Short Communication

Enhanced Collagen Renaturation in the Presence of a Lyotropic Agent

By A. E. RUSSELL and D. R. COOPER

Leather Industries Research Institute, Rhodes University, Grahamstown, Republic of South Africa

(Received 17 March 1969)

The lyotropic effect of urea and certain neutral salts on protein structures in solution is well established and is generally evident in a lowering of the characteristic thermal transition temperature at which the native ordered conformation is transformed into a random-coil form (Steven & Tristram, 1962; Hippel & Wong, 1963; Tanford, 1967). In the present communication, preliminary results obtained when solutions of acid-soluble calf-skin collagen were renatured in the presence of urea are reported. These studies suggest that at moderate urea concentrations an appreciable increase in the rate and extent of reversion as measured by physico-chemical techniques is effected.

Renaturation kinetics were followed by optical-rotation and reduced-viscosity measurements at 15° by using procedures described by Davidson & Cooper (1967). Acid-soluble calf-skin collagen (Cooper & Davidson, 1965) in 0.15M-potassium acetate buffer, pH 4.8, was denatured at 45° for 15 min. before addition of the required amounts of urea and dilution to volume with buffer to give a final concentration of 0.86 mg. of dry protein/ml.

Increasing urea concentration progressively decreased the initial rate of back mutarotation (Fig. 1a), although the rates of the subsequent slow mutarotation after 2 hr. were greater than that of the control. Thus the reaction curve with 0.5M-urea crossed that of the control after approx. 8 hr., giving reversion values after 32 hr. slightly in excess of the control. It was apparent that a similar situation would arise at higher urea concentrations if the renaturation periods were to be extended. Although there was evidence of progressive decrease in the initial rate of reduced-viscosity recovery with increasing urea concentration similar to that noted in the mutarotation reaction profiles, this effect was largely obscured by the subsequent increase in the rate and extent of recovery in urea concentrations up to 2.0M, with maximum effect at about 1.0M (Fig. 1c). The optimum regain noted with 1.0M-urea was particularly striking, since, in terms of optical-rotation recovery, reversion after 32 hr. was less than that of the control. The effect of urea concentration on the 32 hr. optical-rotation and reduced-viscosity recovery values is shown in

Figs. 1(b) and 1(d), from which the occurrence of an optimum concentration range in each case is apparent.

The experimental observations appear to be consistent with the renaturation mechanism proposed previously in which the initial relatively rapid event involves the nucleation and propagation of the poly-L-proline II-type secondary structure along individual polypeptide chains, accompanied by chain association through the formation of lateral hydrogen bonds at a lower rate (Hippel & Harrington, 1959; Harrington & Hippel, 1961a,b; McBride & Harrington, 1967). On this basis the action of urea appears to be twofold in nature. First, the decrease in the initial mutarotation reversion rate may be attributed to the effect of urea in retarding the nucleation phase involving localized configurational changes in the imino acid-rich regions of the individual chains by decreasing normal rotational restriction about the backbone α -carbon to carbonyl-carbon bonds in these regions (Harrington & Hippel, 1961a). Decrease in mutarotation recovery rates by urea is also consistent with previous work demonstrating a negative temperature coefficient for mutarotation recovery, the initial rates being directly dependent on the difference between the renaturation temperature employed (variable) and the denaturation temperature of the native collagen in the same medium (fixed) (Flory & Weaver, 1960; Hippel & Wong, 1963; Beier & Engel, 1966). In the present study the effective differential between the renaturation temperature (fixed at 15°) and the denaturation temperature (variable with urea concentration) decreased with increasing urea concentration and a progressive decrease in initial mutarotation rates could be expected.

Secondly, it is suggested that the effect of moderate urea concentrations in enhancing the reaction step responsible for the subsequent increases in optical rotation and, to a greater extent, in reduced viscosity may be attributed to competitive hydrogen-bond formation by the amide at the peptide carbonyl and/or imino groups on the exposed chains of the denatured collagen. These sites are thereby shielded from ready recombination

Fig. 1. Renaturation of heat-denatured acid-soluble collagen (0.86 mg./ml.) at 15° in the presence of urea: specific-rotation recovery as a function of (a) time and (b) urea concentration; reduced-viscosity recovery as a function of (c) time and (d) urea concentration. Concentration of urea: $\circ$, none (control); Δ , 0.5 M; $\square$, 1.0 M; $\bullet$, 1.5 M; $\blacktriangle$, 2.0 M; $\blacksquare$, 2.5 M. Upper and lower limits (broken lines) denote specific-rotation and reduced-viscosity values for collagen (15°) and gelatin (43°) respectively.

through hydrogen-bond formation with complementary sites on segments of the same or adjacent chains. Solvation by urea molecules in this way would tend to restrict selectively the incidence of random hydrogen-bond associations that would retard development of the systematic

co-operative hydrogen-bond structure of collagen. Selectivity may be attributed to the fact that the binding of a urea molecule as a type of peptide-bond structural analogue is a transient phenomenon governed by mass-action considerations and lacking the co-operative features of multiple hydrogen-

bonding. Thus propagation of the inherently more stable collagen structure will still be energetically favoured at moderate urea concentrations. Under these conditions more controlled reversion to collagen-type forms would permit a correspondingly larger proportion of the original structural hydrogen-bonding to be recovered, reflected in the enhanced rate and extent of reduced-viscosity and optical-rotation recovery compared with the control over the subsequent reaction stage. The mechanism proposed also envisages an optimum effect at intermediate concentrations beyond which the increase in urea binding due to mass action progressively decreases the extent of all structural hydrogen-bonding.

Evidence for the occurrence of random inter- or intra-chain interactions inhibiting development of the fully native structure in renaturing collagen systems has been presented by previous workers who point out that the effective chain-segment concentration within the domain of a flexible single-strand gelatin molecule is about 2mg./ml. in the vicinity of any particular segment so that decrease in protein concentration below this value could not be expected to decrease the occurrence of intra-chain intersegment interactions (Drake & Veis, 1964). The progressive improvement in reduced-viscosity recovery (without comparable increase in mutarotation reversion) effected by temperature cycling during renaturation has also been related to the dissociation of less stable randomly hydrogen-bonded structures with subsequent reorientation to give structures of collagen type (Kühn, Engel, Zimmermann & Grassmann, 1965). In renaturation at various fixed temperatures Beier & Engel (1966) have demonstrated the presence of high-molecular-weight pepsin-sensitive aggregates of intermediate thermal stability apparently arising from random hydrogen-bond formation between chain segments and occurring together with re-formed collagen structures of higher thermal stability. The occur-

rence of an optimum in reduced-viscosity recovery was noted at intermediate renaturation temperatures at which the alternative structural forms were no longer stable and hence no longer produced, permitting greater orientation to more stable collagen-type structures with higher reduced viscosity. A similar mechanism would suffice to explain the optimum effect noted at intermediate urea concentrations in the present communication.

The use of lyotropic agents as mechanistic probes in collagen renaturation thus affords a further insight into the nature of the events involved in the renaturation of collagen.

The technical assistance of Mr R. Kohl is gratefully acknowledged. This work was supported by the annual grants of the South African Livestock and Meat Industries Control Board and the Council for Scientific and Industrial Research.

- Beier, G. & Engel, J. (1966). *Biochemistry*, **5**, 2744.
Cooper, D. R. & Davidson, R. J. (1965). *Biochem. J.* **97**, 139.
Davidson, R. J. & Cooper, D. R. (1967). *Biochem. J.* **105**, 965.
Drake, M. P. & Veis, A. (1964). *Biochemistry*, **3**, 135.
Flory, P. J. & Weaver, E. S. (1960). *J. Amer. chem. Soc.* **82**, 4518.
Harrington, W. F. & Hippel, P. H. von (1961a). *Advanc. Protein Chem.* **16**, 1.
Harrington, W. F. & Hippel, P. H. von (1961b). *Arch. Biochem. Biophys.* **92**, 100.
Hippel, P. H. von & Harrington, W. F. (1959). *Biochim. biophys. Acta*, **36**, 427.
Hippel, P. H. von & Wong, K.-Y. (1963). *Biochemistry*, **2**, 1387.
Kühn, K., Engel, J., Zimmermann, B. & Grassmann, W. (1965). *Arch. Biochem. Biophys.* **105**, 387.
McBride, O. W. & Harrington, W. F. (1967). *Biochemistry*, **6**, 1499.
Steven, F. S. & Tristram, G. R. (1962). *Biochem. J.* **85**, 207.
Tanford, C. (1967). In *Physical Chemistry of Macromolecules*, p. 624. New York: John Wiley and Sons, Inc.