systems as compared to the chromatographic profiles. Thus, in sequence, butane-1, 4-diol, butane-1, 3-diol and butane-2, 3-diol show progressively diminishing effectiveness as promoters of chromatographic mobility at high solvent concentrations, but progressively greater ability to destabilize the native structure of collagen. This supports the suggestion that direct polar interaction between organic solvent molecules and the protein might not be the only, or even the dominant, destabilization mechanism.

In terms of the theoretical approaches adopted in the foregoing discussion, there appear to be two possible mechanisms which may account for the observed effects of the isomeric propanediols and butanediols. The first is that described in relation to the

chromatographic behaviour of catechin at low to moderate concentrations of organic solvents. Thus, perturbant effects could be regarded as a function of the influence of the polar atom concentration of the liquid environment on the ability of water molecules to exchange with that environment. The second mechanism that must be considered is one involving apolar interactions between organic solvent molecules and hydrophobic side chains of the protein. The introduction of sterically accessible non-polar groups into the environment would competitively reduce the tendency for apolar side chains of the protein to stabilize the triple helix through mutual hydrophobic interaction. In those cases where steric accessibility of perturbant hydrocarbon chain is reduced by the presence of terminally situated polar groups, as in butane-1, 4-diol and propane-1, 3-diol, the tendency towards destabilization by apolar interaction is diminished, and may in fact disappear.

It seems likely that both mechanisms outlined above might complement one another at low concentrations of organic solvents, since the concentration of water in the system will be relatively high. At high solvent molarities, however, the virtual elimination of the perturbant effects of water would lead to an opposing tendency, which may reverse the observed trends, owing to the enhancement of co-operative polar interactions within the triple helix.

The remaining problem is to assess the relative magnitudes of the contributions of these two mechanisms, and thereby estimate the extent to which hydrophobic interactions are involved in the stabilization of the triple helix. In Chapter 1, it was suggested that the stabilization of the triple helix by hydrophobic interactions seemed unlikely, in view of the orientation of all apolar side chains away from the helix axis. This argument will now be examined in relation to the implications arising from Table 7. 12.

TABLE 7.12

Comparison between the relative effects of isomers on Tm of collagen and Rf of catechin on cellulose

Solvent	Tm at 5 M (°C)	△ Tm	Rf at 5 M	△ Rf
propane-1, 3-diol	41.55	3.51	0.72	0.06
propane-1, 2-diol	38.04		0.66	
butane-1, 4-diol	39.73	5.43	0.79	0.01
butane-1, 3-diol	38.56		0.78	
butane-2, 3-diol	34.30		0.78	

The values △ Tm and △ Rf represent the total range over which the respective parameters vary when comparing the most effective and least effective isomers.

A notable feature of Table 7.12 is the contrast between the large variations in Tm and the very small variations in Rf arising from isomerism. The situation suggests fundamental differences in the mechanism of interaction of the organic solvents with the substrates in the two types of systems. Moreover, if polar interaction mechanisms were the dominant processes in both cases, the expected order of effects would be the reverse of that observed, since the co-operatively hydrogen-bonded structure of the triple helix is assumed to be less sensitive to polar-group mediated destabilizing influences than is the simpler catechin-cellulose system.

Finally, a comparison between the effects of diethylene glycol and butane-1, 4-diol on the Tm of collagen and the mobility of catechin on cellulose (Figures 4.5 and 5.5, respectively) confirms the tendencies described above. It is clear that diethylene glycol

strongly stabilizes the native state of collagen at low perturbant concentrations, whereas butane-1, 4-diol does not (Figure 4. 5). In contrast, diethylene glycol destabilizes catechin-cellulose interactions to a considerably greater extent than does butane-1, 4-diol (Figure 5. 5). The latter situation implies increased polar interaction potential in the case of diethylene glycol, which has a higher polar atom/carbon atom ratio than does butane-1, 4-diol. It appears anomalous, therefore, that diethylene glycol should stabilize native collagen molecules to such a marked degree. This suggests that apolar interaction may be the critical factor in such cases. The longer unbroken butylene chain of the 1, 4-diol may be capable of a higher order of apolar interaction with non-polar amino acid side chains of the protein than are the short ethylene chains of diethylene glycol. Thus, destabilization of the protein through apolar interactions between perturbant molecules and amino acid side chains might render butane-1, 4-diol a more powerful destabilizer than is diethylene glycol, despite the apparently lower polar interaction potential of the former.

The foregoing evidence appears to support the contention that apolar interactions between organic solvent molecules and amino acid side chains may be an important factor in the perturbation of the collagen triple helix. The necessary corollary to this conclusion is that hydrophobic interactions contribute significantly to the stabilization of the monomeric tropocollagen macromolecule. Recent work (Bianchi et al., 1970) tends to substantiate the suggestion that hydrophobic stabilization mechanisms might contribute to the stability of the collagen monomer in solution.

Uncertainty with respect to the above interpretation arises in connection with the possibility that collagen molecules in solution may exist in forms other than the simple monomeric state. (Davison and Drake, 1966; Davidson and Cooper, 1967; Davidson and Cooper, 1968). If dimers and higher aggregates are

present, and if the stabilization of such aggregates is partly due to apolar interactions among the peripherally disposed amino acid side chains, explanation of the experimental findings would not require the assumption that apolar interactions contribute significantly to the stabilization of the monomeric triple helix. It seems more probable, however, that the existence of dimers in dilute solution is a function of electrostatic, rather than hydrophobic interaction mechanisms. Consideration of phase equilibria involving highly asymmetric rodlike polymer units (Flory, 1961) indicates that, in dilute solution, apolar interparticle interactions between native three-stranded helices are of a very low order. Transition from the native to the denatured state appears to be largely a function of the collapse of the triple helix itself.

PART II

THE KINETICS OF RENATURATION

In order to interpret the significance of changes in the kinetic parameter, log $y_{\infty}^2 . k$, made use of in the present work, regard must be had to the likely sequence of events during the early stages of renaturation. The three-stage model for renaturation, consisting of (1) nucleation of the poly-L-proline II type helix, (2) propagation of the helical form along individual chains, and (3) formation of the triple helix by specific hydrogen bond formation between chains, has been shown to be subject to certain serious objections (Chapter 3). In particular, the work of Beier and Engel (1966) and Piez and Carrillo (1964) suggests that renaturation to the most completely native state is a concentration-dependent phenomenon. This implies that steps (1) and (2) are functions of step (3), and not independent of it, as proposed by von Hippel and Harrington (1959). Thus, the exact mechanism of the renaturation process is imprecisely defined. Various possible implications arising out of the kinetic observations recorded in Chapter 6 will now be examined.

The marked improvement in the correlation between 1 hour $[\alpha]$ values and log zero-time reversion rates, upon raising the renaturation temperature from 15°C to 20°C, suggests a modification of the mechanism of renaturation. In terms of the proposals of Beier and Engel (1966), this could imply a movement towards the formation of a higher proportion of truly native collagen helices, as opposed to other possible uni- or multi-stranded arrangements of poly-L-proline II type structures. It seems possible that the more uniform reversion to the strictly native-type structure, which might result from renaturation at higher temperatures, could account for an improved linear relationship between fixed-time $[\alpha]$ values and zero-time reversion rates. In contrast, the kinetic situation appears to be more complex at the lower renaturation temperature. This is assumed to result from an increase in the degree of competitive equilibrium between the re-forming native triple helical structures and non-native

aggregates of poly-L-proline II type helices. Conceivably, this could destroy the register between zero-time reversion rates and the attenuated situation at finite time.

The second point of major interest is the apparent non-linearity of the log initial rate v. perturbant concentration plots. There are two distinct groups into which the perturbants may conveniently be divided, namely, stabilizers and destabilizers (as defined in Chapter 6). These terms refer to the effects of perturbants on collagen in the native state, however, and it becomes necessary to reconcile their use with the observed effects of these solvents in renaturing systems. It has been shown (Harrington and von Hippel, 1961b; von Hippel and Wong, 1963a) that changes in the nature of the solvent that result in an increased initial mutarotation rate are associated with an increase in Tm in certain cases. In the present work, however, although initial rate v. concentration curves showed an apparent acceleration of mutarotation rate at low concentrations of certain perturbants, this was followed by sharp trend reversals. Thus, an initial enhancement of the rate of poly-L-proline II type helix formation is indicated, but this is subsequently overcome by a supervening effect as perturbant concentration is increased. This pattern may be interpreted by assuming that perturbants that elevate Tm above the control value, do so because they are less powerful polar-bonding agents than is water. Hence a simple diluting effect, which lowers the dielectric constant of the solvent mixture, dominates at low concentrations of the organic component. The resulting stabilization of the native collagen structure is due to (1) enhanced interchain hydrogen bonding within the triple helix, and (2) reduced frequency of polar interactions between molecules in the environment and peptide carbonyl and imide groups in the protein, thus further restricting the already limited rotation about peptide bonds.

Extending the above approach to renaturing systems, it is to be expected that the reduced polar atom content of the environment should result in enhanced formation of the poly-L-proline II conformation both by virtue of statistically reduced bonding of environmental molecules to peptide carbonyl and imide groups, and by diminished competitive disruption of the native and non-native tertiary structures which probably serve to stabilize the poly-L-proline II type configuration. At higher concentrations of stabilizing perturbants, however, it is conceivable that random intrachain folding could become so prevalent as to reduce the extent and the rate of formation of the poly-L-proline II type helix. Thus, the formation of doubled or tripled back, non-native conformations of individual polypeptide chains (Drake and Veis, 1964), might progressively give way to multiple self-association of chains. In the resulting pseudo-globular structures, the high degree of coiling might actually reduce the potential for the formation of poly-L-proline II type helices. There is also the possibility that this type of inhibition process could arise due to random <u>inter</u>chain aggregation, on much the same basis as that suggested above for intrachain association.

This theoretical approach is consistent with the explanation of the effect of stabilizers with respect to the molecule in the native state. The pre-existing native triple helix would be progressively stabilized by reducing the polar perturbational potential of the environment. In contrast, a sufficiently large change of this nature, in the constitution of the environment, could have completely different consequences for a renaturing system by promoting random aggregation processes to an extent incompatible with the formation of poly-L-proline II type helices.

Different considerations arise with respect to the effects of

perturbants that destabilize the native state of the protein. The destabilizers most closely studied in the present work were propane-1, 2-diol, butane-1, 4-diol and 2-ethoxy-ethanol. Since these perturbants lower the Tm of the native state, it is to be expected that they would retard the renaturation process, which they clearly do (Figures 6.6, 6.7, 6.8 and 6.10, respectively). The apparent non-linearity of the log initial rate v. concentration curves, however, implies the operation of a mechanism other than, or additional to, direct interaction between perturbant and protein. A linear relationship between rate and concentration, that is the absence of any lag phase, may be taken to imply the existence of a direct binding mechanism (von Hippel and Wong, 1963a). Therefore, the appearance of a noticeable lag in the curves at present under consideration, appears to require the postulation of an indirect or dual mechanism. Such a mechanism could possibly be formulated in the following terms. (1) In the presence of low concentrations of a destabilizing perturbant, in a renaturing system, retardation of the rate of formation of poly-L-proline II type helices could simply be due to direct polar interactions between perturbant hydroxyl groups and polar groups in the polypeptide chains. This inference seems the more likely in view of the predominantly aqueous nature of the environment, when the concentration of organic component is low, since the tendency of perturbant molecules to re-enter the bulk of the solution, after associating with protein molecules, could be expected to be reduced. This, essentially, is the hydrophobic shielding mechanism discussed in detail in Chapter I. (2) The additional factor which might account for the accelerating rate of lyotropic activity, as perturbant concentration is increased, is the increased polar interaction capacity of water molecules as the general dielectric constant of the medium is lowered. Thus

the rate of exchange with the environment, of water molecules at polar sites in the polypeptide chains, would decrease as the polar atom content of the environment is reduced. This protraction of the duration of water-polypeptide associations would result in further retardation of the renaturation process.

The most obvious objection to the above approach arises out of mass action considerations, since the concentration of the increasingly active species, namely water, is decreasing at the same time as the hydrogen bonding capacity of water molecules is increasing. It seems reasonable to suppose, however, that the mass action effect might become dominant only at low water concentrations, at which a trend deceleration should appear. In the present study, this effect was not observed, since perturbant concentrations could not conveniently be increased to include such limiting cases in the renaturing systems. It has already been noted, however, that denaturing systems frequently show trend reversals of this nature at high proportions of organic solvents.

In relation to the foregoing suggestions, work on the effects of solvent composition on the helix content of ribonuclease is regarded as significant (Weber and Tanford, 1958; Atsushi and Noda, 1968). It has been shown that the effects of the addition of 2-chloroethanol to aqueous solutions of ribonuclease may be divided into two clear stages. The first stage involves a decrease in helix content with increasing chloroethanol concentration, reaching a limiting (low) value when the concentration of the organic component is 10 to 20 moles percent (30 to 50% by weight). At higher chloroethanol concentrations, however, a trend reversal appears and helix content begins to rise, becoming progressively higher as the chloroethanol content of the system approaches 100%. The helicity of ribonuclease in almost pure 2-chloroethanol is of the order of 70%, in contrast to the less than 20% helix content

of the protein in pure water. These findings may readily be interpreted in terms of the mechanism discussed above. Thus, when the concentration of 2-chloroethanol is increased, the hydrogen-bonding activity of water is enhanced. The effect reversal beyond an optimum concentration of organic component is simply a function of the increasing scarcity of environmental water, mass action considerations dominating the primary process beyond this stage.

The final possibility that must be considered, in relation to these systems, is that perturbant effects are a direct function of apolar association between perturbant molecules and protein side chains, and that direct <u>polar</u> perturbant-protein interactions are of secondary importance. Thus, with respect to the influence of 2-chloroethanol on the helix content of ribonuclease, the following model seems possible. (1) Addition of the organic solvent results in perturbation of the tertiary structure of the protein through hydrophobic interaction between perturbant molecules and apolar side chains of the protein. (2) Destabilization of the tertiary structure of ribonuclease reduces the stability of the secondary structural elements, thus reducing the α-helical content. (3) Simultaneously, the reduction in dielectric constant, which accompanies the increase in concentration of the organic component of the solvent mixture, would result in enhanced inter-peptide hydrogen bonding. This would favour the formation of extensive regions of α-helix, that is secondary structure, despite the absence of a significant degree of tertiary structural organization. Tertiary structure is assumed to be greatly reduced or absent, since its primary causation, the unfavourable free energy of interaction of apolar side chains with water (Tanford, 1970), must tend to disappear as the composition of the environment approaches the pure organic solvent extreme.

The above model may readily be adapted to account for the observed trends in the renaturing collagen systems. Thus, destabilizing perturbants might retard renaturation by direct apolar interaction with collagen side chains. Assuming the formation of poly-L-proline II type helical elements to be a co-operative rather than a stepwise process (Beier and Engel, 1966), the apparent non-linearity of the initial rate v. concentration profiles is readily explained in terms of a suggestion by Bello (1963). This postulates that partial denaturation may render protein structures more labile, resulting in apparently greater lyotropic effectiveness as perturbant concentration is increased. It seems reasonable to expect, therefore, that the partial inhibition of the renaturation process, in a co-operatively forming structure, might cause an increased sensitivity to the action of additional amounts of destabilizing perturbants.

ADDED IN PROOF

Since the completion of this work, a series of papers reporting kinetic data for various natural and modified collagens has appeared (Harrington and Karr, 1970; Harrington and Rao, 1970; Hauschka and Harrington, 1970a, b, c). The findings support the suggestion (Beier and Engel, 1966) that inter or intra-chain interactions are necessary for the generation of poly-L-proline II type helical elements in collagen, in contrast to the three-stage mechanisms proposed by Harrington and von Hippel (1961b) and Flory and Weaver (1960). In addition, it is confirmed that renaturation at low temperatures ($< 15^{o}C$) favours random polar aggregation processes that reduce the extent to which poly-L-proline II type helices are formed. This substantiates the approach adopted in the present work, where the apparently

anomalous action of high concentrations of stabilizing perturbants in reducing mutarotation rates, was interpreted in terms of the formation of inter and intra-chain aggregation states in which the degree of folding is in excess of that which is compatible with the regeneration of poly-L-proline II type helices.

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