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The Role of Aliphatic Alcohols on the Stability of Collagen and Tropocollagen*

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SUMMARY

The role of aliphatic alcohols on the temperatures of the helix-coil (T_{II}) and of the melting transformation (T_I) for collagen in water solutions was investigated. Alcohols used were CH_3OH, C_2H_5OH, and C_3H_7OH and the role of chloroethanol was also investigated.

The results indicate that on increasing alcohol concentration both T_I and T_{II} are initially depressed (*i.e.* the random coil form is favored with respect to both the helical and the crystalline form) the order for increasing alcohol effectiveness being $C_2H_4ClOH > C_3H_7OH > C_2H_5OH > CH_3OH$. In the case of the melting transition a subsequent reincrease of T_I with further increase of alcohol content was observed, and in this region the order for increasing effectiveness for depressing the melting temperature was $CH_3OH > C_2H_5OH > C_3H_7OH$.

Measurements of the equilibrium degree of swelling for cross-linked collagen membranes indicates that the over-all degree of swelling decreases with increasing alcohol concentration. However, measurements of the selective absorption of the alcohols by the membrane reveal a behavior which can be correlated to the melting behavior; *i.e.* the absorption goes through a maximum on increasing alcohol content and the order for increasing absorption is $C_3H_7OH > C_2H_5OH > CH_3OH$ below the maximum and $CH_3OH > C_2H_5OH > C_3H_7OH$ above the maximum.

The depressing effect on T_I and T_{II} observed in diluted alcohol solutions is attributed to a solvation of the side chain apolar groups which become exposed during the transitions.

The conformational stability of native and of helical structures of proteins and synthetic polypeptides is a function of external variables such as temperature, pH, ionic strength, and solvent composition. A quantitative analysis of the role of these variables is a requisite for a description of the forces responsible for conformational stability.

Recent studies (1–4) indicate that alcohols destabilize the native (tertiary) structure of globular proteins, whereas they stabilize the α-helical (secondary) structure of both globular proteins and synthetic polypeptides.

In this work we investigate the role of simple aliphatic alcohols on the stability of the triple helical structure of both fibrous and soluble collagen. Following an approach previously used (5) (for a study of the role of pH and ionic strength on conformational stability), we have determined the role of alcohol concentration on the temperatures of transformation between pairs of the three possible collagen conformations, *i.e.* soluble random coil, soluble triple helix, insoluble crystalline product.

EXPERIMENTAL PROCEDURE

Materials—Tropocollagen stock solutions (*i.e.* dissolved helical collagen) have been obtained by solubilizing rat tail tendons, as described by Dumitru and Garrett (6) and by us in a previous communication (5). The concentration of these solutions was about 0.4%.

Alcohols used (methanol, ethanol, propanol, and chloroethanol) were of analytical grade.

Alcohol solutions were prepared by mixing appropriate quantities of water and alcohol: a suitable part of the stock collagen solution was added to the mixture in order to obtain a polymer concentration of about 0.01% for viscosity measurements and of about 0.03% for melting point determinations. The water-alcohol composition is usually expressed in terms of the molar fraction of alcohol, N_A; the reported result did not depend upon the way of expressing alcohol concentrations.

For the swelling measurements collagen membranes, obtained by casting a dispersion of steer tendons and by cross-linking with *p*-benzoquinone, were used. The characterization of these membranes is reported elsewhere (7).

Determination of Transformation Temperatures—Depending upon pH, temperature, and percentage of alcohol, the initial tropocollagen solution may undergo (5) a transformation to random coils (*i.e.* H → RC, Transformation II)[1] or to a crystalline precipitate (*i.e.* H → C, Transformation III). The precipitate may be transformed (5) into a solution of random coils (*i.e.* C → RC, Transformation I), for example, by increasing the temperature.

Transformation II was investigated through viscosity measurements by determining the temperature variation of the reduced

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[1] The abbreviations used are: H, soluble triple helix; RC, soluble random coil; C, insoluble crystalline product. H → RC is Transformation II; H → C is Transformation III; C → RC is Transformation I.

specific viscosity, η_{sp}/c, at constant pH and solvent composition. A suspended level viscosimeter thermostated to ±0.05° was used, flow times being always greater than 100 sec. The temperature, T_{II}, at which Transformation II occurs was taken as the midpoint of the large decrease of viscosity associated with the H → RC transition (5).

The temperature, T_I, of Transformation I was taken as the temperature at which the precipitate dissolved and was determined visually and by measuring the viscosity of the resulting solution (5). Invariably, the latter corresponded to that of gelatin, indicating the occurrence of Transformation I (rather than III).

Heating rates were 1° per 30 min. Storage periods of solutions and of precipitates for at least 24 hours in the cold room were used. Time effects were negligible.

No extensive investigation of Transformation III was made owing to the occurrence of large time effects which accompanied the H → C transition (5). In the few instances in which this transformation was investigated, the method previously used (5) was followed.

pH Measurements—pH measurements in water-alcohol binary solvents are complicated by the existence of a liquid-liquid junction potential between the water-alcohol mixture and the KCl-saturated solution of the calomel electrode. It is known (8) that one can define:

$$pa_H^* = pH - \bar{E}_j + \log {}_m\gamma_H = pH - \partial \quad (1)$$

and

$$pa_H = pH - \bar{E}_j \quad (2)$$

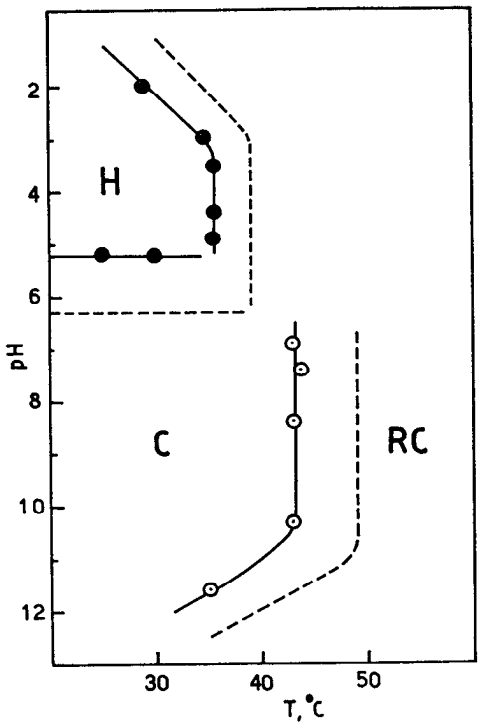

FIG. 1. Pseudo-phase diagram for collagen in a H_2O/C_2H_5OH solution. C_2H_5OH concentration is 30% w/w (= 0.145 molar fraction). The temperature of transition of the helical (*H*) or the crystalline (*C*) forms into the random coiled form (*RC*) is plotted as a function of pH. The *dotted line* represents the collagen water system previously reported (5).

where: a_H^* = hydrogen ion activity referred to the standard state in the mixed solvent; $\bar{E}_j$ = liquid-liquid junction potential; ${}_m\gamma_H$ = free energy variation in the transfer of a proton from water to the water-alcohol mixture (this variation is related to the H^+ activity coefficient in the standard state in water, ${}_w\gamma_H$, and in the mixed solvent, ${}_s\gamma_H$, by the relation ${}_w\gamma_H = {}_m\gamma_H \cdot {}_s\gamma_H$); a_H = hydrogen ion activity referred to the standard state in water. ∂ values for CH_3OH/H_2O and C_2H_5OH/H_2O solutions up to 80% alcohol (N_A = 0.69 and 0.61 for methanol and ethanol, respectively) have been found (8) to be very low. Thus, the pH meter reading may give a correct pa_H^* value. However, if we have to compare pH measurements in different solvents, we must refer the activity to the same standard state, *i.e.* to the water solution. In other terms we must use Equation 2, correcting the pH meter readings by $\bar{E}_j$. Junction potential values are known only for a few systems. Gutbezhal and Grunwald (9) have measured $\bar{E}_j$ for water-ethanol solutions at different composition. $\bar{E}_j$ values are quite low up to 30% of alcohol (N_A = 0.145) but increase up to 0.4 pH unit when the alcohol content is 50% (N_A = 0.28).

Swelling and Solvent Disproportion Measurements for Collagen Membranes—Swelling measurements were performed by equilibrating membrane samples (of about 80 to 100 mg) against 1 cc of water-alcohol mixture in a thermostatic vessel at 20°. After a 48-hour storage time, the membrane was wiped on the surfaces and quickly weighed. The swelling was characterized by the ratio between the increment of weight after equilibration and the initial weight of the dry membrane, multiplied by 100.

The composition of the external solvent in equilibrium with the swollen membrane was determined by gas-chromatography, with a model A 350 Varian Erograph. The columns were of the Poropack type (100 to 200 mesh). Operating temperatures were 130° for methanol, 165° for ethanol, and 210° for propanol mixtures. Helium was the carrier gas. From knowledge of the initial quantity (p_0) and composition (x_0) of the external solvent and of the final quantity (p_f) and composition (x_f) after equilibration, it is possible to obtain the quantity of alcohol absorbed per g of membrane, with the relation $(p_0x_0 - p_fx_f)/g$ where g is the weight of the dry membrane.

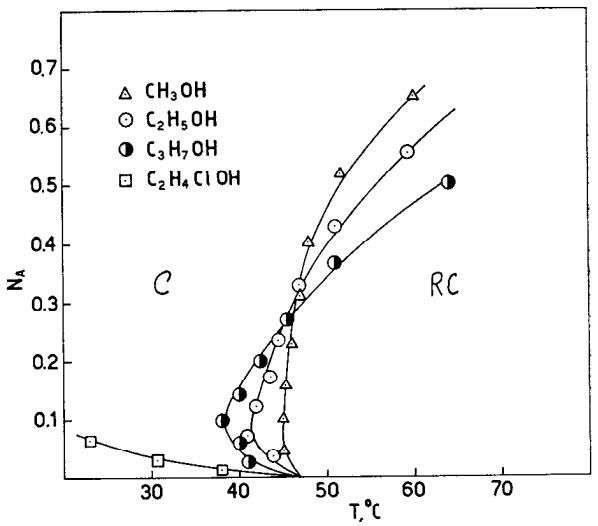

FIG. 2. Variation of the temperature of Transformation I (C → RC) with the molar fraction, N_A, of several alcohols. pH 6. The field of stability of each phase is indicated.

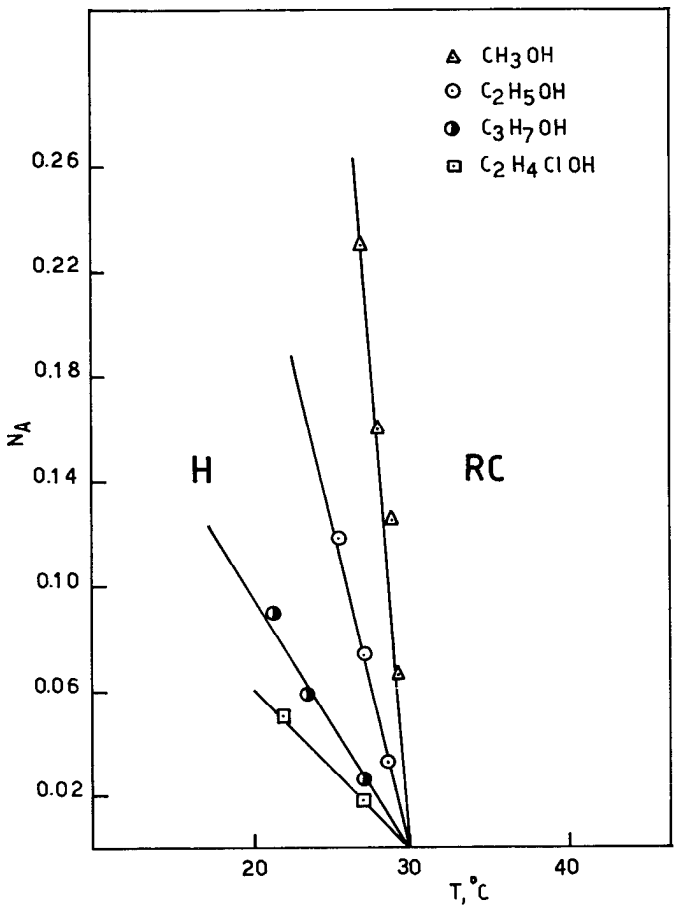

FIG. 3. Variation of the temperature of Transition II (H → RC) with the molar fraction, N_A, of several alcohols. pH 2.3. The field of stability of each phase is indicated.

RESULTS

Pseudo-Phase Diagrams—Fig. 1 shows the behavior of collagen in 0.145 N_A ethanol (30% in weight) at different pH (no salt added), together with the collagen-water system previously studied (5) (*dotted line*). The effects observed in the water-ethanol mixture are similar to those observed in water with the difference that the transition temperatures T_I and T_{II} are lowered by the alcohol. In the case of Transformation III (H → C), the pH of precipitation does not seem to depend on temperature.

In Fig. 2 the variation of T_I with the molar fraction of several alcohols at constant pH is reported. As discussed above, in order to obtain comparable data in different solvents, it might be desirable to operate at constant pa_H. The necessary correction (*cf.* Equation 2) cannot, however, be made owing to the lack of experimental data of $\bar{E}_j$. Nevertheless, we believe that this difficulty does not seriously affect the interpretation of the results shown in Fig. 2 since Transformation I does not depend upon pH in the pH range under consideration (*cf.* Fig. 1).

The data in Fig. 2 indicate that, for methanol, ethanol, and propanol, T_I at first decreases and then increases with N_A, the effect being more evident for the last two alcohols. We have not observed the reversal for chloroethanol, probably, because transformation temperatures are too low. Up to values of $N_A < \sim 0.2$ the order for depressing T_I, for the series of aliphatic alcohols, is

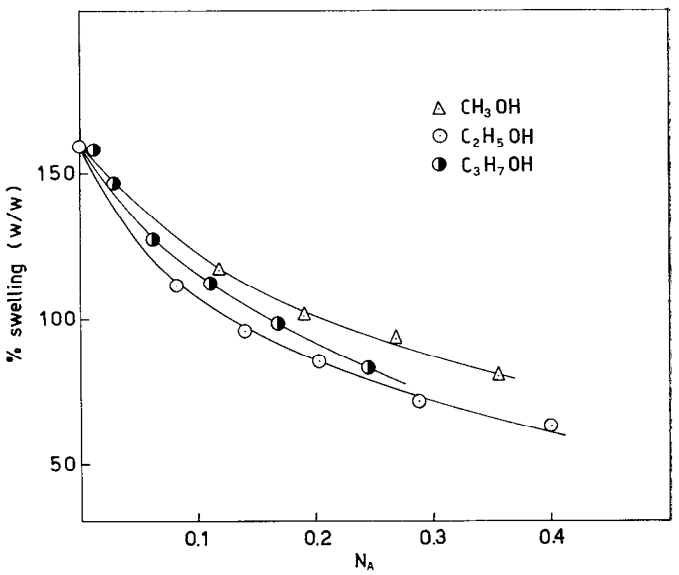

FIG. 4. Variation of the equilibrium degree of swelling of a cross-linked collagen membrane with the molar fraction, N_A, of several alcohols. Temperature, 20°; pH ~6.

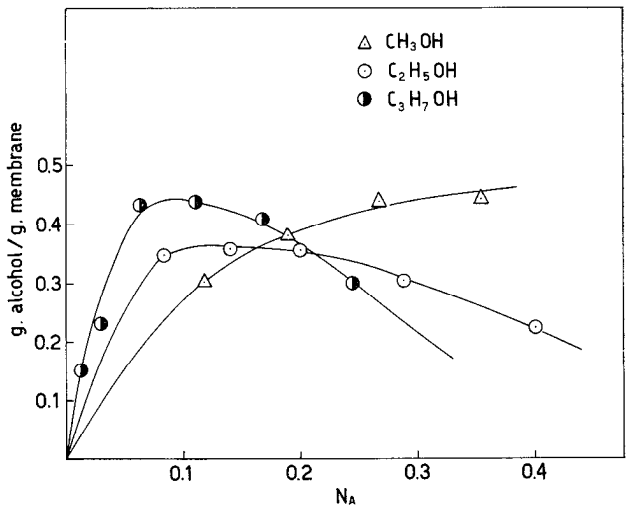

FIG. 5. Selective absorption of several alcohols by a cross-linked collagen membrane as a function of the molar fraction of alcohol. Temperature, 20°; pH ~6.

$$C_3H_7OH > C_2H_5OH > CH_3OH \quad (3)$$

and the effectiveness of chloroethanol is even greater than that of propanol. However, when $N_A > \sim 0.3$, the order becomes:

$$CH_3OH > C_2H_5OH > C_3H_8OH \quad (4)$$

Finally, the transformation H → RC has been studied up to a maximum concentration of alcohol of 30% ($N_A \sim 0.1 \div 0.2$ depending on the alcohol). A depression of T_{II} with increasing N_A is observed from the results reported in Fig. 3. The pH that in this compositional range can be considered coincident with pa_H was kept equal to 2.3. The order for depressing T_{II} agrees with Series 3.

Additional experiments were performed with a 0.2 M KCl solution. No significant alterations of the results obtained in the absence of added salt were noticed.

Swelling and Solvent Disproportion—In Fig. 4 the percentage

weight increase of collagen membranes equilibrated with different alcohol solutions is reported. The order for increasing deswelling is $CH_3OH > C_3H_7OH > C_2H_5OH$. We have also determined the degree of swelling of collagen membrane in pure alcohols. The results are: 60% in CH_3OH, 15% in C_2H_5OH, and 3% in C_3H_7OH.

The swelling data presented above were planned in order to assess an independent measure of the polymer-solvent interaction (10). The bases on which this can be done have been previously analyzed for both crystalline and amorphous collagen tendons (11).

Results for the water-alcohol disproportion are collected in Fig. 5. On increasing the molar fraction of alcohol the amount of alcohol absorbed by the membrane goes through a maximum in the case of C_2H_5OH and C_3H_7OH. For composition less than that corresponding to the maximum the order for increasing absorption is: $C_3H_7OH > C_2H_5OH > CH_3OH$.

DISCUSSION

In the case of synthetic polypeptides, alcohols increase the stability of the α-helical conformation with respect to the random coiled one (2–4). The order for increasing stabilization of the α-helical form found by Conio, Patrone, and Brighetti (4), in the case of poly-L-glutamic acid and poly-L-ornithine, is $C_4H_9OH > C_3H_7OH > C_2H_5OH > CH_3OH$. Thus, in simple polypeptides, the effect of alcohols is apparently opposite to that observed for collagen ($N_A < \sim 0.2$) in which the random coiled form is generally stabilized with respect to both the soluble and insoluble triple helix, the order for increasing random coil stabilization being given by Series 3.

The stabilization of the α-helical form for polypeptides in diluted alcohol solutions has been generally interpreted (2) in terms of a greater ability of the amide group to form hydrogen bonds in a solvent less polar than water. However, as indicated by Conio *et al.* (4), the data of Klotz and Franzen (12) (H_2O to CCl_4 transfer) indicate that the latter effect should be enthalpic in nature, whereas an increase of the entropy for the coil → helix transition plays the determinating role in the stabilization of the α-helix by increasing alcohol concentration (4). Since Series 3 coincides with the series for increasing order in the water structure (13), Conio *et al.* (4) suggested that the increase of α-helix stabilization, according to Series 3, could be related to the difficulty for a diluent of increasing structurization to be a good solvent for the random coiled form. Any contribution from the solvation of the side chains is probably negligible in the case of the α-helix → random coil transition since the side chains are completely exposed to the solvent in both conformations.

The failure of the corresponding alcohol solutions to stabilize the helical form of collagen could then tentatively be ascribed to the fact that Series 3 characterizes diluents which (for $N_A < \sim 0.2$) possess increasing solvating power toward the apolar side chains which become exposed during conversion of helical, or crystalline form, to random coil. A similar interpretation was advanced by Schrier, Ingwall, and Scheraga (14) for explaining the denaturation of ribonuclease in alcohol solutions, when binding of the nonpolar portion of the alcohol to the apolar groups of the denatured protein was postulated to occur.

An alternative interpretation could be based on the ordering effect of the water structure that alcohols of increasing size might have, in line with the suggestion made by von Hippel and Wong (15) also in connection with the behavior of the ribonuclease-alcohol system. If an interpretation based on the water structure is considered, one should explain why alcohols of increasing size have opposite effects toward the stabilization of the ordered conformation of the polypeptides mentioned above and of fibrous or globular proteins. While such a justification could be conceived (*e.g.* competition between the alcohol and the polymer for formation of "icebergs" (15) around hydrophobic groups which would be unconsequential only in the case of polypeptides in which side chains are completely exposed to the solvent in both conformations), we feel that our swelling and solvent disproportion data offer deciding support to the prevailing role of the effect based on a direct protein-alcohol interaction. In fact, the over-all degree of swelling decreases (Fig. 4) with increasing alcohol concentration, and if a property of the water-alcohol mixture (regarded as a whole diluent) determined the melting behavior, this decrease should correspond to an increased stabilization (11, 16) of the helical form, contrary to what was observed. (Decreasing degree of swelling implies a decrease of polymer-solvent interaction in terms of the χ_1 parameter (17) which enters the melting point depression equation valid for single component diluents (11, 17).) Simultaneous with the decrease of the over-all degree of swelling, however, there is an increase (Fig. 5) of the total amount of alcohol absorbed (and, necessarily, a decrease of absorbed water) which indicates a preferential selective interaction of the polymer with alcohols. Moreover, the order for increasing alcohols absorption at a given molar fraction of alcohol ($N_A < \sim 0.2$) is well correlated with Series 3, and the inversion in the trend of the curves of absorption plotted against N_A (Fig. 5) for C_2H_5OH and C_3H_7OH is well correlated with the pronounced inversion in the trend of the curves of melting plotted against N_A (Fig. 2). As shown by Peller (18), by Orofino, Ciferri, and Hermans (16) and by Katchalski and Oplatka (19), such an enrichment by the polymer of one solvent component can theoretically justify the variations of T_I and T_{II} with N_A experimentally observed.

The interpretation of the present results in terms of the above theories requires that an increase of binding (or preferential absorption) occurs during the H → RC and the C → RC transformation. This condition may certainly be met by the peptide bonds which become more exposed to the solvent during both transformations. There is indeed evidence (20) (*cf.* also Reference 21) that binding of —OH groups of the alcohols to polar peptide bonds occurs. However, in order to justify Series 3, and the different effect that alcohols have on collagen and polypeptides such as poly-L-lysine (4), a selective interaction of the apolar sections of the alcohol with the apolar groups of the protein should be advocated. There is little doubt that this latter interaction may increase during the C → RC transformation. Somewhat more complex appears, however, the situation prevailing during the H → RC transformation if, as suggested by Russel and Cooper (21), hydrophobic interactions do not play an important role in the stabilization of the tropocollagen helix. A more detailed analysis of the nature of the selective alcohol-collagen interaction, experimentally evidenced by data such as those in Fig. 5, would certainly be appropriate.

The contribution of water to the over-all melting behavior illustrated in Fig. 2 should also be emphasized. The latter is reflected in the reversal of the melting curve, particularly for CH_3OH (when there is no evidence of site saturation by the alcohol; *cf.* Fig. 5), and in the fact that for pure alcohols the thermodynamic affinity toward collagen decreases in the order

CH_3OH, C_2H_5OH, C_3H_7OH (*cf.* swelling data), contrary to what is found in dilute alcohol solutions.

Finally, we note that Conio *et al.* (4) have successfully applied to their data the concept of the effective methylene molarity, as suggested by von Hippel and Wong (15). They find that the excess of the thermodynamic parameters of helix formation in water-alcohol solutions relative to water per unit of methylene molarity is a constant independent of alcohol type, alcohol concentration, and side chain of the polypeptide. Thus the molarity of methylene groups seems to be a determining factor for the ability of alcohols to depress the denaturation temperature of ribonuclease (15) and to increase the stability of the helical conformation of some synthetic polypeptides (4). In the case of our melting curves of collagen, which exhibit the reversal and crossover illustrated in Fig. 2 (*cf.* also the similar results obtained by Schnell and Zahn (22) for collagen tendons), a simple description in terms of the effective methylene molarity has not been possible over a wide composition interval. Even in a small composition interval the results are rather modest. For instance, the melting temperature is ~43° in 3 M C_2H_5OH (N_A ~0.06), 42° in 2 M C_3H_7OH (N_A ~0.04), and 46° in 6 M CH_3OH (N_A ~0.15).

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