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# Effects of the Aliphatic Carboxylate Series of Salts on the Conformation of Proteins†

Victorio S. Ibanez and Theodore T. Herskovits*

ABSTRACT: The effects of the aliphatic acid series of salts, formate, acetate, propionate, butyrate, valarate, and caproate, on the conformation of sperm whale myoglobin, human hemoglobin A, and horse heart cytochrome *c* were investigated by spectral measurements in the Soret region, optical rotation, and intrinsic viscosity measurements. The effectiveness of the aliphatic acid salts as unfolding reagents for proteins is found to increase with increasing hydrocarbon content of the alkyl chains of the salts, which is analogous in behavior to effects of the urea, amide, and alcohol series of protein denaturants. The denaturation midpoints, $S_m$, as a function of the unfolding reagent were analyzed using the equations of Peller (Peller, L. (1959), *J. Phys. Chem. 63,* 1199) and Flory (Flory, P. J. (1957), *J. Cell. Comp. Physiol. 49,* 175) with binding constants based in part on the Scheraga–Nemethy theory of hydrophobic bonding or evaluated from free-energy transfer data of nonpolar amino acid side chains from aqueous to nonaqueous solvents. The summation of the polar $K_P$ and hydrophobic $K_{H\Phi}$ contributions of solvent to protein amino acid side chain interactions were found to give best account of the protein denaturation data. Intrinsic viscosity and optical rotation data obtained on hemoglobin and myoglobin at high salt concentrations, above the unfolding transition regions, indicate that the product of denaturation by the aliphatic acid salts is less unfolded than in 6 M guanidine hydrochloride solutions. Residual elements of the helical regions of the proteins seem to either escape unfolding or are reformed at high concentrations of the denaturing salts.

A variety of organic solutes and electrolytes are known to alter the native conformation of proteins and other biopolymers by producing changes in the structure in the supporting natural solvent, water, or by preferential interaction with the biopolymer constituents (Kauzmann, 1959; Tanford, 1970; Noelken, 1970; Cann, 1971). Studies from our laboratory in the past several years have dealt with both the effects of the hydrophobic series of organic solutes, i.e., the ureas, the amides, and the alcohols, on the native conformation of proteins (Herskovits and Jaillet, 1969; Herskovits et al., 1970a–c; Elbaum et al., 1974; Herskovits and Solli, 1975; Herskovits and Harrington, 1975) and their effects on the state of association of subunit proteins such as the hemoglobins (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975; Harrington and Herskovits, 1975). The theories of Peller (1959) and Flory (1957) developed to account for the effects of salts and neutral additives on the temperature transitions of biopolymers were used in our denaturation studies in conjunction with binding constants based on free-energy transfer data and the Scheraga–Nemethy theory of hydrophobic bonding (Schrier et al., 1965). Group additivity of the polar and hydrophobic constituents of the denaturant, suggested by the model studies of Schrier and Schrier (1967) and Nandi and Robinson (1972a,b), was assumed for the evaluation of the binding constants in these studies (Herskovits et al., 1970c; Elbaum et al., 1974) as well as in our studies on DNA denaturation (Herskovits and Harrington, 1972; Herskovits and Bowen, 1974) and hemoglobin dissociation (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975; Herskovits and Harrington, 1975).

As a means of bridging the effects of electrolytes and hydrophobic solutes on the conformation and subunit organization of biopolymers and as a further test of group additivity of

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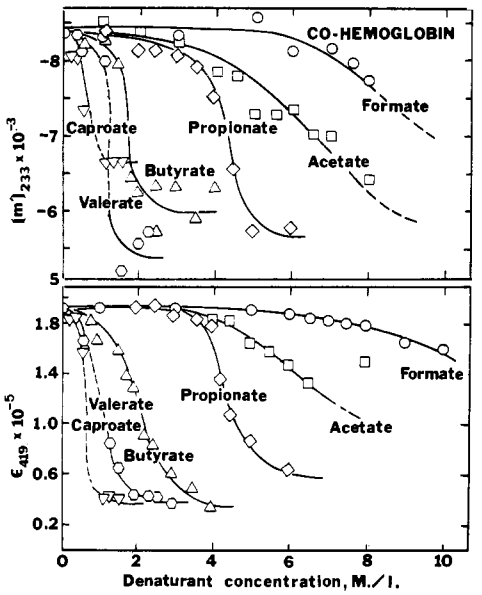


FIGURE 1: The denaturation of carboxyhemoglobin by the sodium salts of the aliphatic acid series at neutral pH (7.0) and 25 °C followed by changes in ORD at 233 nm and in absorbance at the 419-nm Soret band. All the solutions were buffered by 0.01 M phosphate (pH 7). The protein concentrations used were 0.064–0.066%.

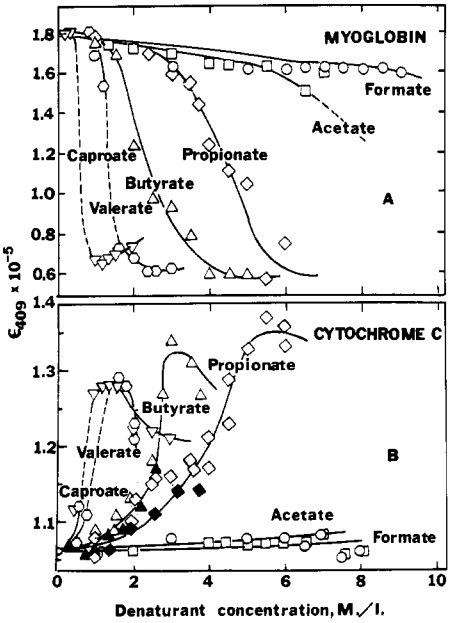


FIGURE 2: The denaturation of ferrimyoglobin and ferricytochrome *c* by the sodium salts of the aliphatic acids at neutral pH (7.0) and 25 °C followed by absorbance changes at the 409-nm Soret band. The data points represented by the filled symbols for cytochrome *c* have been reversed by dilution from 3 M butyrate and 6 M propionate solutions. The protein concentrations used ranged from 0.082 to 0.084% for myoglobin and from 0.008 to 0.01% for cytochrome *c*.

solvent effects related to these two phenomena, we have extended our investigations to the carboxylate series of organic salts, formate, acetate, propionate, etc. This paper reports our findings on the conformation of three globular proteins investigated by absorbance, optical rotation, and viscosity methods, while the companion paper (Ibanez and Herskovits, 1976) presents the results of our subunit dissociation studies on human hemoglobin made by light-scattering measurements.

## Experimental Section

*Materials.* Sperm whale myoglobin and horse heart cytochrome *c* were Sigma Chemical Co. products. Human hemoglobin A was prepared by Drabkin's procedure followed by chromatography on DEAE[1]-Sephadex A-50 (Pharmacia) or DE-52 diethylaminoethylcellulose (Whatman) columns as previously described (Elbaum and Herskovits, 1974). The salts and acids employed were reagent grade or the purest commercially available quality. The valeric and caproic acids were vacuum distilled, with the central fractions being retained for use. The stock solutions of the various salts used (2 to 12 M) were prepared gravimetrically from the neutral salts or the acids, with the latter being neutralized by use of concentrated NaOH solutions followed by dilution to the desired volume. For absorbance, ORD, and CD measurements protein solutions were usually prepared by serial dilution in 5-ml stoppered volumetric flasks from common stocks of protein of the same concentration containing increasing amounts of carboxylate salt and 0.01 M pH 7 phosphate buffer. Where necessary the pH was readjusted to 7, with the measurements being made after 0.5 to 2 h of equilibration at room temperature.

*Methods.* Absorbance, ORD, and CD measurements were made in Cary 14 and Cary 60 recording instruments. Protein concentrations were based on absorbance measurements using the following absorption coefficients, $A_{1cm}$ (1%): cytochrome *c*, 8.36 at 528 nm (Margoliash and Schejter, 1966); sperm whale ferrimyoglobin, 100.6 at 409 nm (Harrison and Blout, 1965); carboxyhemoglobin, 9.04 at 539 nm (Elbaum and Herskovits, 1974); and cyanmethemoglobin, 7.76 at 540 nm (Drabkin and Austin, 1935). At the Soret maxima the molar extinction coefficients expressed per mole of heme iron based on these values are: $1.06 \times 10^5$ for cytochrome *c*, and $1.79 \times 10^5$ for myoglobin both at 409 nm, and $1.92 \times 10^5$ for carboxyhemoglobin at 419 nm. The concentrations of renatured hemoglobin and myoglobin were based on measurements of the refractive index increment using the values of specific refractive index increment ($dn/dc$) of 0.194 at 630 nm. Measurements were made in a Wood light-scattering photometer equipped with a differential refractometer. The ($dn/dc$) values for a large number of proteins are within about 5% of 0.190 (Timasheff, 1970), suggesting that this parameter should be essentially independent of the conformation of the protein under investigation, whereas alterations in the conformation of heme proteins will be more strongly reflected in the absorbance properties and the extinction values, making the latter parameter relatively unreliable for concentration determination. Upon denaturation the hemes detached from the protein chains of hemoglobin and myoglobin are known to aggregate in solution (Polet and Steinhardt, 1969). This problem of course is not encountered in the case of nonheme proteins. The ORD and the CD data are reported as mean residue rotation, $[m']_\lambda$, corrected for the refractive index of the solvent and "uncorrected" mean residue ellipticity, $[\theta]_\lambda$, both having units of deg cm$^2$ dmol$^{-1}$. For the various salt solutions investigated, the aqueous Lorentz corrections, $3/(n^2 + 2)$, were multiplied by the approximate value of the solvent factor, $r_s = (n_w^2 + 2)/(n_s^2 + 2)$, where $n_w$ is the refractive index of water and $n_s$ is the refractive index of the particular salt solution, determined at the sodium D line in an Abbe refractometer.

Most of our viscosity data were obtained with Cannon-Ubbelohde dilution viscometers having water flow times at 25

[1] Abbreviation used: Gdn·HCl, guanidine hydrochloride; DEAE, diethylaminoethyl; CD, circular dichroism; ORD, optical rotatory dispersion.

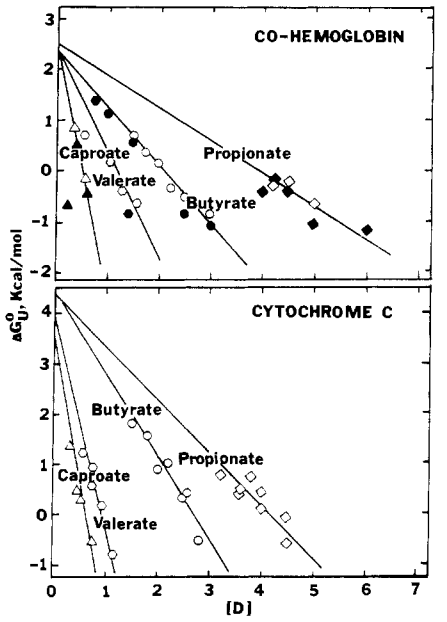


FIGURE 3: $\Delta G_U°$ vs. [D] plots carboxyhemoglobin and cytochrome *c* based on the denaturation data of Figures 1 and 2, represented according to eq 4 of the text. The derived slopes *s* and the $\Delta n$ and $\Delta G_{U,w}°$ parameters are listed in Table I. Open symbols: absorbance data. Shaded symbols: ORD data.

TABLE I: Denaturation Parameters of Carboxyhemoglobin and Cytochrome *c* in Neutral Aliphatic Acid Salt Solutions based on Equations 3 and 4.[a]

| Denaturant Salt | $K_B$ ($M^{-1}$) | *n* | $\Delta n$ | *s*[a] (kcal $mol^{-1}$ $M^{-1}$) | $\Delta G_{U,w}°$ (kcal/mol) |
|---|---|---|---|---|---|
| | | CO-Hemoglobin | | | |
| Propionate | 0.046 | 9.8 | 22 | −0.6 | 2.5 |
| Butyrate | 0.093 | 4.0 | 20 | −1.1 | 2.4 |
| Valerate | 0.217 | 3.3 | 15 | −1.9 | 2.3 |
| Caproate | 0.557 | 4.6 | 13 | −4.4 | 2.3 |
| Average | | | | | 2.4 ± 0.2 |
| | | Cytochrome *c* | | | |
| Propionate | | 8.3 | 30 | −1.1 | 4.4 |
| Butyrate | | 7.4 | 29 | −1.7 | 4.4 |
| Valerate | | 5.4 | 26 | −4.3 | 4.0 |
| Caproate | | 5.1 | 14 | −5.3 | 3.5 |
| Average | | | | | 4.1 ± 0.4 |

[a] The $K_B$ values listed are based on free energies of transfer data of the aliphatic portions of the acid salts listed, $K_{H\Phi}$ plus a polar $K_p$ component (see text in relation to eq 7 and 8). The group $K_{H\Phi}$ values used were 0.029, 0.076, 0.20, and 0.54 $M^{-1}$ for propionate, butyrate, valerate, and caproate, respectively, with a single $K_p$ value of 0.017 $M^{-1}$ used, based on the ln $S_m$ vs. the number of methylene groups plot of Figure 4, extrapolated to zero methylene group. The *n* and $\Delta n$ parameters are based on the slopes of ln $K_{App}$ vs. ln [D] and $\Delta G_U°$ vs. [D] plots. The *s* values listed refer to the slopes of $\Delta G_U°$ vs. [D] plots such as in Figure 3.

°C of approximately 250 s. The viscosities determined using filtered or centrifuged solutions were plotted according to the Huggins (1942) relationship

$$\eta_{sp}/c = [\nu] + k[\eta]^2 c \quad (1)$$

where $\eta_{sp}/c$ is the reduced specific viscosity in ml/g, and *c* is the protein concentration in g/ml. The intrinsic viscosity, $[\eta]$, was based on the extrapolated $[\nu]$ values corrected for solvent density, $\rho_0$, according to the equation of Tanford (1955)

$$[\eta] = [\nu] + (1 - \bar{V}\rho_0)/\rho_0 \quad (2)$$

For some of the concentrated salt solutions, the flow times were found to be longer than 15 min, in which case Cannon-Fenske viscometers (No. 50) of shorter flow times were used.

## Results

*Denaturation Studies.* The denaturation of human CO-hemoglobin, ferrimyoglobin, and cytochrome *c* was investigated by measurements of both the optical activity and absorption properties of these proteins. Figures 1 and 2 present profiles of the unfolding transitions obtained with the various aliphatic acid salts as a function of salt concentration. It is apparent from the location of the midpoints of the transitions that the effectiveness of these salts as unfolding reagents of proteins follows the order of increasing hydrocarbon constant, with the higher members of the series, valerate and caproate, being the most effective reagents. The same order of effectiveness was observed with the parent aliphatic acids in lower pH's investigated by Cann (1971). In the ranges of approximately 0.6 to 1.2 M valerate and caproate, the intrinsic turbidity of these two salts solutions precluded any measurements. Fractional distillation of the acids produced no apparent improvement in the clarity of the solutions in this range of concentration. As a result the transitions in these regions shown in Figures 1 and 2 are denoted by dashed lines.

The reversibility of the effects of salts was examined by dilution of cytochrome *c* solutions exposed to high salt concentrations above the midpoints of the transitions. The data obtained are represented by filled symbols in Figure 2B and show essentially complete reversibility and recovery of the native structure of cytochrome *c*. The reversibility of myoglobin and hemoglobin is complicated by the fact that the detached heme moiety, as well as the polypeptide chains, tend to aggregate (Urry and Pettergrew, 1967; Polet and Steinhardt, 1969; Waks et al., 1973). Nearly complete recovery of the CD and ORD spectra of ferrimyoglobin was obtained following 0.5 h of exposure to 6 M propionate and removal of the reagent by dialysis in the cold against 0.001 M phosphate buffer (pH 6.9). The $[\theta]_{222}$ and $[m']_{233}$ values of the recovered protein were found to be −21 300 and −8000 deg $cm^2$/dmol compared with −22 800 and −9100 deg $cm^2$/dmol, respectively, obtained with the native sample of the protein investigated. The recovery was less extensive with the four-chain human hemoglobin. In addition, the initial exposure to 6 M propionate produced some insoluble material. The clarified dialyzed product was characterized by $[\theta]_{222}$ and $[m']_{233}$ values of −18 000 and −6700 deg $cm^2$/dmol, relative to −23 400 and −8800 deg $cm^2$/dmol of the native cyanmet protein.

The gradual increase in the absorbance profiles of cytochrome *c* as a function of propionate and butyrate concentration seen below the main transition region in Figure 2B and associated with the unfolding process suggests that a simple all-or-none two-state process of unfolding of proteins by the aliphatic series of acid salts must be an oversimplification. However, in view of the fact that a two-state mechanism of denaturation was assumed in our previous investigations of the hemoglobins by the ureas and amides (Elbaum et al., 1974; Herskovits and Harrington, 1975), it is nevertheless instructive to compare the parameters derived on the basis of such an assumption with similar data obtained with these two series of

TABLE II: The Effects of Aliphatic Acid Salts on the Intrinsic Viscosity and the ORD Parameters of Carboxyhemoglobin and Ferrimyoglobin at pH ~7 and 25 °C.

| | $[\eta]$ (ml/g) | Huggin's constant, $k$[a] | $[m']_{233}$ (deg cm$^2$/dmol) | Estimate of $\alpha$-Helix Content (%)[b] |
|---|---|---|---|---|
| | CO-Hemoglobin | | | |
| Water, 0.1 M NaCl, 0.01 M phosphate (pH 6.9) | 3.6 | 0.85 | −8 500 | 68 |
| 4 M formate | 3.4 | 6.0 | −8 500 | 68 |
| 4 M acetate | 4.7 | −0.7 | −8 050 | 63 |
| 4 M propionate | 10.1 | −6.4 | −7 500 | 57 |
| 4 M butyrate | 13.1 | −0.7 | −6 000 | 42 |
| 2.5 M valerate | 14.1 | −2.8 | −5 850 | 40 |
| 2 M caproate | 14.7 | 0.2 | −6 500 | 47 |
| 6 M Gdn·HCl | 18.9[c] | | | |
| | Ferrimyoglobin. | | | |
| Water, 0.1 M NaCl, 0.01 M phosphate (pH 6.9) | 3.3[d] | | −9 400[e] | 77 |
| 4 M formate | 4.7 | −1.8 | −9 040 | 73 |
| 4 M acetate | 4.3 | 8.8 | −8 800 | 71 |
| 6 M propionate | 10.1 | −2.4 | −7 000 | 52 |
| 4 M butyrate | 11.1 | −1.5 | −8 200 | 65 |
| 2.5 M valerate | 20.0 | −0.8 | −7 500 | 57 |
| 6 M Gdn·HCl | 20.9[c] | | | |
| 8 M urea[e] | 21 | | −2 700 | 8 |
| 2-Chloroethanol[e] | 25 | | −10 600 | 89 |

[a] Based on least-squares fit of the viscosity data. [b] Calculated by use of eq 5 with $[m]_{233}$ obtained by dividing the $[m']_{233}$ values listed by 0.764, the $3/(n^2 + 2)$ Lorentz factor for water at 233 nm. [c] Data of Tanford et al. (1967). [d] Data of Crumpton and Polson (1965). [e] Data of Herskovits and Solli (1975).

denaturants. The empirical eq 3 and 4 defining the apparent order of the stoichiometry, $n$, and the difference in the apparent number of binding sites in the unfolded and the native protein, $\Delta n$ (Sturtevant and Tsong, 1969; Aune and Tanford, 1969; Tanford, 1970)

$$K_{App} = C[D]^n \quad (3)$$

and

$$\Delta G_U^\circ = \Delta G_{U,w}^\circ - \Delta nRT \ln (1 + K_B[D]) \cong \Delta G_{U,w}^\circ - \Delta nRTK_B[D] \quad (4)$$

have been employed in our analysis of the denaturation data. In eq 3 and 4 the symbols $C$, [D], $K_B$, $\Delta G_U^\circ$, and $\Delta G_{U,w}^\circ$ represent respectively constants characteristic of the given protein-denaturant system, the concentration of the denaturant, the binding constant of the denaturant to the average amino acid, and the standard free energies of unfolding in the presence and absence of denaturant. Figure 3 shows plots of some of our data based on eq 4. A summary of the $n$, $\Delta n$, and $\Delta G_{U,w}^\circ$ parameters for CO-hemoglobin and cytochrome $c$ based on eq 3 and 4 is given in Table I. Also included in the table are the $K_B$ values used for the estimates of $\Delta n$ for both proteins. The $K_B$ values used were estimated on the basis of eq 7 and 8 of the Discussion, with the hydrophobic and polar group contributions listed in footnote $a$ of Table I. Because of the upswing in the $[m']_{233}$ vs. [D] curves in the posttransition region of ferrimyoglobin (data not shown) and the attendant uncertainties in the baseline extrapolations of the denaturation curves into the transition regions, necessary for evaluation of $K_{App}$ (Sturtevant and Tsong, 1969; Aune and Tanford, 1969; Greene and Pace, 1974), we have not carried out similar analysis of the denaturation data of this protein.

The $n$ values of 5 to 10 obtain on CO-hemoglobin and cytochrome $c$ are to be compared with the values of 5.4 ± 1 and 6.5 ± 2 obtained with the ureas and amides on CO-hemoglobin (Pandolfelli and Herskovits, unpublished results) and 5.8 to 10.8 obtained on RNase with the aliphatic acids at pH's of 2.55 to 3.06 (Cann, 1971). The $\Delta n$ and $\Delta G_{U,w}^\circ$ values are, however, lower than the urea and amide values. For CO-hemoglobin, for example, the values of 18 ± 4 and 2.4 ± 0.2 kcal/mol should be compared with the corresponding values of 32 ± 8 and 3.5 ± 0.4 kcal/mol and 25 ± 2 and 4.0 ± 0.6 kcal/mol, obtained with the ureas and amides. Differences in these derived parameters may reflect differences in the conformational states of the denatured proteins in these three classes of solvents suggested by both the different estimates of helix content (Table II), as well as the deviation in adherence to a simple two-state description of the unfolding process. Poland and Scheraga (1965) have found that, while the transition midpoints will remain unaltered, the enthalpies of unfolding of a gradually unwinding model, such as one would expect with proteins having domains of slightly different conformational stability, will be lower than those of the two-state model. Broadening of the transition due to the latter phenomena would be expected to give a less steep $\Delta G_U^\circ$ vs. [D] plot and a correspondingly lower $\Delta G_{U,w}^\circ$ estimate upon extrapolation to [D] = 0 (Figure 3).

*Viscosity Studies.* The intrinsic viscosity data obtained with the various aliphatic acid salt solutions have been fitted by means of the Huggins relation 1. The corrected $[\eta]$ values based on eq 2 and the Huggins $k$ parameters are summarized in Table II. For reasons of comparison we have also included $[m']_{233}$ values of hemoglobin and myoglobin in these solvents and estimates of $\alpha$-helix content based on the $[m]_{233}$ values calculated by use of the equation of Chen et al. (1972)

$$[m]_{233} = -12\,700 f_H - 2520 \quad (5)$$

with $[m]_{233}$ representing the mean residue rotation uncorrected for the refractive index of the solvent and $f_H$ the fraction of $\alpha$-helix present in the protein.

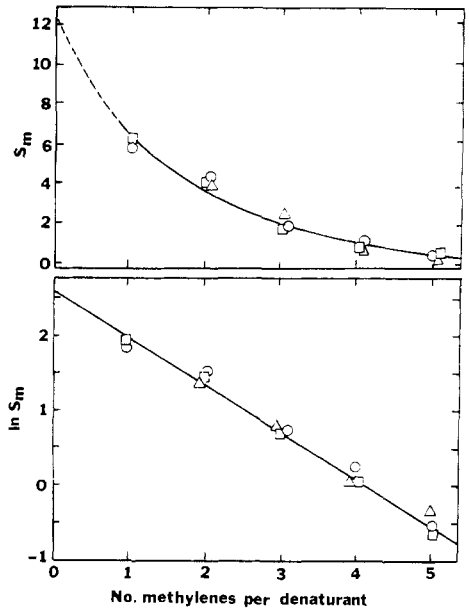


FIGURE 4: A plot of the denaturation midpoints, $S_m$, of carboxyhemoglobin (□), ferrimyoglobin (○), and cytochrome *c* (△), represented as $S_m$ vs. the number of methylene units per denaturant molecule and ln $S_m$ vs. the number of methylene units. The denaturation midpoints $S_m$ for the different salts plotted are the averages of the ORD and the absorbance values given in Table III.

## Discussion

*Denaturation Effectiveness of Aliphatic Acid Salts.* The midpoints of the denaturation transitions, $S_m$, of hemoglobin, myoglobin, and cytochrome *c* obtained with the various salts shown in Figures 1-2 clearly indicate that their effectiveness as protein denaturants increases with increasing hydrocarbon content of the salt. In this respect the behavior of the aliphatic acid salt series is similar to the alcohol, urea, and amide series of denaturants (Schrier et al., 1965; Herskovits and Jaillet, 1969; Herskovits et al., 1970a-c; Elbaum et al., 1974). As in our previous investigations with the latter neutral series of solutes, the equations of Peller (1959) and Flory (1957) can be used to predict the midpoints of the denaturation transitions, provided realistic estimates of the binding parameter $K_B$, representing the interaction of the denaturant with the average amino acid of the protein examined, are available. Equation 6 in the form

$$S_m = (\Delta T_m \Delta h / R T_m T_m^\circ)/\bar{\nu} K_B \qquad (6)$$

was used for calculation of the $S_m$ parameters given in the last three columns of Table III, with $T_m$ and $T_m^\circ$ representing the midpoints of the denaturation transition temperature of the protein in the presence and absence of denaturant, $\Delta T$ the difference in these two temperatures, $\Delta h$ the enthalpy of unfolding per average amino acid, and $\bar{\nu}$ the effective number or fraction of binding sites per average amino acid. As with the amides (Herskovits et al., 1970c) and the ureas (Elbaum et al., 1974), group additivity of the polar, $COO^-$ head group contribution, $K_P$, and the nonpolar contribution $K_{H\Phi}$ due to the hydrocarbon portion of the acid salts to $K_B$ had to be assumed for the prediction of the proper trend in the $S_m$ data as well as the $\Delta n$ data of Table I, with increasing chain length or hydrocarbon content given by the relationship

$$K_B = K_P + K_{H\Phi} \qquad (7)$$

The hydrophobic component of $K_B$ was evaluated by use of the equation

$$K_{H\Phi} = \exp -(\Delta G_{H\Phi}^\circ - T\Delta S_B^\circ)/RT \qquad (8)$$

where $\Delta G_{H\Phi}^\circ$ for the different alkyl groups is based on the Scheraga-Nemethy theory or free-energy transfer data, and $\Delta S_B^\circ$ is the entropy change accompanying the formation of hydrophobic bonds taken as −11 eu (Schrier et al., 1965; Herskovits et al., 1970a). The polar component of $K_B$ was estimated from the extrapolated $S_m$ values of hemoglobin and myoglobin to zero side-chain hydrocarbon content, based on the ln $S_m$ vs. number of side-chain methylene units of the different salts of Figure 4 and eq 6. The extrapolated $S_m$ values of 13.5 and 12.2 M gave average $K_P$ values of 0.010 and 0.017 $M^{-1}$ depending on the choice of $\bar{\nu}$ of 1.0 or 0.6 dictated by the best fit of the data with the Scheraga-Nemethy and free energy of transfer $K_{H\Phi}$ values (see footnote *a* of Table III). It is significant that even without the polar component of $K_B$ the hydrophobic components based on the Scheraga-Nemethy theory or the $\Delta G_{tr}$ data seem to predict reasonable $S_m$ values for valerate or caproate, the higher more hydrophobic members of the series. However, with the lower members of the series, acetate and propionate, the $S_m$ values are clearly overestimated. This is shown by the comparison of the $S_m$ values in the last three columns of Table III. Thus it is apparent that both the polar and hydrophobic contributions of the salt-protein interactions must be considered for the proper assessment of their effects on the stability of proteins.

*Specific Binding vs. Nonspecific Solvent Effects.* Based on the steepness of the denaturation transitions of hemoglobin and myoglobin shown in Figures 1 and 2, one estimates *n* for the various acid salts of 4 to 10 that are not very different from the values obtained by Cann (1971) for the interaction of acetic acid and the related aliphatic acids with ribonuclease A. The assumption of the analysis based on eq 3 is that one deals with a stoichiometric complex of protein P interacting with *n* denaturant molecules producing the complex $P[D]_n$ according to the relationship

$$P + nD \rightleftarrows P[D]_n$$

Cann visualized the binding of acetic and aliphatic acids to side-chain carboxyl groups of poly(L-glutamic acid) and proteins through hydrogen-bond formation, with intrinsic binding constants of the order of 1 $M^{-1}$. With $n = 5$ ($\bar{\nu} = 0.033$) and $K_B = 0.6\ M^{-1}$, eq 6 yields a $S_m$ midpoint of about 7 M for acetate denaturation of hemoglobin or myoglobin, which would be in reasonable accord of what should be expected, considering the approximate nature of both the *n* and $K_B$ estimates. Adding the polar and the hydrophobic contributions to $K_B$ based on group additivities (eq 7), the value of $K_B$ for valerate with the Scheraga-Nemethy value of the contribution of the butyl group of 0.097 (Schrier et al., 1965) would only lead to a lowering of $S_m$ from 7 to 6 M which is far from the observed $S_m$ values of 1.1 to 1.3 M. It is concluded, therefore, that the assumption of specific binding with a few binding sites does not predict the observed trend of increasing effectiveness of denaturation with increasing hydrocarbon content of the acid salts shown in Table III. Thus, the denaturation of proteins by aliphatic acid salts at pH 7 evidently stands in contradistinction to the interaction of aliphatic acid acids with poly(glutamic acid) and proteins at acid pH.[2]

Strong specific interactions via hydrogen-bond formation with the disruptive reagent should be confined initially to the surface areas of proteins.[2] Such interactions could produce

[2] Relatively strong binding interactions of aliphatic acids have also been observed with native serum albumin at neutral pH (Teresi and Luck, 1952).

TABLE III: Comparson of the Theoretical and Experimental Denaturation Midpoints of Human Carboxyhemoglobin, Sperm Whale Ferrimyoglobin, and Horse Heart Cytochrome *c* Obtained with the Aliphatic Acid Salt Solutions at Neutral pH.

| | | | | | | Calcd $S_m$ (eq 6)[a] | | |
|---|---|---|---|---|---|---|---|---|
| | Hemoglobin | | Myoglobin | | Cytochrome *c* | $K_B$ from Scheraga–Nemethy Theory | | $K_B$ from $\Delta G_{tr}$ Data |
| Denaturant | ORD | Soret Abs | ORD | Soret Abs | Soret Abs | $K_{H\Phi}$ Alone ($\bar{\nu}$ = 1.0) | $K_{H\Phi} + K_P$ ($\bar{\nu}$ = 1.0) | $K_{H\Phi} + K_P$ ($\bar{\nu}$ = 0.6) |
| Formate | (13.5)[b] | | (12.2)[b] | | | | 13.6 | 13.3 |
| Acetate | 6.5 | | 6 | | | 10.6 | 6.0 | 8.1 |
| Propionate | 4.4 | 4.3 | 3.7 | 4.3 | 4.2 | 6.4 | 4.4 | 4.9 |
| Butyrate | 2.2 | 1.8 | 1.8 | 2.3 | 2.6 | 3.5 | 2.8 | 2.4 |
| Valerate | 1.1 | 1.1 | 1.3 | 1.3 | 1.0 | 1.4 | 1.3 | 1.0 |
| Caproate | 0.55 | 0.5 | 0.5 | 0.6 | 0.7 | 0.3[c] | | 0.4 |

[a] Parameters employed with eq 6 were $T_m$ = 298 K, $T_m°$ = 355 K, $\Delta h$ = 500 cal/mol of peptide (Elbaum et al., 1974) giving $(\Delta T \Delta h / R T_m T_m°)$ = 0.136. Based on the Scheraga–Nemethy theory of hydrophobic bonding, $K_{H\Phi}$ of 0.0128, 0.0213, 0.0358, and 0.0973 $M^{-1}$ for the acetate, propionate, butyrate, and valerate alkyl side chains and a $K_P$ = 0.010 $M^{-1}$ for the carboxylate moiety were used (the latter based on the ln $S_m$ vs. number of methylene units per denaturant of Figure 5). The free energy of transfer data required a $K_P$ = 0.017 $M^{-1}$ based on the same plot and extrapolation to zero methylene units. The $K_{H\Phi}$ values used for the latter estimates of $K_B$ were 0.011, 0.029, 0.076, 0.20, and 0.54 $M^{-1}$ for the acetate to caproate $CH_3$ to $C_5H_{11}$ alkyl groups, respectively (Herskovits et al., 1970a). [b] Extrapolated value based on the ln $S_m$ vs. number of methylene units of Figure 4. [c] This value is based on $K_{H\Phi}$ of 0.54 $M^{-1}$ based on $\Delta G_{tr}$ data. The value based on Scheraga–Nemethy theory is not available in the literature.

modest swelling or limited changes in the peptide conformation of certain areas of the protein fold. This is in fact suggested by the slight increase in the intrinsic viscosity of both hemoglobin ard myoglobin in 4 M formate and acetate solutions (Table II) and the moderate initial increase in the absorbance profile of cytochrome *c* in the pretransition region, seen with propionate and butyrate (Figure 2B), relative to the much more pronounced changes in both the absorbance properties and the viscosity characterizing the posttransitional states of these proteins investigated. The $\bar{\nu}$ values of 0.6 to 1.0 required to predict the correct trend of increasing effectiveness of denaturation with increasing hydrocarbon content (Figures 1 and 2 and Table III) require the participation of a much larger fraction of the total number of the amino acids of the proteins studied in the solvent–biopolymer interaction than what is suggested by the discrete binding approach. The use of the Scheraga–Nemethy theory of hydrophobic bonding and the free energy of transfer information for the evaluation of the hydrophobic components, $K_{H\Phi}$, of the binding constants suggests nonspecific solvent effects, associated with nonaqueous additives on the structure of water (Kauzmann, 1959; Tanford 1970) as the basic mechanism of unfolding of proteins by the higher members of aliphatic acid series of salts. In this regard it is important to emphasize that the hydrophobic component of the binding constant required for our calculations (based on eq 8) assumes an increasingly more dominant role with the higher members of this series.

*Protein Conformation in Concentrated Salt Solutions.* The high intrinsic viscosities relative to the native state and the changes in optical rotation of hemoglobin and myoglobin in concentrated propionate, butyrate, valerate, and caproate solutions, in the concentration regions substantially above their denaturation midpoints (Figures 1 and 2), summarized in Table II, suggest that their initial folded conformations must have undergone extensive disorganization. While there is a loss of helical organization reflected by the less negative $[m']_{233}$ values, a substantial fraction of the $\alpha$-helix content is retained in these denaturing solvents despite the large increase in the observed intrinsic viscosities (Table II). In this respect the acid salts as protein denaturant have properties similar to the alkyl-substituted higher ureas and amides (Herskovits et al., 1970b–c; Elbaum et al., 1974). The intermediate values of the intrinsic viscosities falling between the native values characteristic of folded globular proteins of 3.3 to 3.7 ml/g and the much higher values of 19–21 and 24–26 ml/g expected in random coil and helix forming solvents, corresponding to the molecular weights of myoglobin and hemoglobin single polypeptide chains (Tanford et al., 1967; Herskovits and Solli, 1975), suggest that the conformation of proteins in neutral solutions of the aliphatic acid salts can be best characterized as that of interrupted helices linked by flexible random coil chain segments. The theoretical calculations of Miller and Flory (1966) suggest that the main dimensions and the subsequent intrinsic viscosities of helical polypeptides with interrupted random coil regions will be significantly reduced relative to what is calculated for fully extended helical rods of the same number of peptide units. The calculated $[\eta]$ value for a fully extended $\alpha$-helical rod of 153 amino acids comprising myoglobin, based on the equations of Simha (1940) or Kirkwood and Auer (1951), is about 21 to 23 (Herskovits and Solli, 1975).

There is evidence accumulating in the literature that certain proteins will retain residual elements of their native structure in the random coil forming denaturants, 6 M guanidine hydrochloride and 8 M urea (Tsong, 1974, 1975; Snape et al., 1974; Hsu and Neet, 1975). With the less pronounced changes in the viscosities and optical rotation, it is likely that some elements of native folding may also be retained in concentrated acid salt solutions. The disruption and unfolding of hydrophobic regions of the internal portions of the proteins suggested by the increasing effectiveness of the denaturing salts with increasing aliphatic groups and the relatively large changes in viscosity do not rule out the possibility that tightly organized regions or regions butressed by disulfide linkages will escape disorganization in these solvents.

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