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Structural and Functional Factors in the Lyotropic Activity of Amides and Alkyl-Substituted Amides on Acid-Soluble Collagen*

Allan E. Russell and Desmond R. Cooper

ABSTRACT: A systematic study of the lyotropic activity of formamide, urea, and alkyl-substituted derivatives on acid-soluble calfskin collagen has been undertaken. These perturbants reduced the rate of mutarotation recovery following heat denaturation and cooling and lowered the transition temperature of the native protein. Activity increased with perturbant concentration, functional group content, and number of potential donor hydrogen atoms, and with increasing linear hydrocarbon chain structure in the molecule. As in the case of polar organic solvents previously examined, these factors are all considered to promote perturbant hydrogen bonding, the hydrocarbon structure serving to exert local "hydrophobic shielding" which stabilizes polar interaction at an adjacent functional group. Further evidence for a largely nonspecific mechanism of perturbant action, consistent with hydrogen-bonding interactions, is apparent from general conformity of the data from the present and preceeding solvent studies with Flory–Weaver reversion kinetics. Thus, renaturation rates were largely determined by the degree of "undercooling" irrespective of the particular perturbant present, although perturbants with hydrocarbon structure deviated progressively from the linear trend found for formamide and urea. The present data support the previous proposal that a direct relationship exists between lyotropic activity and perturbant hydrogen bonding to collagen peptide bonds which, in terms of current structural concepts, results in destabilization due to increased rotational freedom in main-chain bonds and competitive disruption of internal structural hydrogen bonds between peptide links.

In recent studies, we have adopted an approach to the elucidation of lyotropic mechanism in which the effects of related perturbants on a standard collagen–buffer system have been compared systematically in order to correlate activity with perturbant structural and functional factors. (Russell and Cooper, 1969a,b). Compared with globular proteins, the rodlike collagen molecule provides an interesting reference system since structural features are fairly regular, resulting in comparatively isotropic behavior over the extended molecular length and a narrow melting range approximating a single-phase transition. Stabilization of the native conformation appears to be largely due to cooperative interchain hydrogen bonding and chain rigidity conferred by the pyrrolidine residues and rotational restrictions at peptide links (reviewed by Ramachandran, 1967). Since side-chain structures are located externally, interpretation of perturbant effects on collagen is not complicated by considerations relating to the exposure of buried hydrophobic structures to the environment in denaturation. In spite of structural distinctions, however, the relative magnitude of lyotropic effects for a variety of perturbants on collagen is the same as for globular proteins (Von Hippel, 1967; Von Hippel and Schleich, 1969; Von Hippel and Wong, 1962, 1963, 1964, 1965) suggesting that a general interaction mechanism, independent of conformational and compositional details, is operative.

Examination of the effects of aliphatic alcohols, ethers, ketones, and nitriles on collagen renaturation kinetics and thermal stability (Russell and Cooper, 1969a) has shown that concentration, polarity, and hydrocarbon structure are the main factors influencing lyotropic activity. It was concluded that lyotropic activity could be directly related to perturbant hydrogen bonding capacity. The effect of hydrocarbon structure was reconciled with this predominantly polar interaction mechanism by proposing that the pendent hydrocarbon chain exerted local hydrophobic shielding which stabilized interaction at adjacent functional groups in the perturbant. Preliminary examination of urea

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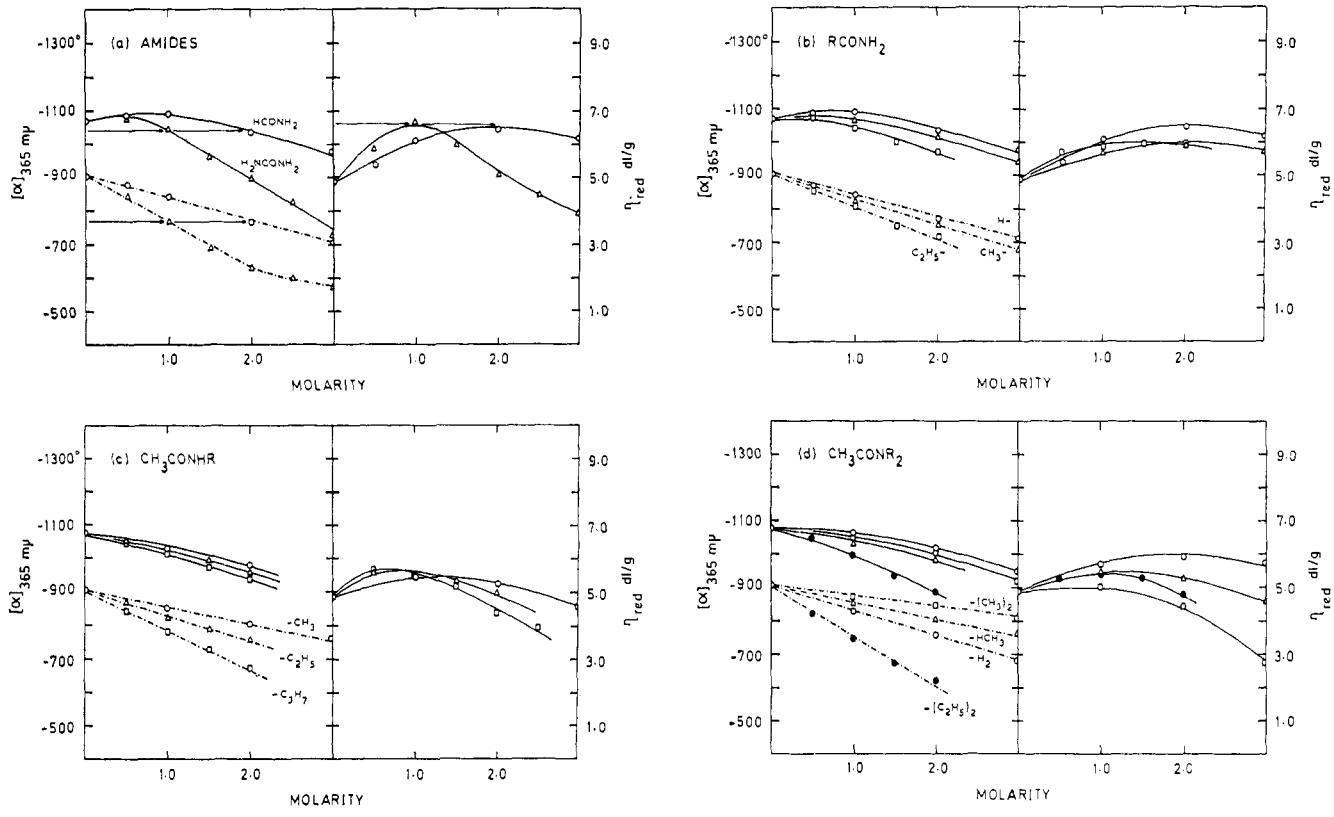

FIGURE 1: Variation in optical rotation (1 hr, broken line; 48 hr, full line) and reduced viscosity (48 hr) recoveries at 15° for acid-soluble collagen (0.86 mg/ml in 0.15 M potassium acetate buffer, pH 4.8) with concentration of perturbant added following heat denaturation (45°; 15 min): (a) formamide, urea (arrows denote lateral shift for formamide twice that for urea); (b) formamide, acetamide, propionamide, (c) methyl-, ethyl-, propylacetamide; (d) *N,N'*-alkylated acetamides.

in collagen renaturation has also shown effects which are qualitatively consistent with those of the solvent perturbants (Russell and Cooper, 1969b).

In the present study, examination of amide lyotropic effects has been extended. In relation to the direct binding mechanism of lyotropic activity, particular interest attaches to the amides as the members of this group may be regarded as peptide-bond structural analogs, predisposed to interact by direct hydrogen bonding at peptide links.

Materials and Methods

The preparation of acid-soluble calfskin collagen, the standard collagen–buffer system (0.86 mg of protein/ml of 0.15 M potassium acetate buffer, pH 4.8) and experimental methods were the same as previously described (Russell and Cooper, 1969a). Reagents were analytical or laboratory grade materials. Propionamide, ethylacetamide, diethylacetamide, and propylacetamide were prepared by reaction of the appropriate acid and amine and purified by distillation or recrystallization.

Results

I. Perturbant Effects in Renaturation. The effects of amides and alkyl-substituted derivatives on the renaturation of acid-soluble collagen are shown in Figure 1a–d in which 1-hr and 48-hr optical rotation and 48-hr reduced viscosity recoveries are plotted as a function of perturbant concentration. As found previously for organic solvent perturbants, 1-hr optical rotation recoveries decreased linearly with increasing concentration while 48-hr optical rotation and viscosity recoveries gave curved plots with maxima at intermediate concentrations. At higher urea concentrations effects were saturating and values approached a limiting level corresponding to the fully denatured state.

FUNCTIONAL GROUP CONTENT. Increase in activity due to an increase in functional group content in the molecule was apparent from comparison of formamide and urea (Figure 1a). Comparison of corresponding optical rotation and viscosity plots indicated that these could be exactly superimposed by halving the scale of the concentration axis in the case of formamide. Thus, urea and formamide differed only in their molar effects and the activity ratio (2:1) corresponded with the increase in amino group content.

HYDROCARBON STRUCTURE. The effect of amide hydrocarbon structure on activity was compared in the homologs, formamide–acetamide–propionamide (increasing linear hydrocarbon chain on the carbonyl atom of the amide group, Figure 1b) and in the monosubstituted acetamides (increasing linear hydrocarbon chain on the imino nitrogen atom, Figure 1c).

Increase in hydrocarbon structure at both substitution positions produced a progressive increase in activity, as assessed from the decreases in 1-hr optical rotation recoveries. The effect of increasing linear hydrocarbon chain structure

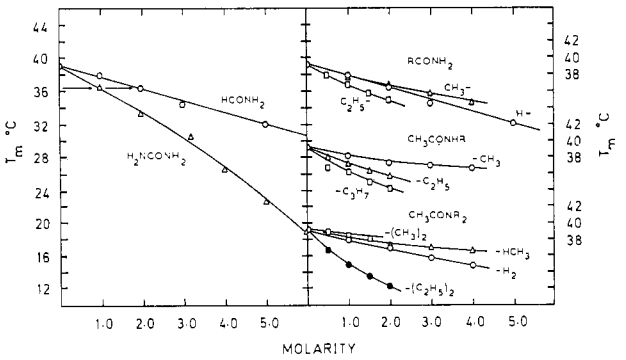

FIGURE 2: Relationship between melting temperature (T_m) corresponding to the midpoint of the optical rotation transition curve for acid-soluble collagen (0.86 mg/ml in 0.15 M potassium acetate buffer, pH 4.8) and perturbant concentration (arrows denote lateral shift for formamide twice that for urea).

in promoting activity was thus similar to that noted previously for the lower homologs of the alcohols, ketones, and nitriles (Russell and Cooper, 1969a). Since the heights of the viscosity and, to a lesser extent, the optical rotation maxima varied with the degree of hydrocarbon substitution, it was evident that superposition of all the plots by lateral rescaling by a fixed factor, as in the case of formamide and urea, was not possible. In general the 48-hr viscosity and optical rotation maxima shifted to lower perturbant concentrations with increasing hydrocarbon content.

The influence on activity of substitution of the two potential hydrogen-bonding protons of the amide group was examined by comparison of effects in mono- and dimethylacetamide. Successive methyl group substitution resulted in a progressive *decrease* in activity, apparent from the increased optical rotation recoveries compared with acetamide (Figure 1d). Replacement of methyl by ethyl substituents to give diethylacetamide, however, resulted in a marked reversal of trend due to the further *increase* in activity which then exceeded that of the unsubstituted acetamide. Thus, the effect of increasing chain length at both substitution positions in promoting activity was consistent with previous observations.

Successive methyl group substitution suppressed the heights of the viscosity recovery maxima particularly which also shifted to lower perturbant concentrations. A corresponding reversal of trend was apparent for diethylacetamide. The pattern of variation in 48-hr optical rotation recoveries appeared to be largely influenced by the 1-hr recovery behavior.

A general pattern of variation similar to that shown in Figure 1d was found for the corresponding mono- and disubstituted formamides (not shown). However, consistently greater activity, attributable to the presence of the additional methyl group in the acetamide series, was evident throughout.

II. Perturbant Effects on Thermal Stability. Perturbant effects on the stability of native, acid-soluble collagen are shown in Figure 2 in which the temperature at the midpoint of the optical rotation transition is plotted as a function of perturbant concentration. As found previously for polar organic solvents (Russell and Cooper, 1969a), the order of lyotropic effects at the one molar level was the same as that found in the renaturation studies. Similarly, urea activity in denaturation was exactly double that of formamide. At low concentrations, formamide and urea activities showed linear concentration dependence, but negative curvature was apparent at higher urea concentrations, reflecting an increasing molar effect.

In contrast with formamide and urea, variation in lyotropic activity with concentration for amides and derivatives with hydrocarbon structure showed *positive* deviation from linearity due to an apparent decrease in molar effect with increasing concentration. Protein precipitation prevented extension of the concentration range, but deviations appeared to be more pronounced in perturbants containing larger amounts of hydrocarbon structure.

Discussion

The role of amide hydrogen bonding as a major determinant of reactivity is supported by consideration of functional and structural effects in the present study. Thus, activity was dependent upon the content of amino groups with potential donor hydrogen atoms as seen in the case of formamide and urea. The twofold increase in activity for urea suggests that for equally accessible locations within the same molecule, individual functional group contributions to total activity are additive. Similar functional group additivity has been demonstrated previously for differing perturbants in the same protein–buffer system (*e.g.*, effects of calcium chloride and ethylene glycol on ribonuclease, Von Hippel and Wong, 1965). The linear increase in activity with perturbant concentration may also be regarded as evidence for additivity of effects due to increments of the same perturbant. Additivity of effects is consistent with the mass action controlled, dynamic nature of hydrogen-bonding interactions in terms of which both perturbant concentration and functional group content may be regarded as synonymous factors since both increase the probability of interaction by raising the number of reactive groups per unit volume of solution. Thus, replotting the formamide and urea data on this basis would give a common plot.

Further evidence in support of the role of perturbant hydrogen bonding through the donor hydrogen atoms of amides follows from consideration of methyl substitution effects. Successive substitution of the two amino hydrogen atoms in acetamide resulted in a stepwise reduction in activity which also suggested an approximately equal and additive contribution to the total activity from each amino hydrogen atom. Residual activity noted in dimethylacetamide containing no donor hydrogen atoms is readily attributable to hydrogen bonding through the carbonyl oxygen or nitrogen acceptor atoms. In contrast with amino hydrogen substitution, methyl substitution of the carbonyl hydrogen atom (*i.e.*, comparing acetamide with formamide) produced no comparable activity decrease. Lack of comparable effect is consistent with the absence of hydrogen-bonding potential in the carbonyl hydrogen atom.

The effect of linear hydrocarbon substituents on the carbonyl carbon or amino nitrogen atoms in promoting lyotropic activity was similar to that reported in the case of the lower alcohols, ketones, and nitriles (Russell and Cooper, 1969a). As distinct from direct hydrophobic interaction, the role of perturbant hydrocarbon structure may be

explained in terms of an increase in perturbant hydrogen bonding capacity due to hydrophobic shielding. Recent studies have provided evidence for an indirect effect of this nature due to local hydrophobic structure (Hammes, 1968; Hammes and Knoche, 1966; Hammes and Lewis, 1966; Hammes and Roberts, 1968; Russell *et al.*, 1968) by which polar interactions between a perturbant and a large protein molecule are stabilized due to shielding by the hydrophobic structure from competing polar molecules in the immediate environment. Since the nonpolar residues in collagen do not appear to be involved in stabilization due to their external location, the mechanism proposed readily accounts for the consistent effects of perturbant hydrophobic structure in enhancing activity in a wide range of polar compounds. Since the shielding volume can be expected to increase rapidly with chain length due to successive carbon–carbon bond rotations (Russell and Cooper, 1969a), the small effect of a methyl group compared with an ethyl group is explained. Thus in the case of diethylacetamide, substitution of the potential donor hydrogen atoms appears to be more than compensated by the shielding effect of the ethyl substituents so that lyotropic activity, actually exceeded that of acetamide.

As in the case of the organic solvent perturbants previously examined, addition of alkyl-substituted amides raised the pH of the protein–buffer system. Comparable pH shifts (pH 4.8 to 5.5) produced by alkali addition had no measurable effect on renaturation, however, indicating that changes in protein charge profile in this range were not responsible for the experimental variation.

The renaturation phenomenon in collagen is complicated by the fact that at least two reversion processes which may be interdependent, are involved, namely, propagation of helical secondary structure along individual polypeptide chains (largely responsible for changes in optical rotation) and varying degrees of aggregation by hydrogen-bond formation between chain segments (reflected in changes in reduced viscosity) to give a modified tertiary structure (Von Hippel and Harrington, 1959; Harrington and Von Hippel, 1961a,b; Drake and Veis, 1964; Beier and Engel, 1966). The significance of the enhanced viscosity recoveries obtained in renaturation at intermediate urea concentrations has been discussed previously (Russell and Cooper, 1969b) and similar maxima were observed for the amide perturbants reported here. The effect is attributed to competitive hydrogen bonding of perturbants to the disordered protein chains. By moderating the rate of chain reassociation, greater reversion to collagen-like structures of higher intrinsic viscosity is possible. At higher concentrations, however, perturbant binding predominates, resulting in decreased recoveries. In general, viscosity maxima shifted to lower concentrations for the more active perturbants (*cf.* urea and formamide), while the heights varied in a complex manner with hydrocarbon substitution and activity.

A feature of the melting temperature concentration dependence for urea was the negative curvature at higher concentrations. This *increase* in molar activity indicates that progressive removal of the cooperative structural hydrogen bonding increases the lability of the residual collagen structure. In contrast, amides with hydrocarbon structure showed positive curvature with increasing concentration, reflecting a progressively *decreasing* molar effect. Similar effects,

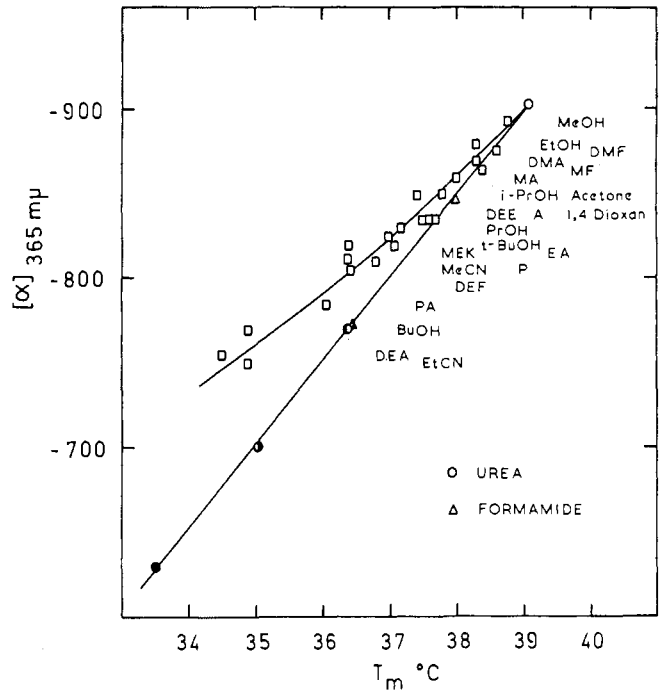

FIGURE 3: Relationship between kinetic (1-hr optical rotation recovery) and equilibrium (melting temperature) data in the presence of various perturbants at 1.0 M concentration (□), formamide (△, 1.0 M; ▲, 2.0 M), and urea (◐, 1.0 M; ◑, 1.5 M; ●, 2.0 M). Abbreviations used in the figures are: DMF, dimethylformamide; DMA, dimethylacetamide; MF, methylformamide; MA, methylacetamide; DEE, diethyl ether; A, acetamide; MEK, methyl ethyl ketone; EA, ethylacetamide; MeCN, acetonitrile; P, propionamide; DEF, diethylformamide; PA, propylacetamide; DEA, diethylacetamide; EtCN, propionitrile.

resulting in a reversal of the initial destabilization at higher perturbant concentrations, have been reported for aliphatic alcohols and ketones on insoluble (Schnell and Zahn, 1965; Russell *et al.*, 1967) and soluble (Herbage *et al.*, 1968; Harrap, 1969) collagen. The stabilization effects at higher concentrations are attributed to increased stability of structural hydrogen bonding due to lowering of the dielectric constant of the medium by the organic solvent component.

Previous investigators (Mandelkern and Stewart, 1964) have demonstrated that a consistent quantitative analysis of the effects of a wide range of neutral salt perturbants on collagen renaturation and stability is obtained by invoking a direct binding mechanism and application of the modified Arrhenius expression introduced by Flory and Weaver (1960). In terms of Flory–Weaver kinetics, reversion rates are independent of the nature of the perturbant, but are determined by the degree of undercooling, the difference between the melting and reversion temperatures in the solvent medium. Conformity of the kinetic data in the presence of organic solvent perturbants with the Flory–Weaver expression has been examined previously (Russell and Cooper, 1969a). It was shown that a closely linear relationship could be expected between 1-hr optical rotation recovery (proportional to the logarithm of the reversion rate constant) and melting temperature (determining the extent of undercooling) for small perturbations, the gradient of the plot being proportional to the apparent activation energy for the reversion process.

Combined data from the present and preceding studies are plotted in Figure 3. Formamide and urea at various concentrations gave a common linear plot consistent with the predictions of the Flory–Weaver treatment. Perturbants with increasing hydrocarbon structure, however, deviated progressively from this linear trend suggesting an effect of the hydrocarbon structure on the apparent activation energy. In spite of the varied nature of the perturbants, these all conformed closely to the average trends shown suggesting that a common interaction mechanism was operative which was subject to quantitative modification in the presence of hydrocarbon structure.

The direct binding mechanism of perturbant activity has received additional impetus from recent studies on perturbant-mediated conformational changes in model polypeptides, particularly poly-L-proline which lacks structural hydrogen bonds (Schleich and Von Hippel, 1969; Steinberg *et al.*, 1958, 1960; Strassmair *et al.*, 1969; Veis *et al.*, 1967; Von Hippel and Schleich, 1969). Conformational changes are attributed to increased bond rotation due to electronic shifts resulting from direct binding of polar solvents and hydrated ions at peptide links. On this basis, perturbant effects on collagen optical rotation and reduced viscosity recovery are both explicable in terms of direct hydrogen bonding at peptide links, a mechanism which is consistent with the effects of the various perturbant structural and functional factors examined. While a possible contribution from hydrophic interaction to perturbant activity in other protein systems cannot be discounted, direct perturbant hydrogen bonding at peptide links possesses the feature of compositional and conformational nonspecificity which would account for the generality of perturbant effects on a variety of proteins.

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