The Effects of Certain Glycols, Substituted Glycols and Related Organic Solvents on the Thermal Stability of Soluble Collagen

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The effects of a number of related diols, substituted diols and glycerol on the thermal stability of acid-soluble calf skin collagen were investigated. Thermal transition temperatures were determined by optical rotation measurement. Short-chain diols with terminal hydroxyl groups, i.e. ethylene glycol and propane-1,3-diol, stabilized the protein at all accessible concentrations. Stabilization was also observed with glycerol and diethylene glycol. Higher homologues in the diol series produced various effects, as did hydroxyl-group positional isomerism. Monoalkyl substitution of diols progressively lowered the denaturation temperature of collagen. Results are discussed in relation to possible mechanisms of perturbant action.

Investigation of the influence of various organic solvents on the thermal denaturation temperature (T_m) of soluble collagen provides information about the possible mechanisms involved in perturbational activity. The theoretical basis for the use of optical rotation as a parameter for monitoring the collagen-gelatin transition has been discussed by Cohen (1955). Optical rotation is related to polypeptide chain conformation as follows.

(1) in the denatured state chain conformation is completely randomized, the observed rotation being attributable to the additive contributions of the individual L-amino acid residues, and (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 that 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 variation in the amount of collagen-type helical structure existing in the main polypeptide chains.

The possibility of a significant contribution by hydrophobic interaction mechanisms to the stabilization of the collagen triple helix has been invoked to explain the effects of certain lyotropic agents (Schnell, 1968; Harrap, 1969; Bianchi, Rampone, Tealdi & Ciferri, 1970). The well-established role for such interactions in relation to the globular proteins and model systems (reviewed by Tanford, 1970) appears to have led to the assumption that similar processes might be operative with respect

to collagen stability at the level of the monomeric macromolecule. However, examination of the fine structure of the tropocollagen monomer casts serious doubt on the validity of this approach. The Ramachandran (1967) model for the collagen triple helix requires all side chains to be directed away from the central helix axis. Thus side chains are, in effect, externally disposed around the outside of the macromolecule, apparently fully exposed to the solvent. Other collagen molecular models, such as that proposed by Rich & Crick (1955), do not differ in this respect since β -, γ - and δ -carbon atoms could not be internally accommodated.

The literature indicates that there is no consensus of opinion about the probability of hydrophobic interaction being a contributor to the stabilization of the collagen triple helix. For example, Harrington (1964) assumes that the only forces involved in the stabilization of the tropocollagen monomer are peptide hydrogen bonds between polypeptide chains, in addition to the stereochemical restrictions associated with the pyrrolidine-ring residues. In contrast, von Hippel (1967), in an assessment of Harrington's (1964) 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. In an attempt to clarify this point, and to relate perturbant molecular structure to possible mechanisms of protein-perturbant interaction, we have investigated the influence of a number of glycols and alkyl-substituted glycols on

the thermal stability of acid-soluble calf skin collagen.

EXPERIMENTAL

Reagents. All solvents and reagents were A.R. grade or Laboratory grade. The components of the primary buffer were: acetic acid, 7.384 g/l (component A); potassium acetate, 17.372 g/l (component B). Equal parts of components A and B produced a solution of pH 4.65–4.70. A secondary buffer system, five times more concentrated than the primary system, was prepared containing: acetic acid, 36.920 g/l (component A'); potassium acetate, 86.860 g/l (component B'). Equal parts of components A' and B' gave a solution which when diluted fivefold was identical with the primary buffer.

Preparation of solutions of acid-soluble collagen. Purified acid-soluble calf skin collagen was prepared as described by Cooper & Davidson (1965). A concentrated stock solution of collagen (3.44 mg/ml) was prepared by dissolving the estimated amount of freeze-dried material in the acetic acid component of the primary buffer (component A) by means of gentle stirring with a magnetic stirrer for approx. 30 min at 10°C. The potassium acetate solution (component B) was then added and the mixture was left overnight in a refrigerator at 4°C. The solution was then centrifuged at 32000g for 1 h at 5°C (MSE model 18 high speed centrifuge) to remove any undissolved collagen. A concentration check was done by polarimetry and the preparation adjusted to the required concentration by the addition of primary buffer. The specific rotation of a completely heat-denatured gelatin solution was taken as $[\alpha] = -460^\circ$ at $\lambda = 365$ nm, and that of soluble native collagen as $[\alpha]_{365}^{25} = -1330^\circ$ (after Drake, Davison, Bump & Schmidt, 1966). The calculated quantities of the concentrated collagen solution, the concentrated secondary buffer solution, the organic solvent and water were then mixed to produce the final systems, the protein concentrations being between 0.25 and 0.86 mg/ml. The final ionic concentration of the buffer and the buffer-glycol mixtures was 0.15 M in all cases.

The effect of pH and of protein concentration. In systems containing glycerol, ethylene glycol, propane-1,3-diol or hexane-1,6-diol the pH variation in the final protein perturbant-buffer solutions was between 4.7 and 5.1 at the extremes of the perturbant concentration. With the remaining perturbants the pH varied between 4.7 and 5.7, the only exception being butane-1,4-diol where a pH of 6.0 was recorded at the highest solvent concentration. According to von Hippel & Schleich (1969) the T_m of soluble collagen is essentially independent of pH over the range 3–11, and Hauschka & Harrington (1970) have shown that the T_m of native ichthyocol increases only about 1°C per pH unit in the pH range 4.7–5.7. At the perturbant concentration of 1 M, where many of the present comparisons were made, the pH variation was very small and in no case did it exceed the limits of pH 4.71–4.81.

We have also examined the effect of collagen concentration on T_m . Controls at collagen concentrations of 0.86 and 0.43 mg/ml gave T_m values of 38.9°C and 39.0°C respectively, which are in agreement with the value of

38.6°C obtained at concentrations of 1.2–1.4 mg/ml (Cooper & Russell, 1969). At the highest concentrations of perturbants having large molar volumes it was necessary to decrease the protein concentration to 0.25 mg/ml (with 10 M-glycerol; 10 M-propane-1,2-diol; 7 and 8 M-butane-1,4-diol; 5 and 6 M-butane-2,3-diol). Flory & Weaver (1960) have shown that T_m is independent of collagen concentration over the entire range from 0.087–2.176 mg/ml. Therefore T_m is considered to be unaffected by protein concentration within the limited concentration range used in the present study.

Optical rotation. Optical rotation was determined by means of a Perkin-Elmer model 141 read-out polarimeter by using the mercury lamp at a wavelength of 365 nm. Polarimeter cells (10 cm) were water-jacketed and temperature controlled to $\pm 0.05^\circ\text{C}$.

Determination of T_m . Collagen-gelatin transitions were monitored by polarimetry by using the '30 min method' of von Hippel & Wong (1963) in which systems are incubated for 30 min before the reading, thus allowing a pseudo-equilibrium state to be attained. von Hippel & Wong (1963) 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 h. However, the 30 min procedure leads to an increase of only about 1°C in the apparent T_m and to 'melting' curves that do not have 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 perturbants on the stability of the collagen

Fig. 1. Typical 'melting' profiles showing progressive elevation of T_m of acid-soluble collagen (0.86 mg/ml in 0.15 M-potassium acetate buffer, pH 4.7) with increasing concentration of propane-1,3-diol: ○, 1 M; △, 2 M; □, 3 M; ●, 4 M; ▲, 5 M.

triple helix and hence the small differences arising from the use of the 30 min method were not considered significant. The T_m values were read at the transition midpoints in all cases.

RESULTS

Typical 'melting' profiles illustrating progressive elevation and depression of the thermal transition temperature of tropocollagen by the addition of

Fig. 2. Typical 'melting' profiles showing progressive lowering of T_m of acid-soluble collagen (0.43 mg/ml in 0.15 M-potassium acetate buffer, pH 4.7) with increasing concentrations of butane-2,3-diol. Symbols are as in Fig. 1.

perturbants are shown in Figs. 1 and 2 respectively. The T_m values as a function of perturbant concentration with glycerol, ethylene glycol, propane-1,3-diol and propane-1,2-diol are shown in Fig. 3. The results with acid-soluble calf skin collagen at pH 4.7 and the diols ethylene glycol, propane-1,2-diol and propane-1,3-diol are very similar to, and serve to confirm, those obtained by Harrap (1969) with soluble rat tail-tendon collagen at pH 3.0.

The effects on protein stability of ethylene glycol and all its homologues up to hexane-1,6-diol are compared in Fig. 4. The pattern of effects is maintained with the higher members of the series. The longer hydrocarbon chains of butane-1,4-diol, pentane-1,5-diol and hexane-1,6-diol progressively decrease the thermal stability of collagen, and after this there is a reversal of the trend at high solvent concentrations.

The relative effects of the two propanediol isomers were shown in Fig. 3. Butanediol isomers are even more versatile for comparisons of this nature, since there are three of them, each having a different lyotropic activity. The effects of hydroxyl-group position and hydrocarbon-chain stereochemistry are clearly demonstrated in Fig. 5. The slight initial destabilization produced by butane-1,4-diol gives way to a somewhat greater effect when the organic solvent molecule contains a terminal methyl group (butane-1,3-diol). The presence of two terminal methyl groups (butane-2,3-diol) produces an even more marked decrease in the thermal stability of the protein. Fig. 5 also shows the useful comparison between the effects of butane-1,4-diol and diethylene glycol. Diethylene glycol differs from butane-1,4-diol in having a centrally situated oxygen atom. This additional

Fig. 3. Comparison of effects of isomeric propanediols (Δ , propane-1,3-diol; $\square$, propane-1,2-diol), ethylene glycol ($\circ$) and glycerol ($\bullet$) on the T_m of acid-soluble collagen in 0.15 M-potassium acetate buffer, pH 4.7.

Fig. 4. Comparison of effects of homologous diols on the T_m of acid-soluble collagen in 0.15M-potassium acetate buffer, pH 4.7: $\circ$, ethylene glycol; $\triangle$, propane-1,3-diol; $\blacktriangle$, butane-1,4-diol; $\blacksquare$, pentane-1,5-diol; $\bullet$, hexane-1,6-diol.

Fig. 5. Comparison of effects of isomeric butanediols ($\blacktriangle$, butane-1,4-diol; $\square$, butane-1,3-diol; $\bullet$, butane-2,3-diol) and diethylene glycol ($\circ$) on the T_m of acid-soluble collagen in 0.15M-potassium acetate buffer, pH 4.7.

Table 1. *Effects of monoalkyl derivatives of ethylene glycol on T_m of soluble collagen*

The collagen concentration was 0.86 mg/ml, in 0.15M-potassium acetate buffer, pH 4.7.

Solvent	T_m (°C)
Ethylene glycol (1M)	39.3
2-Methoxyethanol (1M)	39.0
Control	38.9
2-Ethoxyethanol (1M)	38.1
2-Butoxyethanol (1M)	35.3

DISCUSSION

Effect of hydroxyl groups. The first point of note is the relationship between glycerol and the isomeric propanediols. Propane-1,3-diol, which corresponds to glycerol minus its central hydroxyl group, stabilizes the protein to a lesser extent than does glycerol. This is contrary to what might be expected if it is assumed that perturbant action is of an exclusively polar nature. If this were so the solvent with the greater number of polar groups (glycerol) should be the less powerful stabilizer of the protein, owing to competitive disruption of the hydrogen-bond network of the collagen molecule. Thus interactions other than hydrogen bonding may be involved in maintaining the conformational stability of the triple helix.

Polar-group position also has a significant effect on solvent activity. Fig. 3 shows that propane-1,2-diol is a fairly powerful destabilizer of the collagen macromolecule, in contrast with its isomer, propane-1,3-diol, which raises the T_m . The

polar atom eliminates any destabilizing influence, diethylene glycol being a highly effective stabilizer of the native protein. It appears that a decrease of the length of unbroken hydrocarbon chain of the organic solvent molecule destroys its ability to destabilize partially the triple helix.

Effect of alkyl substitution. The effects of monoalkyl derivatives of ethylene glycol on T_m are shown in Table 1. Precipitation of the protein occurred at concentrations of perturbant above 1M. It is clear, however, that alkyl substitution of the parent compound results in progressive lowering of T_m as the size of the hydrocarbon substituent is increased.

terminal methyl group of the 1,2-diol should be sterically more accessible to neighbouring molecules in the liquid than would the centrally situated methylene group of the 1,3-diol. This suggests the possible existence of a role for hydrophobic interaction processes involving the non-polar methyl group of the 1,2-diol and non-polar side chains of the collagen macromolecule. Support for this contention is provided by comparing the effects of propane-1,2-diol with those of ethylene glycol (Fig. 3).

Dielectric effects. A trend reversal occurs at high concentrations of propane-1,2-diol (Fig. 3). It appears that a sufficient decrease in the environmental dielectric constant overcomes the destabilization tendencies by enhancing the strength of the co-operatively hydrogen-bonded network within the protein molecule.

The stabilizing effect of ethylene glycol may be regarded as being a result of the lowering of the dielectric constant of the medium, thus decreasing the susceptibility of the co-operative system of hydrogen bonds of the triple helix to perturbation by polar molecules in the liquid environment. On the basis of this assumption the greater stability-promoting power of propane-1,3-diol is readily explained. Propane-1,3-diol has, because of its additional methylene group, a lower polar atom/carbon atom ratio than has ethylene glycol. Hence propane-1,3-diol will, on a molar basis, lower the dielectric constant of the solvent-water system more than ethylene glycol would.

There is also the possibility that collagen molecules in solution may exist in forms other than the simple monomeric triple helical state (Davison & Drake, 1966; Davidson & Cooper, 1967, 1968). Thus, lowering the dielectric constant of the solution by adding organic solvents might be expected to enhance intermolecular electrostatic interactions to an extent that could appreciably raise the T_m . Hence this process could also contribute to the stabilization of the native protein and accentuate the effects that lowering the dielectric constant would have on intramolecular interactions.

Considering the first member of this homologous series to cause destabilization of soluble collagen (butane-1,4-diol, Fig. 5), it appears that the four-carbon butylene chain is long enough to allow a limited measure of hydrophobic interaction with non-polar groups of the protein. The trend is soon reversed, however, as the effect of decreasing dielectric constant with increasing solvent concentration becomes dominant.

The experimental evidence discussed above suggests that the destabilization of the collagen triple helix may be viewed as a manifestation of hydrophobic interaction phenomena. There remains, however, the major objection to this

approach, that crystallographic studies indicate that all the side chains in the collagen molecule are directed away from the helix axis, presumably eliminating any potential for extensive formation of hydrophobic micelles within the macromolecule. Thus, contact between contiguous non-polar side chains in the tropocollagen monomer would appear to be at most a minor contributor to macromolecular stability.

In an attempt to overcome the theoretical difficulties outlined above, we have developed an alternative interpretative approach. This consists of an elaboration on suggestions made by Russell & Cooper (1969) and Hart (1971) and does not incorporate the idea of direct hydrophobic interaction between collagen and perturbant molecules. Destabilization of the protein is regarded as a function of direct hydrogen-bond formation between organic solvent polar groups and potential hydrogen-bonding sites of the macromolecule. The model incorporates the concept of water structure formation (Frank & Evans, 1945; Drost-Hansen, 1969; Hatefi & Hanstein, 1969; Hertz, 1970) in order to facilitate the explanation of the influence of hydrocarbon chain length and isomerism. We suggest that structuring of water in regions immediately adjacent to the non-polar part of a perturbant molecule, hydrogen bonded to a collagen molecule, could be expected to inhibit the freedom of approach of all other molecules in the vicinity. This would serve to prolong the lifetime of the transient polar bond between solvent molecule and protein. If such water structures have appreciable lifetimes, relative to the frequency of thermal oscillation of the hydrocarbon chain, a considerable build-up of water structure seems feasible. The greater mobility and extended sweep-out volume of linear isomers and alkyl derivatives could thus be expected to produce larger volume elements of ordered vicinal water than would their more compact branched-chain counterparts or lower homologues (Russell & Cooper, 1969). Similarly, organic solvent molecules in which the hydrophobic alkyl groups are sterically more accessible to the aqueous environment would be structure-promoting to a greater extent than isomers in which the accessibility of the hydrocarbon chain is decreased as a result of interference by polar groups.

The above interpretation may be applied with equal facility to the effects of aliphatic alcohols on the thermal stability of soluble collagen (Harrap, 1969; Russell & Cooper, 1969). It is of note that where comparisons are possible, a given polyol has a smaller destabilizing effect than the corresponding monohydric compound. The polyols often show a marked tendency to stabilize the protein (Table 2). Thus the pattern shown in Table 2 supports the

Table 2. *Comparison of effects of alcohols and polyols on thermal stability of soluble collagen*

Experimental conditions were as described in the Experimental section. The ΔT_m is the increase or decrease in T_m relative to the control value. Perturbant concentration was 1.0M in all systems. Results for alcohols are taken from Russell & Cooper (1969).

Alcohol	ΔT_m (°C)	Polyol	ΔT_m (°C)
Ethanol	-1.3	Ethylene glycol	+0.4
Propanol	-2.4	Propane-1,2-diol	-0.2
		Propane-1,3-diol	+0.4
		Glycerol	+1.0
Butanol	-4.3	Butane-2,3-diol	-0.9
		Butane-1,3-diol	-0.3
		Butane-1,4-diol	-0.3

suggestion that simple hydrogen-bonding alone does not account adequately for perturbant action. On a mass-action basis, polyols could be expected to be more lyotropic than the corresponding alcohols if polar bonding was the only effective mechanism.

Further support for the mechanism outlined above is provided by comparison of the effects of acetamide, *N*-methylacetamide, *NN'*-dimethylacetamide and *NN'*-diethylacetamide (Russell & Cooper, 1970). The successive replacement of the donor amino hydrogen atoms by methyl groups progressively decreases lyotropic activity. The *NN'*-diethyl derivative, however, is more lyotropic than the parent amide. In terms of the hypothesis outlined above, this suggests that the water-structure promoting properties of the ethyl substituents increase the polar interaction potential of the nitrogen atom to an extent that more than compensates for the removal of the donor hydrogen atoms. Notably, such a mechanism does not presuppose the existence *ab initio* of any hydrophobic contribution to the stability of the collagen triple helix. It does suggest, however, that the extent to which vicinal structuring of water molecules is promoted by the pendant hydrocarbon chains of perturbant molecules has a critical bearing on the hydrogen bonding potential of the perturbant.

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