Structural and Functional Factors in the Hydrogen Bonding of Polar Organic Solvents to Acid-Soluble Collagen. Effect on Renaturation Kinetics and Thermal Stability*

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ABSTRACT: The effect of a number of polar organic solvents, including aliphatic alcohols, ethers, ketones, and nitriles, on the renaturation kinetics and stability of acid-soluble calfskin collagen has been investigated. The solvents reduced the rate of mutarotation and viscosity recovery following heat denaturation and cooling and lowered the thermal transition temperature of native collagen. These effects increased with solvent concentration, hydrocarbon chain length, and polarity. Final viscosity regain, however, was enhanced progressively in solvents of increasing hydrocarbon chain length, but decreased in solvents with branched-chain structure. Examination of the quantitative effects on the collagen system indicated that a modified Arrhenius expression satisfactorily described the relationship between renaturation rate and thermal stability in the various solvent media, consistent with a generalized mechanism of solvent–protein interaction. As distinct from hydrophobic interaction, a mechanism is proposed in which the solvent hydrocarbon structure exerts local shielding from environmental water at the polar group in the same molecule, thereby stabilizing solvent hydrogen-bonding interactions at polar groups in collagen. In terms of current views of collagen stabilization, increased solvent binding at the carbonyl and imide groups of collagen may result in (a) increased rotational freedom about main polypeptide bonds in addition to (b) disruption due to competitive solvation of structural hydrogen bonding between polypeptide chains, thereby accounting for solvent-mediated changes in thermal stability and renaturation kinetics.

The influence of solvent environment on the nature and stability of the equilibrium conformations adopted by a variety of proteins (Singer, 1962; Von Hippel and Wong, 1963, 1965; Von Hippel, 1967; Ikai and Noda, 1968) and synthetic polypeptides (Engel, 1967; Traub *et al.*, 1967; Veis *et al.*, 1967) is well established. The use of solvent perturbants as structural probes in the study of conformational changes and transformation kinetics arises from the fact that, to a large degree, the forces implicated in protein conformational stability and protein–solvent interactions are similar in nature. Thus, systematic changes effected in the competing solvent environment may be correlated with resultant shifts in protein conformation and stability to provide an insight into the nature of the forces involved. These include hydrophobic and hydrogen bonding in particular, while electrostatic interactions may also be implicated. In spite of differences of detail in conformational changes in various proteins, certain features of nonspecificity with respect to protein have emerged which permit a general ranking of a wide range of electrolytes and nonelectrolytes in order of increasing lyotropic effect (Von Hippel and Wong, 1963–1965). This lack of specificity suggests that a common interaction mechanism may be involved; due to the variety of structures and interactions which may occur, however, little progress has been made toward the establishment of a unified or systematized theory.

The structural regularity and limited number of stabilizing factors involved in the collagen system appear to confer some advantages in the systematic study of protein–environment interactions in comparison with globular proteins. General agreement has been reached on the main structural features (Ramachandran, 1967) and considerable progress has been made toward an understanding of the processes of renaturation and stabilization of the collagen structure (Harrington and Von Hippel, 1961; Von Hippel, 1967; Veis, 1967). The collagen molecule has a comparatively regular, rodlike structure consisting of three poly-L-proline II type helices wound around a common axis to form a superhelix. The main stabilizing interaction in the superhelix or tertiary structure is that due to the cooperative system of lateral hydrogen bonds between the component polypeptide helices, whereas the secondary structure of the individual chains is maintained by the rigidity conferred by the rotational restrictions which exist at the α-carbon to carbonyl–carbon bonds of pyrrolidine residues and the partial double-bond character of the peptide bonds. The distinction between these stabilizing factors is formal since both act cooperatively in determining structural stability. Since the polar and neutral side chains cannot be accommodated within the helical structure, these appear to be largely excluded from participation in direct, stabilizing interactions in individual molecules. In insoluble, fibrous collagen, however, considerable additional stability may be derived from intermolecular interactions attributable to side-chain, electrostatic, and hydrophobic bonding.

Aqueous aliphatic alcohols and other polar solvents have been shown to lower the shrinkage temperature of insoluble collagen (Jordan Lloyd and Garrod, 1948; Schnell and Zahn, 1965) at low solvent concentrations with stabilization occurring at higher solvent proportions. The destabilization effect

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increased with solvent hydrocarbon content, "linear" chains being more effective than "branched" chains, and has been attributed to the disruption by the organic solvent component of intermolecular hydrophobic bonds. Similar decreases in the denaturation temperature of acid-soluble collagen, observed in aqueous alcoholic media, have also been attributed to the influence of the alcohols on hydrophobic interactions implicated in intramolecular stabilization (Schnell, 1968); in the same study, 1-propanol was shown to retard mutarotation recovery observed on cooling heat-denatured, acid-soluble collagen. Herbage *et al.* (1968) have also attempted to correlate the effects of various water–alcohol and water–amide mixtures on transition temperatures in acid-soluble collagens with possible solvent–protein interactions.

Recently, studies have been carried out on solvent interactions in simple amides and substituted amides, serving as peptide-bond analogs, which are themselves capable of exerting conformation-regulating effects on polypeptides and proteins (Chao *et al.*, 1966; Veis *et al.*, 1967). These studies have suggested that direct hydrogen bonding of solvent components to peptide bonds may be responsible for conformational transitions in gelatin by their effect in promoting rotational freedom about the main bonds of the polypeptide chains. Such a mechanism would also be consistent with the solvent-promoted reverse mutarotation in the polypeptide collagen model, poly-L-proline II, lacking intramolecular hydrogen bonds, in which *cis–trans* isomerization at the peptide bonds occurs on addition of 1-propanol and 1-butanol (Steinberg *et al.*, 1958). Further evidence for direct solvent binding at peptide bonds in collagen is derived from the capacity of alcohols and other polar solvents, including amides, to displace bound polyphenolic vegetable tanning constituents known to hydrogen bond to the peptide groups in collagen (Merill *et al.*, 1947; Russell *et al.*, 1968). In view of the growing evidence for the influence of direct peptide bond solvation in protein conformational changes, a systematic examination of the effects of a number of aqueous polar solvents on collagen renaturation and stability has been undertaken in the present study.

Materials and Methods

Preparation of Acid-Soluble Collagen. Purified calfskin acid-soluble collagen was prepared by the method described previously (Cooper and Davidson, 1965) after removal of the neutral salt-soluble fraction.

Reagents. All solvents and reagents were commercial AR grade or Laboratory grade materials.

Optical Rotation. Optical rotation was measured in 10-cm, water-jacketed tubes using a Perkin-Elmer Model 141 polarimeter at 365-mμ wavelength.

Viscosity. Viscosities were measured in Canon-Fenske flow viscometers (sizes 50 and 100, B.S. 188).

Renaturation. Renaturation curves at 15 ± 0.05° were measured by polarimetry and viscometry over 48 hr. Acid-soluble collagen in 0.15 M potassium acetate buffer (pH 4.8) was heat denatured at 45° for 15 min prior to addition of the predetermined volumes of solvent and dilution to volume with buffer to give a final concentration of 0.86 mg of dry protein/ml. The solution was then transferred as rapidly as possible to the polarimeter tube and viscometer and the reversion was monitored at intervals.

Denaturation. Melting curves were obtained by polarimetry using the "30-min method" (Von Hippel and Wong, 1963), the temperature at the midpoint of the transition being taken as the melting point.

Results

Effect of Aliphatic Alcohols. Typical optical rotation and viscosity regain profiles obtained by renaturation in the presence of 1.0 M methanol, ethanol, 1-propanol, and 1-butanol are shown in Figure 1a,b. A progressive decrease in the initial rate of optical rotatory and, to a lesser extent, viscosity regain with increasing linear hydrocarbon chain length was apparent, with a further slow increase evident at 48 hr which suggested that the various optical rotation curves would converge with that of the control in an extended study. Viscosity recovery after 48 hr was lower than the control in the case of methanol, with a progressive increase occurring with increase in chain length. In the case of 1-propanol and 1-butanol, viscosity values exceeded that of the control after 1 hr approximately, and were still increasing appreciably after 48 hr so that a close approach to equilibrium values would require a considerably extended reaction time.

The effect on renaturation profiles of aliphatic alcohols at various low concentrations covering the restricted range of miscibility and protein solubility is shown in Figure 2a,b in which the changes in optical rotation and reduced viscosity after 1- and 48-hr reaction periods, respectively, are plotted as a function of concentration for both the linear and branched-chain homologs. Linear plots were obtained for initial reversion values, but final recoveries showed a curved relationship. The effect of each alcohol increased progressively with concentration over the range examined, the branched-chain members being less effective than the corresponding linear homologs. Initial mutarotation recovery and final viscosity regain were regions of the reaction curves most affected by the presence of alcohols. In general, a large retardation in the initial rate of mutarotation recovery in the presence of straight-chain hydrocarbon alcohols was followed by a corresponding increase in the final reduced viscosity value.

Other Polar Solvents. Qualitatively similar changes in the optical rotatory and viscometric renaturation profiles were apparent in the case of other aqueous polar solvents examined, namely, acetone, methyl ethyl ketone, acetonitrile, propionitrile, diethyl ether, and 1,4-dioxane. These effects are shown in Figure 3a,b in which initial and final values are plotted as a function of concentration as in the case of the alcohols. Linear plots were again obtained for initial reversion rates, although final recoveries showed a curved relationship. The effect of the various solvents increased progressively with concentration, except in the case of methyl ethyl ketone which showed a slight maximum in 48-hr viscosity recovery at intermediate concentration. From comparison of the solvent pairs, acetone–methyl ethyl ketone and acetonitrile–propionitrile, the effect of increasing chain length was found to be qualitatively similar to the alcohols indicating that the effect was independent of the particular functional group present.

Effect of Hydrocarbon Structure. The effect of increasing hydrocarbon content on renaturation and thermal stability in the presence of various solvents at 1.0 M concentration is shown in Figure 4a,b in which the initial mutarotation recovery and the thermal denaturation temperature of native acid-soluble collagen are plotted against the number of carbon atoms in

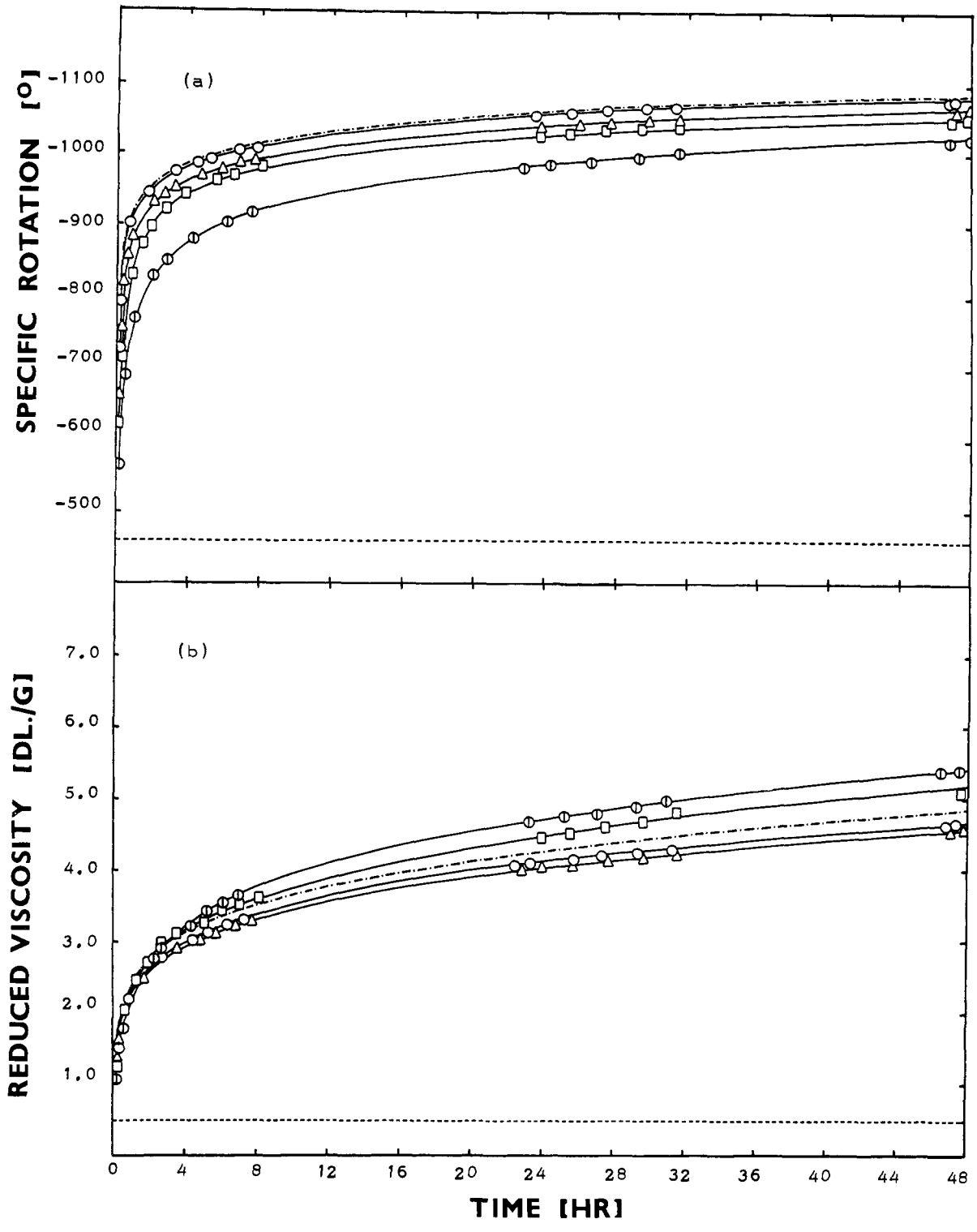

FIGURE 1: Effect of aliphatic alcohols at 1.0 M concentration on renaturation profiles of heat-denatured (45°; 15 min) acid-soluble collagen in 0.15 M potassium acetate buffer. (a) Specific rotation recovery; (b) reduced viscosity recovery. —·—, average control (pH 4.86); ○, methanol (pH 4.92); △, ethanol (pH 4.94); □, 1-propanol (pH 4.97); ◌, 1-butanol (pH 4.99) broken line indicates lower limit for gelatin at 45°. Upper limit: specific rotation, −1330°, reduced viscosity at 0.86 mg/ml, 29.5.

the organic solvent. In the case of the ketones, the number of effective carbon atoms was taken as the content of the longest chain present. A trend was apparent in the homologous alcohol series which showed a progressive reduction in the initial rate of mutarotation recovery with increasing linear chain length (Figure 4a), whereas progressive substitution of methyl groups on the α-carbon atom to give the branched-chain homologs, isopropyl alcohol and *t*-butyl alcohol, was less effective. The effect of increasing linear chain length was also apparent in the solvent pairs, acetone–methyl ethyl ketone and aceto-

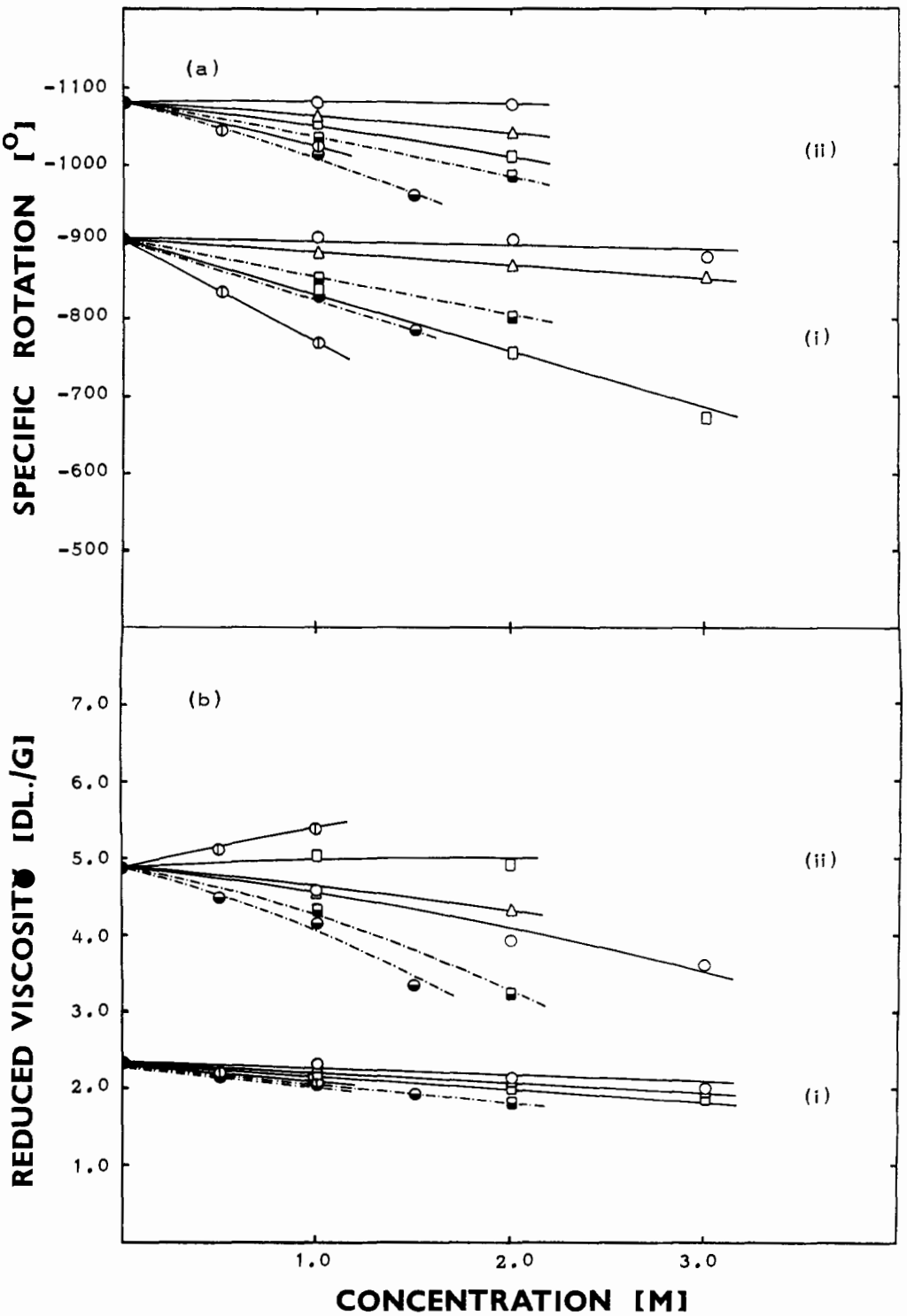

FIGURE 2: Effect of alcohol concentration on (i) initial (1 hr) and (ii) final (48 hr) values for (a) specific rotation and (b) reduced viscosity recovery. (●) Control, (○), methanol, (△) ethanol, (□) propanol, (◪), isopropyl alcohol, (◌) 1-butanol, and (◒) *t*-butyl alcohol.

nitrile–propionitrile, where limited water miscibility did not permit extension of the data. Diethyl ether and 1,4-dioxan with comparable hydrocarbon content were found to be similar in effect.

Parallel effects were found when denaturation temperatures of native, acid-soluble collagen at 1.0 M solvent concentration were plotted on the same basis (Figure 4b), indicating a direct relationship between the 1-hr mutarotation reversion and thermal stability.

The relationship between solvent hydrocarbon content and final reduced viscosity value was examined by plotting the latter against the total number of carbon atoms present (Fig-

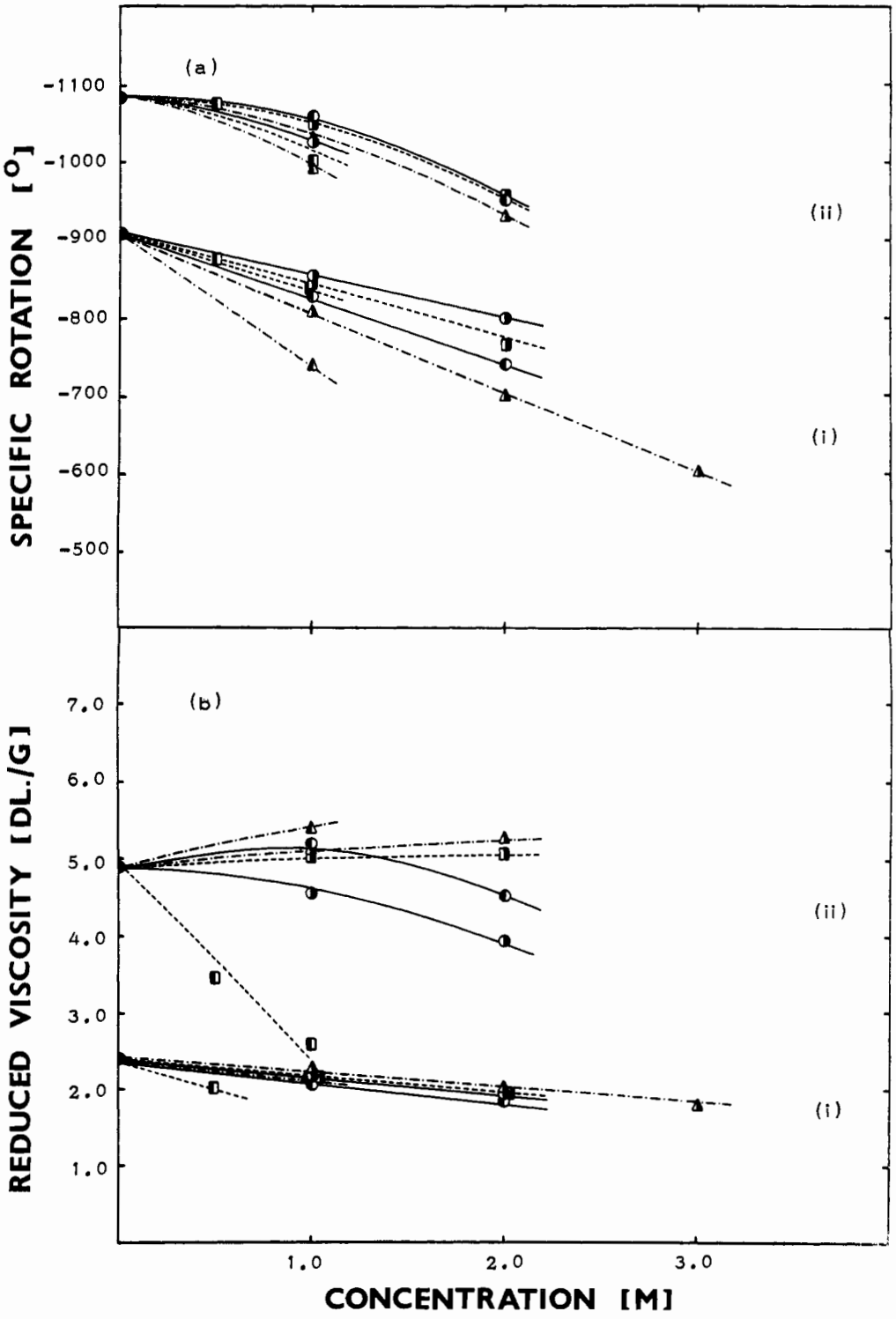

FIGURE 3: Effect of solvent concentration on (i) initial (1 hr) and (ii) final (48 hr) values for (a) specific rotation and (b) reduced viscosity recovery. (●) Control, (◑) acetone, (◐) methyl ethyl ketone, (▲) acetonitrile, (▲) propionitrile, (■) diethyl ether, and (■) 1,4-dioxane.

ure 5). Final values increased progressively with linear chain length while, in the case of the branched-chain (isopropyl alcohol, *t*-butyl alcohol) and double-chain (diethyl ether) solvents, a progressive decrease in final reduced viscosity with increasing hydrocarbon content was apparent. A further feature was the large difference in the effects of diethyl ether and 1,4-dioxane on viscosity which was not apparent in the mutarotation curves.

Effect of Functional Group. Comparison of solvents of comparable hydrocarbon structure (Figure 4a,b) indicated that the particular functional group present exerted a consistent effect on renaturation and thermal stability. In general, functional

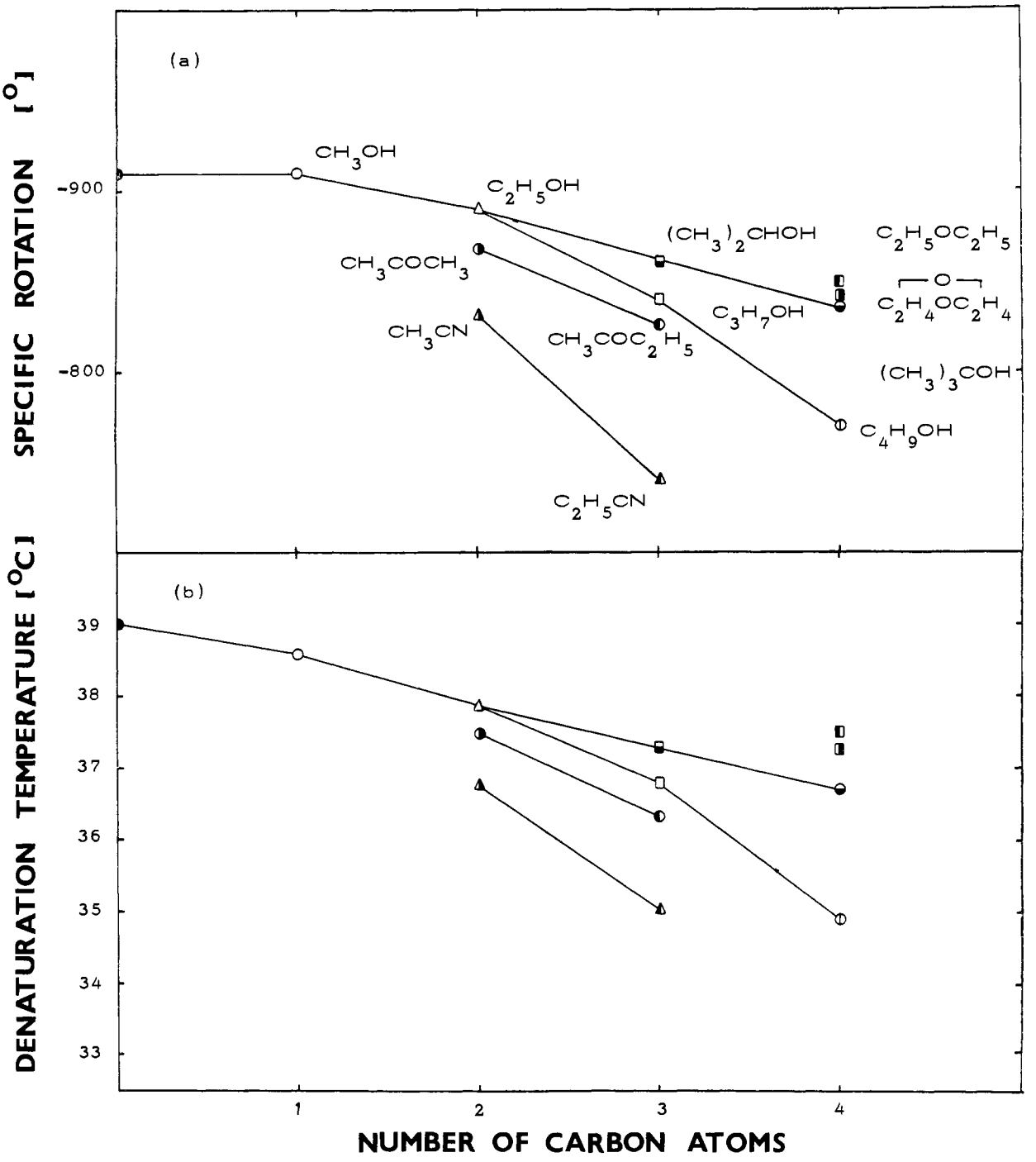

FIGURE 4: Effect of hydrocarbon structure on (a) initial (1 hr) specific rotation recovery and (b) denaturation temperature of native, acid-soluble collagen at 1.0 M solvent concentration.

groups of higher electronegativity had a correspondingly greater effect in reducing renaturation and thermal stability in the order, ethers < alcohols < ketones < nitriles, for solvents of comparable hydrocarbon content and concentration. In Figure 6a,b, initial mutarotation recoveries and thermal stabilities are plotted as a function of the dipole moment of the organic solvent component (McClellan, 1963) as an indication of relative electronegativity of the functional group present. For the simple, monofunctional solvents under consideration, dipole moments are largely determined by the electronegative atom present (Syrkin and Dyatkina, 1950) and an approximate proportionality between electronegativity and dipole moment is assumed.

Linear plots representing average trends have been drawn through the experimental points for solvents of comparable hydrocarbon content to give a succession of lines corresponding to an increasing number of carbon atoms in the linear chain solvents. The distribution of the experimental points

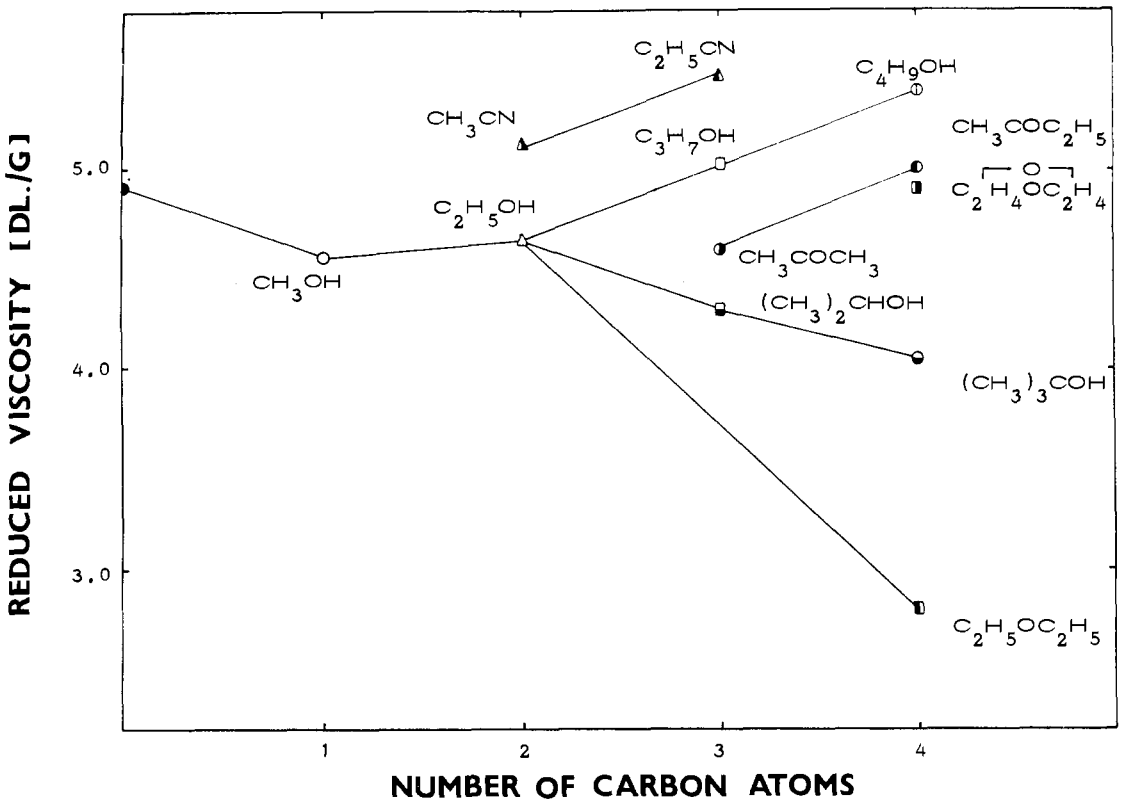

FIGURE 5: Effect of hydrocarbon content on final (48 hr) reduced viscosity recoveries at 1.0 M solvent concentration.

about the average trend lines appeared to support the contention that a direct relationship exists between the observed effects and the degree of electronegativity of the functional group present. Deviations from the linear plots were readily attributable to the influence of branched- and double-chain structure. Thus, isopropyl alcohol and *t*-butyl alcohol were displaced to intermediate positions corresponding to effects smaller than that of the corresponding straight-chain homologs as noted earlier. Similar virtual displacement was apparent in the plotted positions of acetone and methyl ethyl ketone. The position of the double-chain solvent, diethyl ether, also indicated an effect smaller than expected for single-chain compounds of comparable electronegativity and carbon content. In the case of the cyclic ether, 1,4-dioxane, the electronegativity of each oxygen atom was assumed similar to diethyl ether. Comparison with ethanol having the same hydrocarbon to polar atom ratio, suggested an augmented effect conferred by the difunctional character in 1,4-dioxane.

The various solvent effects discussed were similar when either initial mutarotation recoveries (Figure 4a) or denaturation temperatures (Figure 4b) were compared on the basis of solvent dipole moments.

Discussion

Previous workers have reported a progressive increase in the effect of aliphatic alcohols of increasing hydrocarbon chain length in lowering the thermal transition temperature in ribonuclease (Schrier and Scheraga, 1962; Von Hippel and Wong, 1965), the magnitude of the molar effect depending upon the number of effective methylene groups per mole. According to the statistical mechanical treatment developed by Nemathy and Scheraga (1962), an increase in hydrophobic interaction with increasing effective chain length is predictable. This effect has been confirmed in chromatographic studies of organic acid binding to polystyrene where the interaction was shown to be dependent upon the nature and size of the associated nonpolar structure (Steinberg and Scheraga, 1962). These observations have led to the conclusion that the destabilization of globular proteins, observed in the presence of certain polar organic solvents, is attributable to their effect in disrupting intramolecular hydrophobic bonds which contribute to the stability of the native folded conformations, as originally proposed by Kauzman (1959).

Comparable destabilization with increasing hydrocarbon structure has been demonstrated in studies of the effect of aqueous aliphatic alcohols on the thermal transition temperature of insoluble (Schnell and Zahn, 1965) and acid-soluble (Schnell, 1968) collagens and these observations have led to the tentative conclusion that hydrophobic bonding is similarly implicated in collagen stabilization. Although the possibility of intermolecular hydrophobic bonding in fibrous, insoluble collagen cannot be discounted, intramolecular interaction between contiguous nonpolar side chains on the surface of an isolated collagen molecule in solution would appear less probable. In contrast with the globular proteins, the extended, rigid conformation in collagen and the radial disposition of side chains along the length of the molecule, would appear to minimize the possibility of effective, systematic contacts between pairs of nonpolar side chains, particularly as these include a number of compact, branched structures. Moreover, a consistent, quantitative analysis of thermal stability in terms of

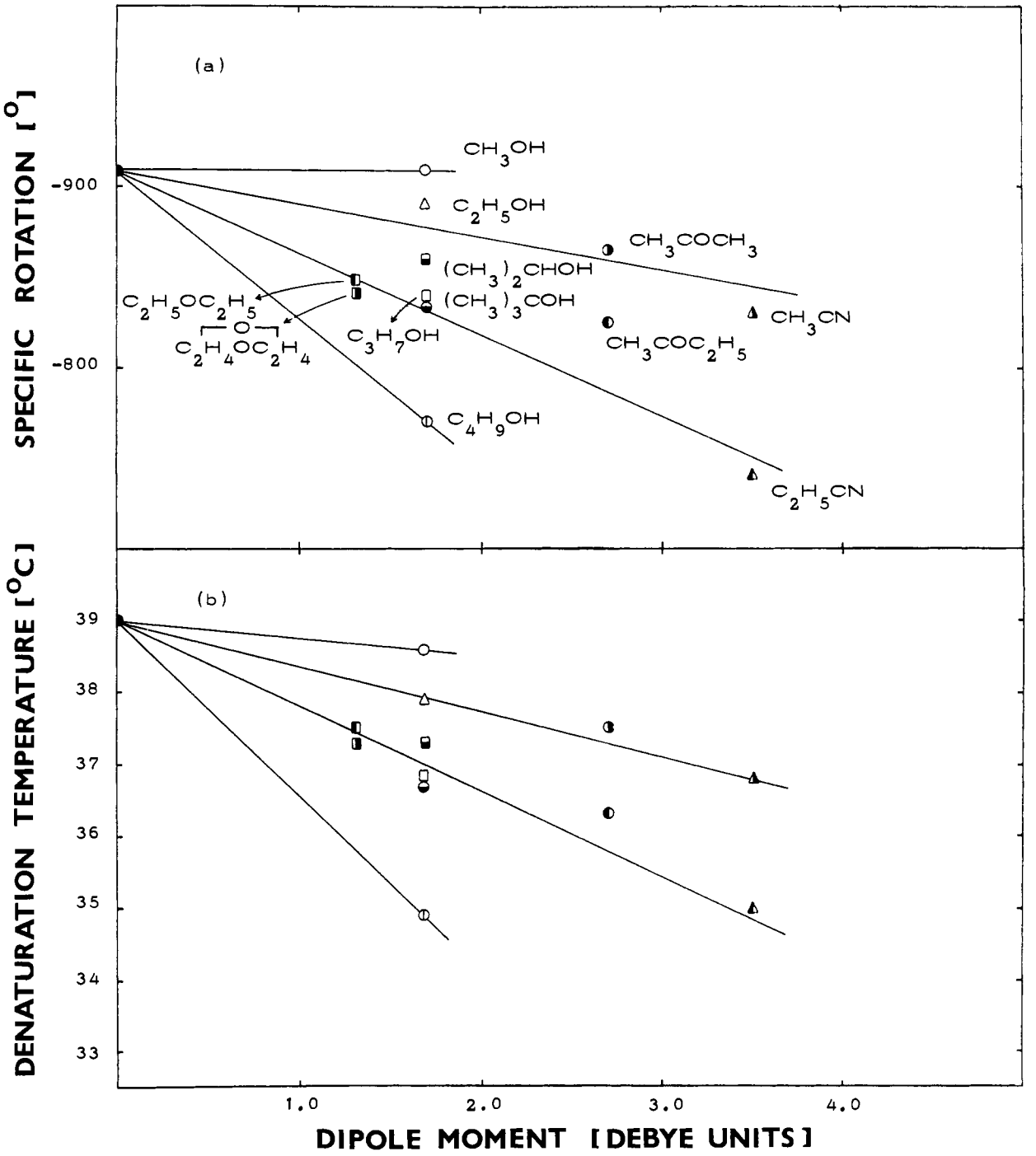

FIGURE 6: Effect of solvent polarity on (a) initial (1 hr) specific rotation recovery and (b) denaturation temperature of native, acid-soluble collagen at 1.0 M solvent concentration.

pyrrolidine residue content has been demonstrated for a range of soluble collagens with a variety of compositions without reference to hydrophobic bonding (Harrington and Rao, 1967).

Recent studies have provided evidence for an indirect effect of local hydrophobic structure on polar interactions which offers an alternative explanation for the effects of aqueous organic solvent media on protein structure. Ultrasonic attenuation measurements of solvent–macromolecule interactions in aqueous polyethylene glycol (Hammes and Lewis, 1966; Hammes and Roberts, 1968) and similar studies in water–dioxane mixtures (Hammes and Knoche, 1966; Hammes, 1968) have led to the proposal that local hydrophobic structure exerts an influence on the immediate aqueous environment which stabilizes hydrogen-bond formation at neighboring polar groups. The effect may be considered to arise from the repulsion of environmental water by the hydrophobic structure thereby shielding polar atoms participating in hy-

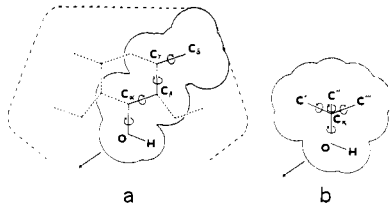

FIGURE 7: Planar projections of carbon skeletons for (a) 1-butanol and (b) *t*-butyl alcohol, showing various extreme chain conformations. Molecular outlines derived from van der Waals' radii for constituent atoms are shown in projection. Arrows denote approximate hydrogen-bonding direction with solvent as proton acceptor. Broken surround (1-butanol) indicates approximate cross-section of volume accessible to hydrocarbon chains due to bond rotations.

drogen bonding from competing water molecules. This effect is consistent with the proposal that hydrophobic structures have a localized, ordering action on water structure, which increases with chain length, and which is also largely responsible for the entropic stabilization of hydrophobic bonds (Nemathy and Scheraga, 1962). Thus, factors which increase hydrophobic interaction can also be considered to enhance hydrogen-bond formation at adjacent polar groups.

Further evidence for the effect of hydrophobic shielding in enhancing hydrogen-bonding activity in polar molecules is derived from studies of the reversible binding to collagen of the polyphenolic constituents of natural vegetable tannins (Russell *et al.*, 1968). The large number of hydroxyl groups present, and their insolubility in nonpolar solvents, predispose these constituents to hydrogen-bond formation at the carbonyl or imide groups of the collagen polypeptide chains. Aqueous mixtures of alcohols, ketones, and nitriles were found to be effective in removing bound tannins, the amounts removed increasing with concentration, hydrocarbon chain length, and polarity. From these studies, it was concluded that the pendant hydrocarbon structure stabilized solvent hydrogen bonding to collagen due to local shielding, thereby influencing indirectly the amounts of tannins competitively displaced.

Examination of the effects of aqueous alcohols and other polar solvents in the present study has shown similarly that the main variables affecting renaturation rates and thermal stability are solvent concentration, hydrocarbon structure, and polarity, factors which, from the above observations, can be considered to influence the extent of solvent hydrogen bonding. Accordingly, as an alternative to hydrophobic interaction, we propose that direct solvent binding at the carbonyl oxygen or imide nitrogen atoms of the constituent polypeptide chains in collagen is responsible for the observed effects of the solvent environment on renaturation and stability. In terms of current views of collagen structure, the effects of such binding may be twofold resulting in (a) reduction in rotational restrictions at main bonds in the polypeptide chains and (b) disruption of structural hydrogen bonding between peptide groups. Both these effects would be consistent with a decrease in renaturation rate and thermal stability of acid-soluble collagen. Evidence for solvent-mediated changes in bond rotation is to be found in studies on conformational changes in synthetic polypeptides, particularly poly-L-proline which lacks hydrogen bonds (Harrington and Von Hippel, 1961; Veis *et al.*, 1967). In order to account for lyotropic effects of neutral salts on keratin, Mandelkern *et al.* (1962) have proposed that direct hydrogen bonding of hydrated ions at peptide carbonyl or imide groups results in electronic shifts in the adjacent peptide bond, to bring about a reduction in double-bond character and an increase in rotational freedom.

Examination of the effect of the lower aliphatic alcohols on collagen renaturation and stability showed that "linear" hydrocarbon chain alcohols had greater lyotropic activity than the corresponding branched-chain homologs. This observation is also consistent with increased hydrogen-bonding activity due to hydrophobic shielding. In Figure 7, the planar projections of the various extreme chain conformations in 1-butanol are compared with a similar projection for *t*-butyl alcohol. Clearly, the time-average shielding volume, arising from random gyration of the linear hydrocarbon chain, increases progressively with chain length in the series, ethanol–propanol–butanol, whereas the increase in shielding is minimal in the branched-chain series.

The possibility that the effects of the various solvents were attributable to changes in electrostatic interaction between charged side chains was examined, since the addition of solvents of low dielectric constant produced pH shifts in the systems due to suppression of carboxyl group dissociation. Accordingly, pH values in the control system were adjusted by alkali addition to cover the range, pH 4.8–5.2, representing the limits of pH variation encountered in the experimental systems. No differences were found in the optical rotatory and reduced viscosity reversion curves in this pH range, indicating that changes in charge profile on the polypeptide chains had no significant effect. Similar lack of effect due to changes in electrostatic interaction between charged side chains in collagen renaturation has been confirmed by Rauterberg and Kühn (1968) in studies on modified collagens.

Examination of 48-hr viscosity recovery values in the present study indicated that these *increased* with solvent concentration, hydrocarbon content, and polarity in the case of the straight-chain alcohols and nitriles. Thus, the effect of these solvents on viscosity recovery is similar to that noted in the case of strongly lyotropic agents such as urea at intermediate concentrations (Russell and Cooper, 1969). The enhanced recovery is attributed to the action of lyotropic agents in retarding random hydrogen-bond formation between chain segments, particularly over the initial phase of rapid renaturation. Under these conditions, a greater degree of reversion to the more specific, cooperative hydrogen-bond system of collagen can be expected, reflected in higher viscosity recoveries. Similar reasoning has been applied to account for increased collagen renaturation observed at slightly elevated renaturation temperatures (Beier and Engel, 1966).

Viscosity recoveries in the presence of the branched-chain solvents showed a tendency to *decrease* progressively with concentration and hydrocarbon content. This was particularly evident in the alcohol series. In order to account for this apparent anomaly, it is suggested that in the absence of strong hydrogen-bonding activity, an inert diluent effect predominates in these solvents. Since water itself must be considered an important reactant species moderating hydrogen-bond formation in the renaturing systems, a reduction in water concentration due to greater displacement with increasing solvent volume and concentration, can be expected, thereby increasing the rate of formation of random hydrogen-bonded structures of lower viscosity.

Quantitative comparison of initial renaturation rates and thermal transition temperatures in various solvents (Figures 4 and 6) indicated a proportional relationship between these variables. Previous renaturation studies conducted at various fixed temperatures (Flory and Weaver, 1960; Harrington and Von Hippel, 1961; Von Hippel and Wong, 1963; Von Hippel, 1967) have shown that the temperature dependence of the reversion rate as measured by optical rotation is described by a modified Arrhenius equation in which the apparent activation energy increases with temperature. Similarly, Mandelkern and Stewart (1964) in examining quantitative changes in renaturation rate at fixed temperature and corresponding thermal transition temperatures, in the presence of lyotropic neutral salts (Von Hippel and Wong, 1962, 1963), have shown that the modified Arrhenius expression accounted equally satisfactorily for the experimental data, consistent with a common ion-binding mechanism in which the apparent activation energy increased with neutral salt concentration.

Accordingly, the applicability of the modified Arrhenius expression to the changes produced by nonelectrolytes in the present study was investigated. When 1-hr mutarotation values were compared with the logarithm of the zero-time reversion rates calculated for a number of curves, a linear relationship resulted. The following average linear trend was established for 1-hr mutarotation values from −600 to −900° covering the range of variation encountered

$$\ln r/r_0 = -7.2 \times 10^{-3}\ \text{deg}^{-1}[\alpha]^{15°}_{265\ m\mu,\ 1\ hr} - 6.6 \qquad (1)$$

where r is the zero-time mutarotation reversion rate and r_0 is the rate in the absence of solvent. The 1-hr mutarotation values in the range 25–75% of the control final recovery value thus served as a convenient measure of the logarithmic zero-time reversion rate and the linear relationship between the former and solvent concentration (Figures 2 and 3) was consistent with a similar relationship found for neutral salts (Von Hippel and Wong, 1962) and urea (Von Hippel and Wong, 1963). Mandelkern and Stewart (1964) have pointed out that the form of the empirical relation proposed (Von Hippel and Wong, 1962) is supported by theoretical considerations based on a direct binding mechanism.

The temperature dependence of the reversion rate constant, K, at various fixed temperatures, T, and for a particular denaturation temperature, T_m^*, in the absence of a perturbant is defined by the modified Arrhenius expression as (Flory and Weaver, 1960)

$$K = \text{Const}\exp\left[\frac{-A}{kT\,(T_m^* - T)}\right] \qquad (2)$$

where A is the apparent activation energy and k is the Boltzman constant.

Assuming that the same reversion mechanism applies in the presence of a solvent perturbant which lowers the denaturation temperature, T_m, at fixed protein concentration and reversion temperature, it follows that (Mandelkern and Stewart, 1964)

$$\ln r/r_0 = \beta\left[\frac{1}{T_m^* - T} - \frac{1}{T_m - T}\right] \qquad (3)$$

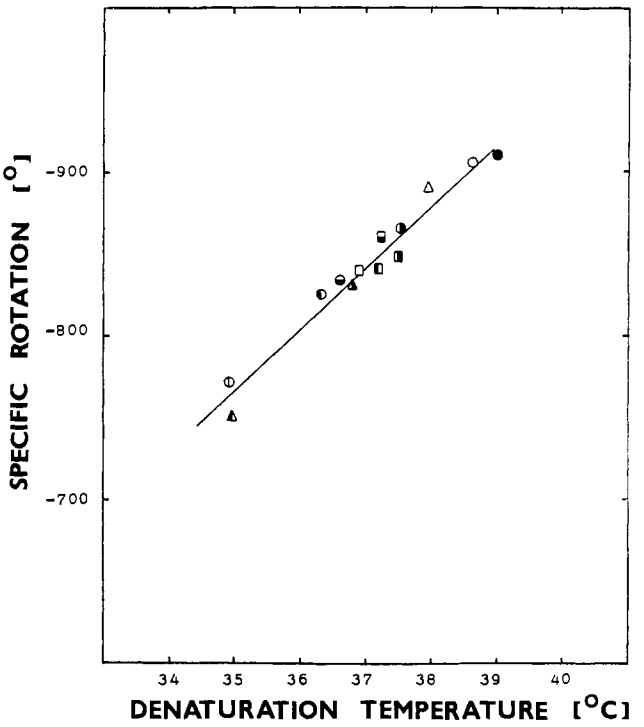

FIGURE 8: Relationship between 1-hr mutarotation reversion and denaturation temperature at 1.0 M solvent concentration (symbols as for Figures 2 and 3).

where β equals A/kT. From eq 3 it can be shown that

$$\ln r/r_0 = \frac{\beta(T_m - T_m^*)}{(T_m^* - T)^2\left[1 + \dfrac{T_m - T_m^*}{T_m^* - T}\right]} \qquad (4)$$

or

$$\ln r/r_0 \approx \frac{\beta(T_m - T_m^*)}{(T_m^* - T)^2} \qquad (5)$$

for small perturbations. From eq 1 and 5

$$[\alpha]^{15°}_{365\ m\mu,\ 1\ hr} \approx -\frac{\beta(T_m - T_m^*)}{7.2 \times 10^{-3}(T_m^* - T)^2} - 917 \qquad (6)$$

A plot of 1-hr mutarotation reversion as a function of denaturation temperature at 1.0 M solvent concentration is shown in Figure 8. A reasonably linear relationship was apparent, consistent with eq 6, suggesting that the modified Arrhenius expression (eq 2) was applicable in the presence of solvent perturbants.

A consistent, quantitative analysis of the lyotropic effects of a range of solvents and neutral salts (Mandelkern and Stewart, 1964) in terms of the modified Arrhenius expression can thus be demonstrated, suggesting that a common interaction mechanism is operative. The observations of the present study have been shown to be consistent with the proposal that the solvent perturbants examined exert lyotropic effects by direct hydrogen-bonding interactions at the peptide groups of collagen, thereby reducing the reversion rate constant with a resultant decrease in the equilibrium melting temperature. Al-

though the detailed mechanism whereby solvent binding brings about conformational destabilization is not apparent, in terms of current views of collagen structure, changes in the rotational freedom of main polypeptide bonds as well as the breaking of structural hydrogen bonds may be implicated. A direct binding mechanism involving peptide bonds has the features of nonspecificity with respect to protein composition and conformation required to account for the common order of lyotropic effects which various electrolytes and nonelectrolytes exert toward a number of fibrous and globular proteins.

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