The Effects of Glycols on the Renaturation of Soluble Collagen

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The effects of a number of related glycols and substituted glycols on the renaturation kinetics of acid-soluble calf-skin collagen have been investigated. Optical rotation recovery was monitored at a fixed temperature in the presence of perturbants and the initial rates of reaction were determined. The effects of perturbants on stability of the native protein are compared with their action in the renaturing systems. The relationship between initial recovery rates and fixed-time $[\alpha]$ -values is shown to be dependent upon the renaturation temperature. The influence of perturbant concentration on recovery rates is discussed in terms of present theories of the mechanism of collagen renaturation.

Much work has been directed towards the elucidation of the mechanism involved in the re-formation of the collagen fold after heat denaturation. Until recently the polymerization of polypeptide strands during collagen renaturation was thought to involve three distinct steps: (1) a nucleation process occurring in individual strands in regions of high pyrrolidine residue content; these regions would be favoured as nucleation sites because of decreased chain mobility resulting from the steric constraints imposed by the pyrrolidine ring structures; (2) the propagation of the helical form along individual polypeptide chains; (3) combination of three chains to give the stable triple-helical arrangement (Flory & Weaver, 1960; von Hippel & Wong, 1963a). A central requirement of mechanisms of this type is the existence of individual poly-L-proline II-type helices before the trimerization step.

The validity of the above theory depended largely on the finding that the renaturation process, as monitored by optical rotation recovery, was independent of protein concentration below about 2 mg/ml. It was this apparent concentration-independence that supported the suggestion that the nucleation process could occur in isolated polypeptide chains. In direct contradiction of the evidence upon which the three-stage mechanism is based, Beier & Engel (1966) and Piez & Carrillo (1964) have shown that renaturation to the most nearly native form is not independent of protein concentration. This ambiguity has been thoroughly explored by Harrington & Rao (1970) who have shown that the earlier findings suggesting concentration-independence were artifacts and that

true concentration-independence exists only below 0.1 mg of protein/ml. Harrington & Rao (1970) consider that helix formation does not follow the three-step mechanism discussed above and suggest that the nucleation step cannot occur in an isolated polypeptide chain unless it is mediated by the formation of intramolecular hydrogen bonds. Thus, at very low protein concentrations (below 0.1 mg/ml), helix formation is considered to be the result of a triple folding of individual polypeptide chains, as proposed by Drake & Veis (1964). In contrast, at high concentrations of protein (above 2 mg/ml) the dominant process would be the formation of triple-stranded structures via the association of three separate chains. At intermediate concentrations, such as those used in the present study, the observed kinetic order will represent an 'average' process in which both types of reactions are major participants. It has nevertheless been shown (Harrington & von Hippel, 1961a) that under suitable conditions the initial stages of renaturation in the intermediate concentration range (approx. 1 mg/ml) are satisfactorily described by a second-order rate equation ($n = 2.2 \pm 0.15$).

The acid-soluble calf-skin collagen used in the present study has been shown to contain a significant proportion of β -components (Cooper & Davidson, 1965). Despite this complication we have found it practical to describe our initial rate values in terms of a second-order equation. The mechanisms of renaturation of cross-linked and non-cross-linked collagens have, in fact, been shown to be similar in a number of respects (Harrington & Karr, 1970; Hauschka & Harrington, 1970a).

In the studies referred to above, the methods were

based largely on the isothermal or 'undercooling' technique that makes use of buffered aqueous solutions of protein. This involves keeping the denatured protein solutions at various fixed temperatures below the denaturation temperature (T_m) during the course of renaturation. We have adopted a different approach: by varying the composition of the solvent environment, rather than the degree of 'undercooling', it has proved possible to demonstrate distinct maxima in the renaturation rate at intermediate concentrations of added organic solvent. This has led us to conclude that the mechanism of renaturation in the systems we have examined is substantially the same as that suggested by Harrington & Rao (1970) in explanation of the findings in their 'undercooling' experiments.

A number of studies (Flory & Weaver, 1960; Harrington & von Hippel, 1961a; von Hippel & Wong, 1962, 1963b; von Hippel, 1967) have shown that a modified expression based on the Arrhenius equation adequately describes the effects of temperature on mutarotation rates under suitable conditions, in the absence of added perturbants. Following on from this, it has been shown (Mandelskern & Stewart, 1964) that the same reversion mechanism may be assumed to apply in the presence of a lyotropic agent that lowers the T_m . The approach has been further extended by Russell & Cooper (1969) to show that, with perturbants that lower the T_m , there is a linear relationship between the log of zero-time reversion rates and the optical rotation recovery values at 1 h after the commencement of renaturation. The use of 1 h $[\alpha]$ -values was developed to overcome the difficulties associated with obtaining accurate initial rate values during the early stages of a relatively fast reaction. The existence of a linear relationship between 1 h $[\alpha]$ -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. In other cases, however, where the recovery decrease was not large and systematically related to perturbant concentration, no linear relationship was observed.

MATERIALS AND METHODS

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; potassium acetate, 17.372 g/l. Equal parts of these components produced a solution of pH 4.65–4.70. A secondary buffer system, five times more concentrated than the primary system, was prepared and used in the preparation of the protein–buffer–glycol mixtures as described by Hart, Russell & Cooper (1971).

Preparation of acid-soluble collagen. Purified acid-soluble calf-skin collagen was prepared by the method of

Cooper & Davidson (1965). The procedure for preparing a concentrated stock solution from freeze-dried collagen and of checking concentrations has been described by Hart *et al.* (1971). The protein concentration was between 0.84 and 0.86 mg/ml and the acetate ion concentration in the final buffer–protein–glycol mixtures was 0.15M in all cases.

Optical-rotation recovery measurements. Optical-rotation recovery was monitored 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 the temperature was controlled to $\pm 0.05^\circ\text{C}$. Concentrated solutions of collagen in the buffer were denatured by heating at 45°C for 15 min, after which the systems were made up to final volume by the addition of predetermined quantities of organic solvent, concentrated buffer and distilled water at ambient temperature. Solutions were then transferred as quickly as possible to the polarimeter cells and allowed to equilibrate for approx. 5 min before taking the first readings. Readings were taken at 5–10 min intervals for the first h of renaturation and at 20–30 min intervals thereafter.

Effect of pH. In a preliminary investigation we found no significant effect on renaturation on raising the pH from 4.7–5.1. In most cases in the present investigation the final pH was between 4.7 and 5.1, the exceptions being with butane-1,4-diol and 2-ethoxyethanol where pH values of 5.4 and 5.5 respectively were recorded at the highest concentrations of perturbant. This latter variation is regarded as being well within the acceptable range, as a control renaturing at pH 5.5 showed an increase in optical rotation of only 3% after 48 h, as compared with a control at pH 4.7.

Calculation of initial rates. The use of a second-order equation in the present work provided a convenient means of determining initial rate values. The most suitable form was found to be:

$$\frac{1}{[A]} = \frac{1}{[A]_0} - \nu_A k'(T)t$$

(from Gardiner, 1969) where $[A]_0$ is the concentration of A at the start of the reaction; $[A]$ is the concentration of A at the variable upper limit; T is the absolute temperature; ν_A is negative and equals -1 or -2 ; t is the time; and $k'(T)$ is the rate constant, k ; T is included to indicate the temperature dependence of the rate constant.

The equation may be modified to give:

$$\frac{1}{y} = \frac{1}{y_\infty} + \frac{1}{y_\infty^2 \cdot k} \cdot \frac{1}{t} \quad (1)$$

(Hart, 1971) where $y = ([\alpha] \text{ at time } t) - \text{observed value of } [\alpha] \text{ at zero time } ([\alpha] \text{ at } t=0 \text{ is } -460^\circ, \text{ i.e. the specific rotation of fully denatured collagen})$ and y_∞ is assumed to be the maximum (i.e. equilibration) value for $[\alpha]$.

RESULTS

In order to test the accuracy with which a second-order rate equation of the form used in the present study (eqn. 1) could be fitted to experimental profiles, a comparison between experimental values and theoretical curves was made by using a Hewlett–

Fig. 1. Application of a second-order rate equation to experimental values for renaturation of acid-soluble collagen (0.84 mg/ml in 0.15M-potassium acetate buffer) at 15°C. Theoretical second-order recovery profiles predicting the course of the first 3 h of renaturation 1, in the absence of perturbant, and 2 in the presence of 4M-2-ethoxyethanol. Symbols show experimental points: ■, without perturbant; ▲, with 4M-2-ethoxyethanol.

Packard model 9100B programmable desk-top calculator. Basing calculations on the results during the first three hours of renaturation, the theoretical curves obtained from the rate equation were compared with the experimental values as shown in Fig. 1. The experimental points coincide very well with the theoretical profiles both in the absence of perturbant and in the presence of a relatively high concentration of one of the more strongly lyotropic perturbants studied, namely 2-ethoxyethanol. This is particularly so in the initial stages of renaturation, which are of special interest in the present study. The correlation coefficient relating the experimental results and the theoretical curves in both cases shown in Fig. 1 is 0.999, indicating a very good fit. The agreement between experimental values and theoretical profiles was checked in similar manner for all the perturbants studied and was found to be satisfactory in every case, correlation coefficients better than 0.99 always being obtained.

Relationship between $[\alpha]$ at 1h and the log of the initial rate. After having established the utility of the second-order kinetic equation in relation to the renaturing systems under investigation, the rate equation was further modified to yield the zero-time relation $dy/dt = y_{\infty}^2 \cdot k$ (Hart, 1971). An assessment of the value of 1h rotation readings, as a measure of initial mutarotation rates, was then made by plotting $\log(y_{\infty}^2 \cdot k)$ as a function of $[\alpha]$ at 1h. Fig. 2 illustrates the use of the method, the values for $\log(\text{initial rate})$ being plotted against $[\alpha]$ at 1h

Fig. 2. Relationship between 1h optical rotation recovery values and $\log(\text{initial mutarotation rate})$ for heat-denatured (45°C; 15 min) acid soluble collagen (0.84–0.86 mg/ml in 0.15M-potassium acetate buffer) renaturing at 15°C in the presence of various concentrations of 2-ethoxyethanol (▲) and diethylene glycol (○).

for various concentrations of 2-ethoxyethanol and diethylene glycol in systems renaturing at 15°C. In the case of 2-ethoxyethanol there is a linear relationship between the rate parameter and the 1h $[\alpha]$ value, the correlation coefficient being 0.997. This serves as the model case in which there is a significant and systematic decrease in mutarotation rate as the perturbant concentration is increased. Diethylene glycol (Fig. 2) exemplifies the case where there is no linear relationship between $[\alpha]$ at 1h and $\log(\text{initial rate})$, the correlation coefficient being 0.30. There appears to be a relationship between the effects of perturbants on T_m and the $[\alpha]$ at 1h versus $\log(\text{initial rate})$ correlation coefficient. Table I shows that perturbants that stabilize native soluble collagen show low correlation coefficients with respect to the $[\alpha]$ at 1h versus $\log(\text{initial rate})$ relation, whereas 'destabilizers' show high correlation coefficients. It appears that $[\alpha]$ at 1h is a simple function of the initial mutarotation rate only with perturbants that destabilize the native collagen molecule.

Effect of temperature. As the mutarotation rate depends markedly on the temperature at which renaturation takes place (Flory & Weaver, 1960; Harrington & von Hippel, 1961b), it was considered to be of importance to determine the effect on the $[\alpha]$ at 1h versus $\log(\text{initial rate})$ relation of slowing the overall reaction by raising the temperature at which renaturation was done. This also allowed for more precise monitoring of the early stages of the process. Thus 2-ethoxyethanol, a powerful destabilizer, and diethylene glycol, a highly effective stabilizer of the native protein,

Table 1. Relationship between the correlation coefficient for $[\alpha]_{365}^{15}$ at 1h versus log (initial rate) and the effect on the T_m of collagen at a perturbant concentration of 1M

ΔT_m is the change in T_m of native soluble collagen relative to the control.

Solvent	Correlation coefficient for $[\alpha]_{365}^{15}$ at 1h versus $\log(y_{\infty}^2 \cdot k)$ at 15°C	ΔT_m (°C)
(a) 'Stabilizers'		
Glycerol	0.878	+0.96
Diethylene glycol	0.301	+0.91
Propane-1,3-diol	0.739	+0.38
Ethylene glycol	0.599	+0.35
2-Methoxyethanol	0.770	+0.05
(b) 'Destabilizers'		
Butane-1,4-diol	0.974	-0.19
2-Ethoxyethanol	0.990	-0.33
	0.997	-0.85

were examined for their effects on renaturation kinetics at 20°C. When renaturation is monitored at 15°C, $[\alpha]$ at 1h, in the absence of perturbant, is approx. -900° . A comparable reaction stage for a control renaturing at 20°C occurs at 3h, as $[\alpha]$ is then in the region of -900° . We therefore compared the correlation between $[\alpha]$ at 3h and the log(initial rate) for renaturations at 20°C with that between $[\alpha]$ at 1h and the log(initial rate) for renaturations at 15°C. Table 2 shows that in the case of the destabilizer, 2-ethoxyethanol, the renaturation temperature does not appear to be a decisive factor affecting the correlation between fixed-time $[\alpha]$ values and initial rates. In contrast, systems containing the stabilizer, diethylene glycol, show a much improved correlation between the fixed-time values and the initial rate parameter when the reaction is slowed by renaturing at the higher temperature.

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 & Wong (1962, 1963a). A similar linear relationship between perturbant concentration and recovery rates has been found in studies with monohydric alcohols (Russell & Cooper, 1969) and various amides and alkyl-substituted amides (Russell & Cooper, 1970). In the present study, however, there have arisen a number of examples of an apparently non-linear relationship between perturbant concentration and zero-time reversion rates. Fig. 3 shows the effects of several perturbants on recovery rates: with diethylene glycol there is a marked acceleration in the log of the initial mutarotation rate as the perturbant concentration is increased. A maximum is shown

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

Solvent	Correlation coefficient for $[\alpha]_{365}^{15}$ at 1h versus $\log(y_{\infty}^2 \cdot k)$ at 15°C	Correlation coefficient for $[\alpha]_{365}^{20}$ at 3h versus $\log(y_{\infty}^2 \cdot k)$ at 20°C
2-Ethoxyethanol	0.997	0.988
Diethylene glycol	0.301	0.946

Fig. 3. Effect of perturbant concentration on log (initial mutarotation rate) of heat-denatured (45°C; 15 min) acid-soluble collagen (0.84–0.86 mg/ml in 0.15M-potassium acetate buffer) renaturing at 15°C: ○, diethylene glycol; ■, ethylene glycol; △, 2-methoxyethanol; □, propane-1,2-diol; ●, butane-1,4-diol; ▲, 2-ethoxyethanol.

at about 2M-diethylene glycol, after which there is a phase of rate decrease. Ethylene glycol produces a less marked increase in renaturation rate at low concentrations and a shallower maximum that occurs at about 3M. The pattern of effects produced by 2-methoxyethanol is similar; however, the acceleration of initial rate at low perturbant concentration is even less marked than with ethylene glycol. Acceleration of the initial rate is not observed with propane-1,2-diol, although there is the suggestion of a period of induction at low concentration, after which the mutarotation rate decreases sharply. The tendency towards progressive retar-

Fig. 4. Effect of perturbant concentration on log(initial mutarotation rate) of heat-denatured (45°C; 15 min) acid-soluble collagen (0.86 mg/ml in 0.15M-potassium acetate buffer) renaturing at 20°C: ○, diethylene glycol; ▲, 2-ethoxyethanol.

dation of mutarotation rate is accentuated in the cases of butane-1,4-diol and 2-ethoxyethanol.

The trends shown in the systems renaturing at 15°C were confirmed by an examination of reaction profiles from systems renaturing at 20°C. Diethylene glycol was chosen as the prototype for perturbants showing a strong tendency to stabilize the protein in its native state, and 2-ethoxyethanol as an example of a powerful destabilizer (Hart *et al.* 1971). Fig. 4 shows that the results at 20°C confirm the findings for diethylene glycol at 15°C (cf. Fig. 3); the initial acceleration of mutarotation rate at low perturbant concentration is again followed by a trend reversal, after which rate retardation increases sharply. Similarly, the results obtained at 20°C with the destabilizer, 2-ethoxyethanol (Fig. 4), substantiate the trends shown at 15°C (Fig. 3).

DISCUSSION

The findings reported above provide supporting evidence for the suggestion (Beier & Engel, 1966; Harrington & Karr, 1970; Harrington & Rao, 1970; Hauschka & Harrington, 1970*a,b,c*) that inter- or intra-chain interactions are a fundamental part of the process involved in the generation of poly-L-proline II-type helical segments in collagen. The three-stage mechanisms proposed by Harring-

ton & von Hippel (1961*b*) and Flory & Weaver (1960) now appear to be unsatisfactory.

Despite the fundamental differences that exist between systems containing the protein in its native triple helical form and the more complicated structural situation prevailing in a renaturing system, the effects of perturbants in the two types of system may usefully be compared. Considering first the apparent non-linearity of the log (initial rate) *versus* perturbant concentration plots; it has been shown (Harrington & von Hippel, 1961*b*; von Hippel & Wong, 1963*a*) that changes in the nature of the solvent that result in an increased initial mutarotation rate in renaturing systems are associated with an increase in the T_m of the native protein in certain cases. Our results show, however, that although 'stabilizers' progressively raise the T_m as the perturbant concentration is increased, they produce distinct rate maxima and trend reversals in renaturing systems. Thus the initial enhancement of the rate of optical rotation recovery at low perturbant concentrations appears to be overcome by a supervening effect as the perturbant concentration is increased.

The results described above may be interpreted by assuming that perturbants that stabilize native collagen do so because they are less powerful polar bonding agents than water. Hence a simple diluting effect, which lowers the dielectric constant of the solvent mixture, dominates at low concentrations of organic component. The enhanced stability of the native structure would then be caused by: (1) a diminished tendency towards disruption of interchain hydrogen bonds within the triple helix, and (2) decreased frequency of polar interactions between environmental molecules and peptide carbonyl and imide groups in the protein, thus limiting further the already restricted rotation about peptide bonds. Extending this approach to include renaturing systems, it is suggested that the lower polar-atom content of the environment should result in enhanced formation of 'favourable' nuclei, both by virtue of a statistically diminished bonding of environmental molecules to peptide carbonyl and imide groups and by lessened competitive disruption of the native and non-native tertiary structures that probably stabilize the re-forming helices (Harrington & Rao, 1970). At higher concentrations of stabilizing perturbants, however, it is conceivable that 'unfavourable' intrachain and interchain nucleation events could become so prevalent that they inhibit the rate and extent of recovery of optical rotation. Thus, the formation of doubled- or tripled-back, non-native conformations of individual polypeptide chains (Drake & Veis, 1964), and of disordered aggregates of several chains [defined by Harrington & Karr, (1970) as the 'freezing in' of disordered conformational

patterns during the nucleation process], might be enhanced to an extent that is incompatible with the optimum regeneration of the collagen fold. This approach is analogous to the explanation offered by Harrington & Karr (1970) and Hauschka & Harrington (1970a) for the results of their 'under-cooling' experiments, namely, that at very low quenching temperatures the 'freezing in' process results in the formation of imperfect structures that do not have the optical rotatory power of the better-ordered conformations. In addition, the effect of perturbants on the initial mutarotation rates provides support for the suggestion (Hauschka & Harrington, 1970b) that even the very earliest stages of nucleus formation involve interaction of a number of polypeptide chains or segments of the same chain.

A simpler kinetic situation appears to prevail in relation to the effects of perturbants that destabilize the native form of the protein. The destabilizers most closely studied in the present work were butane-1,4-diol, propane-1,2-diol and 2-ethoxy-ethanol. As these perturbants lower the T_m of the native state (Hart *et al.* 1971) it appears likely that they should retard the renaturation process, which they clearly do (Fig. 3). In these and similar cases it seems practical to use $[\alpha]$ values at suitable fixed times, e.g. 1 and 48 h, as relative indicators of mutarotation kinetics and equilibrium recovery values respectively (Russell & Cooper, 1969). Finite-time $[\alpha]$ -values have further uses as readily accessible indicators of the effects of destabilizing perturbants (D. R. Cooper, A. E. Russell & G. J. Hart, unpublished work).

Also of interest is the marked improvement in the correlation between fixed-time $[\alpha]$ -values and the log of the zero-time reversion rate, on raising the renaturation temperature from 15° to 20°C. This suggests a modification of the processes involved. In terms of the proposals of Beier & Engel (1966), this could imply a movement towards the formation of a higher proportion of truly native-type collagen triple helices, as opposed to other possible uni- or multi-stranded structures. The more complex kinetic situation that seems to prevail at the lower renaturation temperature may result from an increase in the extent to which a competitive equilibrium exists between the re-forming native-type structures and non-native, back-folded or aggregated helical conformations.

The above arguments are consistent with the suggestions we have made previously in relation to the effects of perturbants on the thermal transition temperature of native collagen (Hart *et al.* 1971). It was proposed that perturbant action might be dependent upon the water-structure-promoting properties of perturbant molecules and the effect this could have on perturbant-protein hydrogen-

bonding processes. Notably, such a mechanism does not require any role for direct hydrophobic interactions in perturbational activity. This is in agreement with the approach adopted by Harrington & Rao (1970) in which the formation of a hydrogen-bonded nucleus is considered to be the rate-determining step. Thus an entirely polar-bond-mediated mechanism for perturbant action appears to be consistent with all the results discussed above.

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