Effect of Alcohols and Neutral Salt on the Thermal Stability of Soluble and Precipitated Acid-Soluble Collagen

By ALLAN E. RUSSELL

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

(Received 5 July 1972)

The effects of mono- and poly-hydric alcohols in the presence of KCl on the intrinsic stability of collagen molecules in dilute acid solution were compared with corresponding solvent and salt effects on the increased stability of the aggregated molecules in salt-precipitated fibrils. Salt addition decreased solubility and increased the thermal stability of fibrils, but progressively decreased the stability of collagen molecules in solution. In contrast, the alcohols enhanced solubility and decreased fibril stability, the effects increasing with solvent hydrocarbon chain length and with decreasing hydroxyl/methylene-group ratio. Molar destabilization of dissolved collagen by alcohols was lower than for fibrils, and at low salt concentration, both ethylene glycol and glycerol were structural stabilizers. Electron-micrograph studies indicated that salt-precipitated fibrils tended to adopt the native aggregation mode, and qualitatively similar solvent effects were observed in insoluble collagens. Implications of the experimental findings are discussed in terms of a model in which electrostatic and apolar interactions mainly govern the excess of stability in collagen fibrils whereas intrinsic stability of single molecules is a function of polar interactions and polypeptide-chain rigidity.

Previous studies from these laboratories have reported the use of related organic perturbants as collagen structural probes in attempts to elucidate lyotropic mechanisms and gain an insight into factors governing the intrinsic stability of collagen molecules in the dispersed state. Perturbant structural and functional features such as linear hydrocarbon chain length, chain isomerism and position and number of potential hydrogen-bonding groups were found to constitute the main factors determining solvent effects on thermal stability and renaturation kinetics of collagen in solution (Russell & Cooper, 1969*a,b*, 1970, 1972; Hart *et al.*, 1971; Cooper *et al.*, 1971). Similar solvent effects have been reported by other workers for soluble collagen (Herbage *et al.*, 1968; Schnell, 1968; Harrap, 1969; Bianchi *et al.*, 1970) and globular proteins (Schrier & Scheraga, 1962; Herskovits *et al.*, 1970*a,b,c*; Kaminsky *et al.*, 1972).

In extending these solvent studies to the molecular aggregates present in fibrous collagen, it was found that certain perturbants differed markedly in their activities in the soluble and insoluble collagen systems. Thus, ethylene glycol, glycerol and propane-1,3-diol progressively stabilized collagen in solution (Harrap, 1969; Hart *et al.*, 1971), but destabilized mature fibrous collagen at the low-solvent extreme (Russell & Cooper, 1971). Although relative activities and the influence of solvent structure on perturbant activity in soluble and insoluble collagens were qualitatively similar for the other solvents examined,

molar activities at infinite dilution were consistently greater in insoluble collagen.

The different response of collagen in the dispersed and aggregated states toward the glycols and glycerol suggests that different factors predominate in the stabilization of the two forms. Accordingly, in the present study, the phenomenon and its structural implications have been further explored. In particular, the effects of the lower mono- and poly-hydric alcohols in the presence of neutral salt have been compared with respect to their effects on the thermal stability of soluble, salt-precipitated and insoluble collagens as a function of solvent composition.

Experimental

Materials

Acid-soluble calf skin collagen was extracted and purified by the method previously described after removal of the neutral-salt-soluble fraction (Cooper & Davidson, 1965). Both the acid-soluble collagen and the insoluble collagen fragments recovered after extraction were neutralized, dialysed free of salts and freeze-dried. An area of intact insoluble collagen for shrinkage-temperature studies was prepared from limed and acetone-dehydrated sheep skin as previously described (Russell & Cooper, 1971) to give a collagen matrix conforming to standard commercial hide powder [*Official Methods of Analysis* (1965)]

Society of Leather Trades' Chemists, Redbourn]. Reagents used were A.R. grade or laboratory-grade materials.

Measurement of thermal-transition temperatures

'Melting' temperature of acid-soluble collagen in solution. 'Melting curves' were obtained by polarimetry by using the '30 min method' (von Hippel & Wong, 1965) on collagen solutions containing approx. 1 mg of dry protein/ml in potassium acetate buffer containing various concentrations of KCl and organic solvent within the range of protein and salt solubility and 0.15 M with respect to total acetate concentration in the final mixture (pH 4.8 in pure aqueous solution). The temperature at the midpoint of the transition was taken as the 'melting' point.

Dispersion temperature of salt-precipitated acid-soluble collagen. Fibres of acid-soluble collagen were precipitated by mixing equal volumes of a solution of acid-soluble collagen (2 mg/ml) in 0.15 M-potassium acetate buffer (pH 4.8) and a solution of 1 M-KCl in 0.15 M-potassium acetate buffer. The collagen was thus allowed to precipitate in 0.15 M-potassium acetate buffer at ambient temperature at a final composition of 1 mg of protein/ml and 0.5 M-KCl, when precipitation was substantially complete in a few minutes. Precipitations were also carried out at lower KCl concentrations (0.25, 0.125 M), requiring correspondingly longer precipitation times. Thermal-stability measurements were carried out as previously described (Russell & Cooper, 1971) by suspending a small portion of the precipitated fibres in aqueous organic solvent media of various compositions incorporating 0.15 M-potassium acetate buffer and various concentrations of KCl. Addition of KCl to the organic solvent-buffer medium was necessary to suppress protein solubility, since in the absence of salt, the precipitate tended to redisperse. The suspension was heated (0.5–1°C/min) in a test tube supported in a beaker of water on a magnetic stirrer/hotplate and the temperature of incipient dissolution of the fibres as gelatin was noted.

Shrinkage temperature of mature collagen fibres. The temperature of incipient length contraction (or 'shrinkage temperature') of mature insoluble collagen strips exposed to various aqueous organic solvent compositions incorporating 0.15 M-potassium acetate buffer and various concentrations of KCl, was measured as previously described [Russell & Cooper (1971); *Official Methods of Analysis* (1965) Society of Leather Trades' Chemists, Redbourn]. With extracted insoluble calf skin fragments recovered after the preparation of acid-soluble collagen, shrinkage temperatures were measured by observing dimensional changes in individual fibres exposed to solvent media by using a Reichert 'Thermopan' microscope fitted with a Kofler hot stage. A portion of dry

collagen was shaken with excess of solvent medium; the wetted fibres were placed in the centre of a ring of light oil on a microscope slide and sealed with a cover slip. The temperature was then raised at a controlled rate (0.5–1°C/min) and the temperature of incipient dimensional change noted.

Electron microscopy

The collagen fibrils precipitated at various KCl concentrations were positively stained in 1% (w/v) phosphotungstic acid adjusted to pH 6.0–6.3 by addition of 1% (w/v) NaOH. A drop of fibril suspension was placed on a formvar-coated grid on filter paper, the grid was flooded twice with water to remove salts and drained. The grid was then held in staining solution for 30–60 s and subsequently washed by immersion in water four times and drained on filter paper. The positively stained fibrils were examined in the Hitachi transmission electron microscope.

Results

Effect of ethylene glycol and glycerol at various KCl concentrations on thermal stability of dissolved and salt-precipitated acid-soluble collagen

Variations in transition temperatures of the dissolved and salt-precipitated forms of acid-soluble collagen, respectively, with solvent concentration at various concentrations of added KCl are shown in Fig. 1. At a given salt concentration, the dispersion temperature of the precipitated collagen decreased as the ethylene glycol or glycerol concentration increased up to the limiting solvent concentration at which the precipitate redissolved at ambient temperature. Similar destabilization by ethylene glycol and glycerol at low solvent proportions in the absence of salt was noted previously with mature sheep skin collagen (Russell & Cooper, 1971). At a given solvent concentration, however, thermal stability of the precipitate increased as the concentration of added KCl increased, and the limiting solvent concentration for dissolution of the precipitate at ambient temperature shifted to higher solvent proportions. Molar destabilization effects at the various salt concentrations examined were similar for both ethylene glycol and glycerol. No significant differences were apparent when effects of ethylene glycol on fibrils precipitated in 0.5 M- and 0.25 M-KCl were compared, although precipitation rates at the higher salt concentration were more rapid (Fig. 1a).

Specific-rotation measurements in the solutions obtained at temperatures fractionally above the fibril-dispersion temperatures yielded values corresponding to those of gelatin ($[\alpha]_{589}^{25} -460^\circ$), indicating that fibril dissolution under these conditions resulted from

Fig. 1. Effect of (a) ethylene glycol and (b) glycerol at various concentrations of added KCl on thermal stability of precipitated and dissolved acid-soluble calf skin collagen

The solvent medium was 0.15M-potassium acetate buffer (pH4.8) containing various amounts of KCl. Open symbols correspond to experimental values obtained for collagen fibrils precipitated initially in 0.5M-KCl (see the Experimental section); identical trends were obtained for fibrils formed in 0.25M-KCl. Closed symbols correspond to values for dissolved collagen. Discontinuities marked by vertical broken lines denote approximate limiting solvent concentrations at which precipitated collagen redissolved. Back-extrapolated broken lines represent hypothetical trends for soluble collagen. Concn. of KCl: ●, ○, 1.0M; ▲, △, 0.75M; ■, □, 0.5M; ▼, ▽, 0.25M; ♦, 0M.

a helix-to-coil transition in the material. On the other hand, optical-rotation measurements at ambient temperature on the material in solution at solvent concentrations above the fibril-dissolution limit gave specific-rotation values corresponding to those of native collagen ($[\alpha]_{589}^{25} - 1330^\circ$), indicating that normal protein dissolution had occurred under these conditions without accompanying internal structural change.

Both solvent and salt effects on the dissolved collagen differed markedly from corresponding effects on the precipitated form. Thus, addition of KCl progressively lowered the stability of the dispersed collagen at all organic solvent concentrations, whereas at low salt concentration, ethylene glycol and glycerol acted as structural stabilizers, as noted previously in the absence of neutral salt (Harrap, 1969; Hart *et al.*, 1971). Solvent stabilization effects decreased with salt addition, however, and at higher concentrations of KCl, ethylene glycol and the isomeric propanediols (1.0M-KCl; Figs. 1a and 4) were structural destabilizers. Comparison of plot gradients on either side of the fibril-dissolution limit indicated that molar destabilization of soluble collagen by these solvents was considerably less than for the precipitated form.

A tentative back-extrapolation of the approximately linear trends observed for soluble collagen to zero ethylene glycol concentration is shown in Fig. 1(a), and a similar extrapolation of the curved plots

obtained with glycerol was attempted in Fig. 1(b). The divergent trends apparent in both cases reflect an increase in molar destabilization by KCl as the concentration of solvent increased.

Comparison of effects of ethylene glycol and KCl on thermal stability of insoluble and precipitated collagens

Qualitatively similar effects were apparent on comparing ethylene glycol activity at various concentrations of KCl (Fig. 2) on precipitated acid-soluble collagen, extracted insoluble calf skin collagen (microscopic methods) and mature sheep skin collagen (macroscopic shrinkage method). As found above for precipitated collagen, thermal stability of the insoluble collagens decreased with solvent addition, but increased with salt addition. With mature sheep skin collagen a converging trend with increasing solvent concentration was apparent, resulting in intersection of the plots at higher solvent proportions. Similar but less pronounced convergence was also evident with the precipitated and insoluble calf skin collagens.

Quantitatively, transition temperatures for the precipitated collagen were approx. 5°C higher than those of the insoluble collagen from which it was extracted. Shrinkage temperatures of the mature sheep skin collagen, however, were approx. 12°C higher than those of the precipitated calf skin collagen at the same salt concentration.

Fig. 2. Comparison of ethylene glycol activity at various concentrations of added KCl on thermal stability of precipitated acid-soluble collagen and insoluble fibrous collagens

The solvent medium was 0.15M-potassium acetate buffer (pH4.8) containing various amounts of KCl. Values for insoluble sheep skin collagen ($\circ$, Δ) were determined by the macroscopic shrinkage method; values for acid-soluble calf skin collagen precipitated in 0.5M-KCl ($\bullet$, Δ) and extracted insoluble calf skin collagen ($\bullet$, Δ) were determined by the microscopic method (see the Experimental section). $\circ$, $\bullet$, 1.0M-KCl; Δ , Δ , 0.5M-KCl.

Electron-microscope studies

Collagen fibrils precipitated at various concentrations of KCl were examined in the electron microscope to determine the effect of salt on aggregation mode. At initial collagen concentrations of 1 mg/ml in 0.15M-potassium acetate buffer, precipitation in 0.5M-KCl was virtually instantaneous, whereas at 0.25M- and 0.125M-KCl, turbidity development was preceded by induction periods of approx. 1 h and 4 h respectively.

In 0.125M-KCl, extended fibrils were obtained with a higher degree of native 64nm (640 Å) striation apparent. Microfibrillar strands comprising the larger fibrils and showing a small degree of twisting were also discernible.

At 0.25M-KCl a mass of entangled, twisted fibrils was obtained in which striation appeared to be obscured. Native striations were apparent in the thinner less-dense fibrils, showing various degrees of twisting.

Fig. 3. Effect of solvent hydrocarbon chain length at constant hydroxyl/methylene-group ratio on thermal stability of precipitated and dissolved acid-soluble calf skin collagen

The solvent medium was 0.15M-potassium acetate buffer (pH4.8) containing 1.0M-KCl. For other details see Fig. 1. Solvents: $\circ$, methanol; Δ , ethylene glycol; $\blacksquare$, glycerol.

At 0.5M-KCl a high yield of tightly twisted fibrils of extended length was obtained without visible striation.

Structural factors in solvent activity

The effects of methanol, ethylene glycol and glycerol on thermal stability of precipitated and dissolved acid-soluble collagen are compared at 1.0M-KCl in Fig. 3. Molar destabilization of precipitated collagen by the three solvents was comparable notwithstanding the change in molecular size. As noted above, however, solvent activity varied significantly with collagen in solution, where glycerol was a structural stabilizer and ethylene glycol a destabilizer under these conditions. The precipitated collagen remained insoluble over the methanol concentration range examined.

The effect of decreasing ratio of solvent hydroxyl/methylene groups is shown in Fig. 4, in which the activities of glycerol, propane-1,2-diol, propane-1,3-diol and propan-1-ol are compared. A progressive increase in molar destabilization of the precipitated collagen was apparent as the hydroxyl/methylene-group ratio decreased. Positional isomerism had little influence on propanediol activities, which were comparable. With collagen in solution, however, propane-1,2-diol was the more active isomer, whereas a progressive increase in destabilization with decreasing hydroxyl-group content was suggested by comparison

Fig. 4. Effect of decreasing solvent hydroxyl/methylene-group ratio at constant hydrocarbon chain length on thermal stability of precipitated and dissolved acid-soluble calf skin collagen

The solvent medium was 0.15M-potassium acetate buffer (pH4.8) containing 1.0M-KCl. For other details see Fig. 1. Solvents: ●, ○, glycerol; ▲, △, propane-1,3-diol; ■, □, propane-1,2-diol; ▽, propan-1-ol.

Fig. 5. Effect of increasing hydrocarbon chain length in monohydric alcohols on thermal stability of precipitated acid-soluble calf-skin collagen

The solvent medium was 0.15M-potassium acetate buffer (pH4.8) containing 1.0M-KCl. No fibril dissolution occurred over the experimentally accessible range. Solvents: ○, methanol; △, ethanol; □, propan-1-ol.

of glycerol and the propanediols. Solvent immiscibility prevented extension of the results for propan-1-ol.

The effect of hydrocarbon chain length on activity

of monohydric alcohols is shown in Fig. 5. A progressive increase in molar destabilization of the precipitated collagen with increasing chain length was apparent, as noted in mature sheep skin collagen (Russell & Cooper, 1971) and acid-soluble calf skin collagen in solution (Russell & Cooper, 1969a). Molar activity decreased progressively as the solvent concentration increased. No protein-solubility limit was apparent over the extended range of solvent concentrations examined, so that solvent effects on soluble collagen under these conditions were not measurable.

Discussion

The results of the present study indicate that collagen aggregation to form fibrils *in vitro* causes a significant gain in thermal stability relative to that for single molecules under the same conditions. An insight into the origin of the stability increase and the underlying factors involved may be gained from consideration of the different responses of collagen in the dispersed and aggregated states to solvent and neutral-salt perturbants.

Salt effects on soluble and precipitated collagens

The effects of KCl in accelerating collagen precipitation and stabilizing the resultant fibrils are suggestive of an increase in protein-protein interaction in acid medium in the presence of salt. Similar correlation between formation rate and fibril stability as influenced by various environmental factors such as pH value, temperature and ionic strength has been noted previously (Cooper, 1970). At physiological pH values, however, neutral-salt effects on collagen interaction appear to differ considerably from those in acid medium. Thus Bensusan & Hoyt (1958) and Wood & Keech (1960) found a progressive decrease in precipitation rate with increasing ionic strength as a result of salt addition in the pH range 7.0–8.5. Similarly in the present study, addition of KCl to precipitated and insoluble collagens in alkaline media produced slight destabilization, but markedly stabilized both forms at acid pH values (Table 1).

These pH-dependent salt effects indicate that charge interactions are implicated in collagen-fibril aggregation and stabilization. In acid media at low salt concentration, mutual repulsion between collagen molecules carrying an excess of positive charge would be expected to minimize aggregation. As the KCl concentration is increased, however, preferential anion binding may decrease the cationic character of the protein, resulting in a progressive precipitation of isoelectric collagen at rates governed by Cl^- ion concentration. Electrophoretic evidence for such preferential binding for Cl^- ions on collagen has been

Table 1. *Effect of KCl on thermal stability of precipitated and insoluble collagens in acidic and neutral pH ranges*

Values reported are the dissolution and shrinkage temperatures for precipitated acid-soluble calf skin collagen and insoluble sheep skin collagen respectively. For details see the text.

Concn. of KCl (M)	Temperature (°C)			
	0.15M-Potassium acetate buffer, pH4.8		0.15M-Potassium acetate buffer, pH8.5	
	Precipitated acid-soluble collagen	Insoluble collagen	Precipitated acid-soluble collagen	Insoluble collagen
0	43.6	60.0	52.0	63.5
0.25	46.0	62.3	52.0	62.3
0.50	48.5	63.0	51.6	61.0
0.75	51.5	66.0	51.2	59.7
1.00	53.5	67.0	51.0	60.5

presented by Bensusan & Hoyt (1958). Conversely, dissolution of precipitated fibrils will also depend on concentration of Cl^- ions available to maintain electrical neutrality. It follows that additional structural stabilization in fibrils can be expected arising from an increase in polypeptide-chain rigidity in the associated molecules, the effect increasing with salt addition. At pH values above the isoelectric point, however, when the protein carries an excess of negative charge, no comparable effects due to Cl^- ion binding can be expected.

A similar mechanism of charge interaction may be invoked to account for the effects of KCl on the thermal stability of insoluble collagens, in which dissolution is prevented by the presence of covalent crosslinks (reviewed by Veis, 1967). Variation in thermal stability with salt concentration was qualitatively similar to that noted for fibrils precipitated in 0.5M- and 0.25M-KCl, the latter showing evidence of the striated, native aggregation mode. The larger number of covalent crosslinks conferring rigidity in mature sheep skin would appear to account for the generally higher stability of this material relative to the precipitated and insoluble calf skin collagens. Reasons for the slightly lower stability of insoluble calf skin collagen compared with the precipitated material are not apparent.

Destabilization of acid-soluble collagen by KCl in alcohol-buffer media is consistent with previous reports of lyotropic effects of neutral salts on collagen (reviewed by von Hippel, 1967) and other proteins (reviewed by von Hippel & Schleich, 1969). Although lyotropic mechanisms remain controversial, neutral-salt effects are currently considered to involve ion-dipole association or hydrogen-bonding of a hydrated species at the peptide bond leading to a decrease in double-bond character and consequent loss in poly-

peptide-chain rigidity. Evidence for such interaction stems from neutral-salt effects on conformational changes in poly-L-proline, which lacks internal hydrogen bonding (Schleich & von Hippel, 1969). Alternatively, for proteins, alteration of water structure by ions has also been suggested to favour the exposure of internal hydrophobic groups to the solvent, resulting in unfolding of the native structure.

For individual collagen molecules in dilute solution, a lyotropic mechanism involving changes in side-chain solvation is less plausible, since all side chains are located on the molecular surface (Ramachandran, 1967) and hence are relatively accessible to the solvent in both the native and denatured states. Alternatively, a mechanism involving binding of ions at peptide bonds in soluble collagen with charge interactions constituting an additional factor in precipitated and insoluble collagen, would account for the distinctive effects of KCl on protein stability in the two states.

Alcohol effects on soluble and precipitated collagens

Destabilization of precipitated acid-soluble collagen and the influence of hydrocarbon structure and hydroxyl-group substitution on solvent activity in the present study were consistent with effects of aqueous alcohols at low proportion on insoluble collagen previously reported (Schnell & Zahn, 1965; Russell & Cooper, 1971). Analogous activity-structure relationships in retardation of collagen precipitation by monohydric alcohols (Bensusan, 1960) suggests that these solvent effects on stability and fibril formation reflect decreases in protein-protein interaction as a result of competitive solvent binding in predominantly aqueous media. Evidence for direct solvent-protein interaction moderated by hydro-

carbon chain length, has been reported in studies of the binding of alcohol homologues to collagen membranes (Bianchi *et al.*, 1970).

The influence of hydrocarbon chain length on lyotropic activity has led previous workers to conclude that alcohols disrupt internal hydrophobic bonding in proteins generally (Kauzman, 1959; von Hippel & Wong, 1965; Herskovits *et al.*, 1970a) and, by analogy, in soluble and insoluble collagens (Schnell & Zahn, 1965; Herbage *et al.*, 1968; Schnell, 1968; Harrap, 1969). For collagen fibrils, the positive temperature dependence of formation (Gross, 1958; Cooper, 1970; Cassel, 1971) is indicative of an endothermic, and hence entropy-driven, process, implicating solvent-labile hydrophobic interactions between apolar side chains in adjacent molecules in aggregation and stabilization.

A qualitative difference between the stabilizing factors predominating in individual molecules and collagen fibrils is suggested by the observation that activities for ethylene glycol and glycerol and for the isomeric propanediols differed substantially for collagen in solution, but were comparable for fibrils. Moreover, molar effects of the solvents for precipitated fibrils were consistently greater than for isolated molecules with reversal of effects occurring in the latter in some cases.

For single collagen molecules in dilute solution, the external location of polar and apolar side chains suggests that structural stability is primarily a function of interpeptide hydrogen bonding and chain rigidity conferred by rotational restrictions at pyrrolidine residues and peptide bonds (reviewed by Ramachandran, 1967). Recent calorimetric studies of denaturation, however, indicate that internal hydrogen bonding does not contribute greatly to tropocollagen stability (Privalov & Tikopulo, 1970; Cooper, 1971). Alternatively, the observation that solvent binding to peptide groups induces conformational changes in poly-L-proline (Strassmair *et al.*, 1969) suggests that as for neutral salts, polar interactions of solvents can induce similar changes in bond rotation in collagen. The role of hydrocarbon structure in the activity of aqueous binary solvents in non-protein systems lacking hydrophobic bonds (Pittz & Bello, 1969; Gerlsma, 1970; Russell & Cooper, 1971) indicates that polar interactions of the solvent may be influenced through the ordering effects of the hydrocarbon chain on local water structure (Russell & Cooper, 1969a, 1970).

Comparison of propanediol activities on collagen in solution with those of ethylene glycol and glycerol in the present study confirmed that molar activity decreased with increasing hydroxyl/methylene-group ratio as noted previously (Harrap, 1969; Hart *et al.*, 1971). Thus, for weakly active solvents of higher polar/methylene-group ratio such as glycerol and ethylene glycol, stabilization of dissolved collagen

with increasing solvent concentration appeared to reflect a further effect, namely, a progressive strengthening of internal hydrogen bonding as a result of a decrease in medium dielectric constant. Significantly, when the dielectric constant was increased by salt addition, stabilization effects decreased and ethylene glycol became an active destabilizer.

In general, the solvent and salt effects observed are consistent with a model in which long-range electrostatic forces and hydrophobic interactions govern aggregation and the resultant excess of stability in collagen fibrils, whereas the intrinsic stability of individual molecules is a function of polypeptide-bond rotational constraints and, under conditions of low dielectric constant particularly, of intramolecular polar interactions.

The assistance of Mr. R. Cross of the Rhodes University Electron-Microscopy Unit 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.

References

- Bensusan, H. B. (1960) *J. Amer. Chem. Soc.* **82**, 4995-4998
- Bensusan, H. B. & Hoyt, B. L. (1958) *J. Amer. Chem. Soc.* **80**, 719-724
- Bianchi, E., Rampone, R., Tealdi, A. & Ciferri, A. (1970) *J. Biol. Chem.* **245**, 3341-3345
- Cassel, J. M. (1971) *Biophys. Prop. Skin* **1**, 63-100
- Cooper, A. (1970) *Biochem. J.* **118**, 355-365
- Cooper, A. (1971) *J. Mol. Biol.* **55**, 123-127
- Cooper, D. R. & Davidson, R. J. (1965) *Biochem. J.* **97**, 139-147
- Cooper, D. R., Russell, A. E. & Hart, G. J. (1971) *Biochem. J.* **125**, 1069-1074
- Gerlsma, S. Y. (1970) *Stärke-Starch* **22**, 3-9
- Gross, J. (1958) *J. Exp. Med.* **108**, 215-226
- Harrap, B. S. (1969) *Int. J. Protein Res.* **1**, 245-252
- Hart, G. J., Russell, A. E. & Cooper, D. R. (1971) *Biochem. J.* **125**, 599-604
- Herbage, D., Hanus, A. & Vallet, G. (1968) *Biochim. Biophys. Acta* **158**, 544-554
- Herskovits, T. T., Gadegbeku, B., & Jaillet, H. (1970a) *J. Biol. Chem.* **245**, 2588-2598
- Herskovits, T. T., Jaillet, H., & Gadegbeku, B. (1970b) *J. Biol. Chem.* **245**, 4544-4550
- Herskovits, T. T., Jaillet, H. & De Sena, A. T. (1970c) *J. Biol. Chem.* **245**, 6511-6517
- Kaminsky, L. S., Yong, F. C. & King, T. E. (1972) *J. Biol. Chem.* **247**, 1354-1359
- Kauzman, W. (1959) *Advan. Protein Chem.* **14**, 1-60
- Pittz, E. & Bello, J. (1969) *Trans. Faraday Soc.* **66**, 537-545
- Privalov, P. L. & Tikopulo, E. L. (1970) *Biopolymers* **9**, 127-139
- Ramachandran, G. N. (1967) in *Treatise on Collagen* (Ramachandran, G. N., ed.), vol. 1, pp. 103-183, Academic Press, New York

- Russell, A. E. & Cooper, D. R. (1969a) *Biochemistry* **8**, 3980-3990
- Russell, A. E. & Cooper, D. R. (1969b) *Biochem. J.* **113**, 221-223
- Russell, A. E. & Cooper, D. R. (1970) *Biochemistry* **9**, 2802-2806
- Russell, A. E. & Cooper, D. R. (1971) *Biochemistry* **10**, 3890-3896
- Russell, A. E. & Cooper, D. R. (1972) *Biochem. J.* **127**, 855-863
- Schleich, T. & von Hippel, P. H. (1969) *Biopolymers* **7**, 861-877
- Schnell, J. (1968) *Arch. Biochem. Biophys.* **127**, 496-502
- Schnell, J. & Zahn, H. (1965) *Makromol. Chem.* **84**, 192-202
- Schrier, E. E. & Scheraga, H. A. (1962) *Biochim. Biophys. Acta* **64**, 406-408
- Strassmair, H., Knof, S. & Engel, J. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1153-1154
- Veis, A. (1967) in *Treatise on Collagen* (Ramachandran, G. N., ed.), vol. 1, pp. 367-439, Academic Press, New York
- von Hippel, P. H. (1967) in *Treatise on Collagen* (Ramachandran, G. N., ed.), vol. 1, pp. 326-338, Academic Press, New York
- von Hippel, P. H. & Schleich, T. (1969) *Struct. Stabil. Biol. Macromol.* **2**, 560-574
- von Hippel, P. H. & Wong, K.-Y. (1965) *J. Biol. Chem.* **240**, 3909-3923
- Wood, G. C. & Keech, M. K. (1960) *Biochem. J.* **75**, 588-598