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The Thermal Denaturation of Human Oxyhaemoglobins A, A_2, C and S

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1. The time-courses of thermal denaturation of human oxyhaemoglobins A, A_2, C and S at 45°C were studied by following the increase in protein fluorescence. Haemoglobins S and C were less stable than haemoglobin A, whereas haemoglobin A_2 was considerably more stable. 2. The time-courses of denaturation did not follow first-order kinetics and could be fitted most simply to a co-operative scheme in which the partial denaturation of the α chain preceded that of the β chain. 3. The denaturation of these haemoglobins was studied as a function of temperature by using optical rotatory dispersion. Haemoglobin A_2 was again more stable than the others. The addition of small quantities of haemoglobin A_2 had a disproportionate effect on the stability of haemoglobin C. 4. The thermodynamic parameters of the denaturation process were calculated.

It has been shown that a number of variants of human haemoglobins are denatured far more readily than haemoglobin A, and that the half-lives of such haemoglobins *in vivo* are decreased (Lehmann & Carrell, 1968). Variations on the surface of the molecule are likely to cause less change in the properties than those that alter the non-polar nature of the haem pockets or participate in the interchain interaction sites (Perutz & Lehmann, 1968). Haemoglobins S and C are of interest in this respect because the substitutions glutamic acid to valine and lysine respectively occur at the same site on the β chain for the two haemoglobins (β-6). Although the residues lie on the surface of the molecule and would apparently have no effect on the three-dimensional structure, their solubilities are markedly different from that of haemoglobin A (Perutz & Mitchison, 1950; Perutz & Lehmann, 1968). Haemoglobin A_2, which contains a δ chain instead of a β chain, contains no variations in the interaction sites between chains (Perutz *et al.*, 1968; Lehmann & Huntsman, 1966), and has seven residues which differ from those in the β chain.

Precipitation at elevated temperatures is frequently used as a test for the presence of unstable haemoglobins, but no detailed study has been reported on the thermal denaturation of the more commonly occurring haemoglobin variants. The present paper reports a comparison of the denaturation of such variants and haemoglobin A.

Materials and Methods

Erythrocytes of normal individuals were used for the preparation of haemoglobins A and A_2, and homozygotes of haemoglobins C and S were used for the preparation of these haemoglobins.

The erythrocytes were washed four times with 0.9% NaCl and once with 1% NaCl. They were then shaken with 3ml of water and 1ml of carbon tetrachloride/ml of packed cells for 2min to cause haemolysis, and then centrifuged. The haemoglobin solutions were purified by paper electrophoresis at pH8.9 for 12h (Cradock-Watson *et al.*, 1959). The haemoglobin bands were cut out and eluted from the paper with cold water, and the electrophoretic procedure was repeated to give improved separation. The haemoglobin solutions were concentrated by vacuum dialysis in the cold. A second electrophoretic procedure, that of Graham & Grunbaum (1963), on paper, was used to separate the haemoglobins from other erythrocyte proteins such as methaemoglobin reductase. The solutions obtained in this way were the oxyhaemoglobins containing a small proportion of methaemoglobin.

The haemoglobins were reduced by passage through a band of sodium dithionite on a deoxygenated column of Sephadex G-25 under N_2 (Dixon & McKintosh, 1967), and were stored in the reduced state under a positive pressure of N_2. They were oxygenated immediately before use by admission of a small amount of air into a sealed spectrophotometer

cuvette. Concentrations and degree of oxygenation were determined spectrophotometrically at 541 and 577nm. The extinction coefficient at 541nm was assumed to be $E^{1\%}_{1cm} = 8.5$ (Chiancone *et al.*, 1968).

Optical rotation at 233nm, determined with a Bellingham and Stanley Polarmatic 62 spectropolarimeter, was used as a probe of the thermal stability of the haemoglobins. A water-jacketed 1.0cm cell was filled with a haemoglobin solution in 10mM-Tris–HCl buffer, pH8.0, containing 10mM-EDTA, which gave a rotation of approx. 0.1° at 233nm and at 20°C; this corresponded to a concentration of approx. 6μM with respect to haem. The haemoglobin solutions were allowed to equilibrate at 15°C for some time, and then heated slowly (approx. 0.2°C/min). The change in optical rotation with temperature was observed between 20° and 60°C. The temperature could not be raised above 60°C because of the proximity of the cell to the temperature-sensitive electronics of the spectropolarimeter. The temperature of the sample was continuously measured with a calibrated thermistor probe connected to a Wheatstone-bridge circuit.

The intrinsic fluorescence of the haemoglobin solutions in 10mM-Tris–HCl buffer containing 10mM-EDTA was measured at 350nm with an exciting wavelength of 280nm by using an Aminco–Bowman SPF spectrophotofluorimeter. The buffer solutions were heated to the desired temperature in a water bath. A sample of buffer was placed in the fluorimeter cell, which was sealed with a serum cap. The cell was then placed in the water-jacketed cell compartment of the fluorimeter and allowed to equilibrate until a thermistor probe showed the temperature to have been constant for several minutes. A small volume of haemoglobin solution in water was then introduced to give a final haemoglobin concentration of less than 1μM with respect to haem. The denaturation of the haemoglobins at 45°C was followed by the increase of the fluorescence with time.

Spectrophotometric measurements of the denaturation at 280 and 415nm were attempted. Changes in the extinction with temperature were very small, and experiments were conducted over the whole temperature range used for the optical-rotation experiments and at 45°C for comparison with the experiments using the intrinsic fluorescence of the proteins. These experiments used a Unicam SP.1800 spectrophotometer. The sample cell was placed as close to the photomultiplier as possible. A reference cell, containing the same haemoglobin solution, was kept at a constant temperature of 20°C throughout.

Results and Discussion

Fluorescence studies

The fluorescence emission of oxyhaemoglobin A on excitation at 280nm was small but measurable. By assuming the fluorescence yield of globin to be 0.14 (Konev, 1967) the fluorescence yield of oxyhaemoglobin A was calculated by the method of Parker & Rees (1960) to be 0.006. The fluorescence yields of oxyhaemoglobins S, C and A_2 were similar to that of haemoglobin A.

At haemoglobin concentrations at which the extinction at 280nm exceeded 0.01 unit (approx. 0.25μM with respect to haem), the variation of emission at 350nm with concentration deviated markedly from linearity (Fig. 1). This deviation was greater than would be expected by assuming the critical transfer distance for non-radiative energy transfer to be 6nm for haemoglobin (Weber & Teale, 1959). The fluorescence emission of the isolated subunits, without haem, deviated from linearity when the extinction at 280nm exceeded 0.04, as would be expected.

On acid denaturation of oxyhaemoglobin A, the fluorescence yield increased to 0.04. This value is similar to the fluorescence yield for semi-haemoglobin found by Cassoly *et al.* (1967), and considerably less than that found for globin. It seems unlikely that loss of haem occurs on denaturation, as there was a rapid return to a fluorescence yield of 0.006 when the solution was readjusted to a neutral pH. Similar

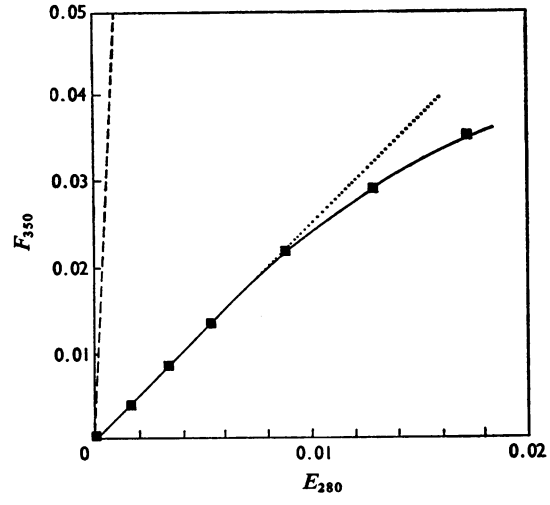


Fig. 1. *Fluorescence emission of human oxyhaemoglobin A at 350nm plotted against the absorbance at the excitation wavelength, 280nm*

Measurements were done in 10mM-Tris–HCl–10mM-EDTA, pH8.0, at 20°C. ····, Deviation from linearity above 0.01 absorbance unit; ---- represents the fluorescence emission of the β chain of haemoglobin, without haem, under the same conditions.

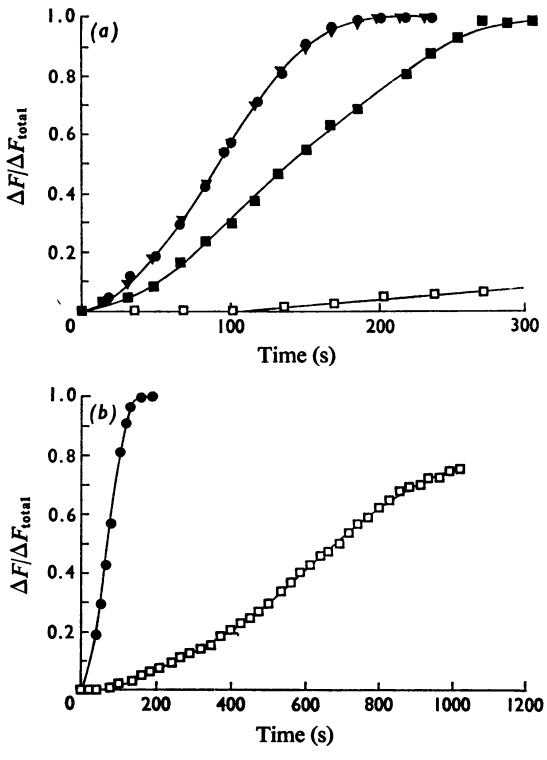


Fig. 2. *Time-course of thermal denaturation of human oxyhaemoglobins A, A_2, C and S at 45°C as determined by the change in fluorescence emission at 350nm*

The haemoglobin concentrations were such as to give an extinction at the excitation wavelength, 280nm, of less than 0.01 extinction unit. The haemoglobin solutions were in 10mM-Tris–HCl–10mM-EDTA, pH8.0 at 45°C. Figs. 2(*a*) and 2(*b*) are similar except that the time-scale on Fig. 2(*b*) is such as to allow more of the time-course for haemoglobin A_2 to be shown. ▼, Haemoglobin S; ●, haemoglobin C; ■, haemoglobin A; □, haemoglobin A_2.

effects were observed when the haemoglobins were heated, with a sevenfold increase in the fluorescence yield at elevated temperatures. It appears that the disordered state formed on heating the haemoglobins is different from that found for acid-denatured haemoglobin, as the return to the native conformation, if it occurred at all, is slow. If the haemoglobin was heated to a temperature exceeding 50°C, a hysteresis effect was observed on cooling, and the optical rotation at 233nm did not return to the same value as that observed at 20°C for the native haemoglobin.

Steinhardt *et al.* (1966) showed that the absorption spectrum of horse carbonyl haemoglobin changes little on acid denaturation, unlike that of ferrihaemoglobin. The thermally denatured oxyhaemoglobins did not show the appearance of an absorption peak at 370nm, which would be characteristic of haematin in the presence of protein (Steinhardt *et al.*, 1966). This suggests that on thermal denaturation the haemoglobins do not release the haem groups, and that a hydrophobic pocket for the haem remains in the denatured protein. It has been shown that myoglobin is only partially disordered on thermal denaturation (Tanford, 1968; Acampora & Hermans, 1967).

The time-courses of denaturation of a number of oxyhaemoglobins at 45°C were observed (Fig. 2). The time-courses of denaturation of haemoglobins S and C were identical within experimental error, and the rates were greater than that observed for haemoglobin A. Haemoglobin A_2 appeared to be considerably more stable than the normal haemoglobin at this temperature.

Steinhardt *et al.* (1966) found that the acid denaturation of carbonyl haemoglobin was approximately first-order. Their results indicated that the subunits were denatured separately and independently if the molecules exist as a tetramer. As they examined the carbonyl haemoglobins at concentrations up to 1.5mM with respect to haem, and dissociation constants for the protein are of the order of μM values, this is probably a fair assumption (Chiancone *et al.*, 1968).

All the haemoglobins examined had the α subunit in common, differing only in the β subunit, or δ subunit in haemoglobin A_2. None appeared to have any gross changes in the structure of the molecules, as the optical rotation at 233nm for all the oxyhaemoglobins was the same, within experimental error. Unlike myoglobin, or horse carbonyl haemoglobin (Acampora & Hermans, 1967; Steinhardt *et al.*, 1966) the curves found for these oxyhaemoglobins are not first-order. At the concentrations used, less than 1μM in haem, the haemoglobins are likely to be in the dimeric state (Guidotti, 1967). It is unlikely that monomers exist since the kinetics observed were not first-order (cf. myoglobin). Similarly it is unlikely that only one of the two chains is denatured under these conditions with resultant loss of haem, as might be expected from the value of the quantum yield when compared with that of semihaemoglobin, because of the sigmoid nature of the curves observed, and the slow return to the fluorescence of native haemoglobin if allowed to cool.

A possible explanation of the results observed is the initial unfolding of one chain followed by a co-operative denaturation of the second. Should a hydrogen bond be broken somewhere in a helical segment, the break tends to propagate itself (Schellman, 1958). Although the conformation of the α chain has been shown by X-ray-crystallographic studies to be affected

by the nature of the other chain, relative differences between the haemoglobins studied here have been found to be small in this respect. On the assumption that these induced changes in α-chain conformation would not cause marked changes in the rates of denaturation of the α chains in the haemoglobins, it can be assumed, for convenience, that the denaturation of the α subunits does not constitute the rate-determining step. Hence:

$$\alpha\beta \xrightarrow[k_1]{} \alpha\beta^* \xrightarrow[k_2]{} \alpha^*\beta^*$$

(* indicates denatured subunit).
This would lead to the following expressions for the intermediate concentrations of each of the species at some time t (s), where A represents the initial haemoglobin concentration:

$$[\alpha\beta] = Ae^{-k_1 t} \quad (1)$$

$$[\alpha\beta^*] = A[k_1/(k_1-k_2)]\cdot(e^{-k_2 t}-e^{-k_1 t}) \quad (2)$$

$$[\alpha^*\beta^*] = [A/(k_1-k_2)]\cdot(k_2 e^{-k_1 t}-k_1 e^{-k_2 t}) \quad (3)$$

It has been assumed, as a first approximation, that these reactions are essentially irreversible, as the rate constants for the reverse reaction are probably considerably smaller than those for the forward reactions at this temperature. Insertion of these reverse-rate constants makes the integrated rate equations far more complex (Benson, 1960) and a reasonable fit to the experimental data would be well-nigh impossible. Observation of the changes in optical rotation at 233 nm, discussed below, suggests that these changes are reversible, but renaturation is indeed considerably slower than denaturation. A further complication is a time-dependent reaction which eventually leads to precipitation. Spectroscopic examination of precipitation suggests that it is a much slower process than the changes observed here, and it has therefore been ignored in the formulation of these expressions.

Let a, b and c be the relative fluorescence yields of each species, $\alpha\beta$, $\alpha\beta^*$ and $\alpha^*\beta^*$. Then aA is the fluorescence yield at zero time, and cA that as time tends to infinity.

The change in fluorescence at any time t would be:

$$\Delta F_t = a[\alpha\beta]+b[\alpha\beta^*]+c[\alpha^*\beta^*]-a\mathrm{A} \quad (4)$$

$$= \mathrm{A}\left(\frac{ck_2-bk_1}{k_2-k_1}+a\right)e^{-k_1 t}+\frac{k_1}{k_1-k_2}\mathrm{A}(b-c)e^{-k_2 t}+(c-a)\mathrm{A} \quad (5)$$

The contribution to the fluorescence of the species $\alpha\beta^*$ is unknown, but it probably lies between that of the native and the denatured ($\alpha^*\beta^*$) species. It is likely to contribute less to the fluorescence changes than the disordered species, because the intramolecular quenching and non-radiative energy transfer from aromatic residues to the haem groups depends on distance and relative orientations in the molecule (Förster, 1959).

If one assumes that the value of b is equal or close to that of c, the expression for the change in fluorescence tends to be hyperbolic rather than sigmoid (eqn. 5), involving only one exponential term. Alternatively, if the value of b approximates to that of a, then eqn. (5) becomes:

$$\frac{\Delta F_t}{\Delta F_{\text{total}}} = 1-\frac{k_1 e^{-k_2 t}-k_2 e^{-k_1 t}}{k_1-k_2} \quad (6)$$

where $\Delta F_t/\Delta F_{\text{total}}$ is the fractional change in fluorescence for any time t. Examination of the experimental curves suggests that the value of b is closer to a than it is to c, and, as a first approximation it has been assumed to equal a.

Attempts to fit these functions to the experimental data by using a Newton–Raphson iteration procedure were unsuccessful, and on contouring of the function for the residual sum of squares:

$$S = [Y_t-(\Delta F_t/\Delta F_{\text{total}})]^2 \quad (7)$$

(Y_t is the observed fractional change in fluorescence at any time t), for various values of k_1 and k_2 for each of the haemoglobins, it was found that the values of k_1 tended to those of k_2, although they were different for the different haemoglobins. Assuming the same arguments as above, one can use different solutions of the differential equations (eqns. 1, 2 and 3) to derive an expression for the fractional change corresponding to eqn. (6):

$$\Delta F_t/\Delta F_{\text{total}} = 1-(1+kt)e^{-kt} \quad (8)$$

and $k = k_1 = k_2$.

If the assumption that the chains are denatured consecutively rather than in parallel is correct, then the denaturation of the second chain is considerably enhanced by that of the first, and appears to be related to it by a rate constant which appears to be the same as that for the original reaction. The rate constants for the individual haemoglobins calculated from the data in Fig. 2 are given in Table 1.

The close fit of the experimental data to eqn. (8) is therefore in accord with a model in which a partial denaturation of one chain (assumed to be the β chain), which causes little change in the haem environment, is followed by an unfolding of the other chain, which has a more drastic effect on the environment of the haem group. It would, of course, be possible to fit these data to more complicated models, but

Table 1. *Rate constants for the denaturation of human haemoglobins*

These were determined at concentrations of approx. 0.25μM in 10mM-Tris–HCl buffer–10mM-EDTA, pH8.0, as observed by the change in fluorescence of the haemoglobins. Standard deviations were calculated by using Newton–Raphson iteration procedures and by using the diagonal elements of the covariance matrix and the residual sum of squares.

Haemoglobin	Rate constant (s^{-1})	S.D.
S	0.02	0.003 (20 points)
C	0.02	0.003 (20 points)
A	0.012	0.001 (25 points)
A_2	0.0019	0.0002 (25 points)

the above represents the simplest model that is consistent with these observations.

Optical rotation studies

The optical rotation at 233nm of the oxyhaemoglobins at 20°C varies linearly with concentration in the range 0–100μM with respect to haem. This indicates that the dissociation of the oxyhaemoglobin tetramers into dimers has no effect on the mean residue rotation (estimates of the dissociation constant for oxyhaemoglobin A vary between 1 and 12μM with respect to haem; Guidotti, 1967). Any variation in the optical rotation owing to temperature cannot therefore be ascribed to this dissociation.

Examination of the spectra of oxyhaemoglobin between 250 and 600nm over a wide temperature range between 20° and 60°C indicated little change in the environment of the haem groups, and precipitation did not occur unless temperatures were maintained above 50°C for a considerable period of time.

The fractional change in the observed optical rotation at 233nm with temperature for haemoglobins A, C, S and A_2 is shown in Fig. 3. With haemoglobins A, C and S the curves obtained reached a plateau at temperatures above 55°C, and the observed rotation at 60°C (α_{60}) was assumed to be the rotation of the disordered haemoglobin. All the haemoglobins showed a plateau in the optical rotation below 25°C, and the rotation at 20°C (α_{20}) was taken to be the rotation owing to the native haemoglobin. The fractional change in the rotation, $F_{obs.}$, was calculated to be:

$$F_{obs.} = \frac{\alpha_{obs.} - \alpha_{60}}{\alpha_{20} - \alpha_{60}} \quad (9)$$

Less than 50% of haemoglobin A_2 is in the disordered state at 60°C if the change in optical rotation

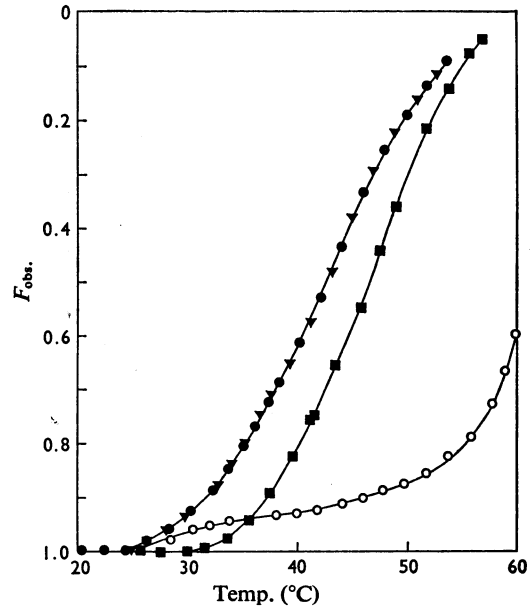


Fig. 3. *Fractional change in optical rotation at 233nm ($F_{obs.}$) with temperature for oxyhaemoglobins A, C, S and A_2*

See the text for calculation of $F_{obs.}$. Each curve on the figure represents the mean of 20 determinations. The haemoglobin solutions were approximately 6μM with respect to haem, in 10mM-Tris–HCl–10mM-EDTA, pH8.0 (at 25°C). The variation in the pH of the solutions on heating were ignored, as the change in optical rotation at 233nm for haemoglobin solutions in the range pH7.5–8.5 was negligible. ■, Haemoglobin A; ●, haemoglobin C; ▼, haemoglobin S; ○, haemoglobin A_2.

is approximately the same as that for the transitions of the other haemoglobins. This was assumed, as the curves obtained for mixtures of the haemoglobins suggested this to be the case. Once again the curves obtained for haemoglobins S and C were identical within experimental error. The changes observed for all the haemoglobins were reversible, unless temperatures were maintained above 50°C for a considerable period of time. The renaturation process, however, appeared to be much slower (about 10-fold) than the denaturation process. Oxyhaemoglobin A_2 appears to have a stable intermediate which has an optical rotation at 233nm of about 93% of that at 20°C (Fig. 3).

The addition of small amounts of haemoglobin A_2 to haemoglobin C had a marked effect on the temperature-dependence of denaturation (Fig. 4*a*).

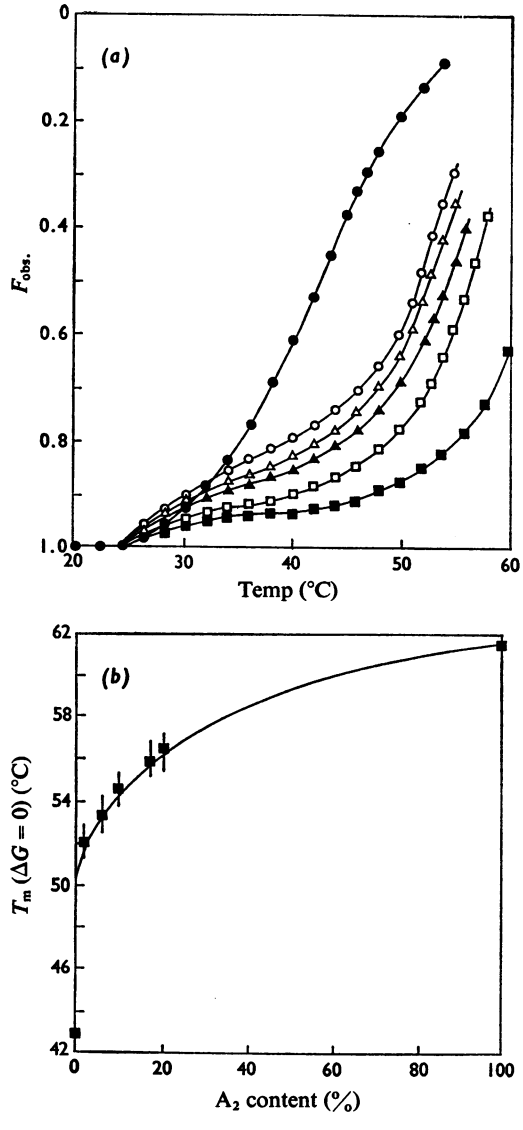
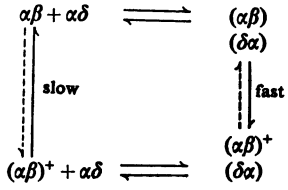


Fig. 4. *Effects of temperature on oxyhaemoglobins C and A_2*

Fig. 4(*a*) shows the fractional change in optical rotation at 233nm ($F_{obs.}$) with temperature for haemoglobins C (●), A_2 (■) and various mixtures of haemoglobins C and A_2 [1% (○), 6% (△), 17% (▲) and 20% (□) haemoglobin A_2 in haemoglobin C]. The experimental conditions were as those for Fig. 3, and the total haemoglobin concentrations were approx. 6 μM with respect to haem. Fig. 4(*b*) is a plot of the temperature at which $F_{obs.} = 0.5$ (ΔG for the transition = 0, see the text) for each of the haemoglobins and mixtures in Fig. 4(*a*), against the percentage haemoglobin A_2 in haemoglobin C.



Scheme 1. *Hybrid formation between $\alpha\beta$ and $\alpha\delta$ chains to give a more stable tetramer*

Fig. 4(*b*) shows a graph of the temperature at which 50% of the total change had occurred ($F_{obs.} = 0.5$; the 'melting temperature') against the percentage content of haemoglobin A_2, from which it can be seen that the presence of 1% of haemoglobin A_2 increased this temperature by about 10°C. The shapes of the curves obtained with mixtures of haemoglobins C and A_2 are markedly different from that obtained with haemoglobin C alone in that they indicate the formation of a stable intermediate. These effects of haemoglobin A_2 on the stability of haemoglobin C obviously cannot be explained simply in terms of the formation of the hybrid $\alpha_2\beta\delta$ and the assumption that this hybrid has the same stability as haemoglobin A_2. A possible explanation would be that hybrid formation between $\alpha\beta$ and $\alpha\delta$ chains results in a more stable tetramer which on dissociation leaves the $\alpha\beta$ dimer in a more stable conformational state $(\alpha\beta)^+$ which reverts to the initial state only relatively slowly (Scheme 1). In this system the transition from the mixed tetramer to that in which the $\alpha\beta$ subunit is in the stable form must be fast as indicated and the stabilizing effect would be achieved if the transition from the stabilized subunit $[(\alpha\beta)^+]$ to the normal subunit were relatively slow. In this manner rapid association and dissociation of the α-subunits might be able to cause a proportionally greater stabilizing effect. Similar but smaller effects were observed on addition of haemoglobin A_2 to haemoglobin A. The significance of this stabilizing effect is not clear but it is noteworthy that haemoglobin A_2 concentrations are frequently raised in homozygotes for haemoglobin S (Wrightstone *et al.*, 1968).

Although analysis of the kinetics of the denaturation at 45°C has indicated that the denaturation of the haemoglobins is not a first-order process, it is possible, as a first approximation, to obtain estimates of the apparent equilibrium constants at each temperature for the single-step reaction:

$$\text{Native protein} \underset{}{\overset{k_{app.}}{\rightleftharpoons}} \text{Denatured protein}$$

These could be obtained from the data:

$$K_{app.} = (1 - F_{obs.})/F_{obs.}$$

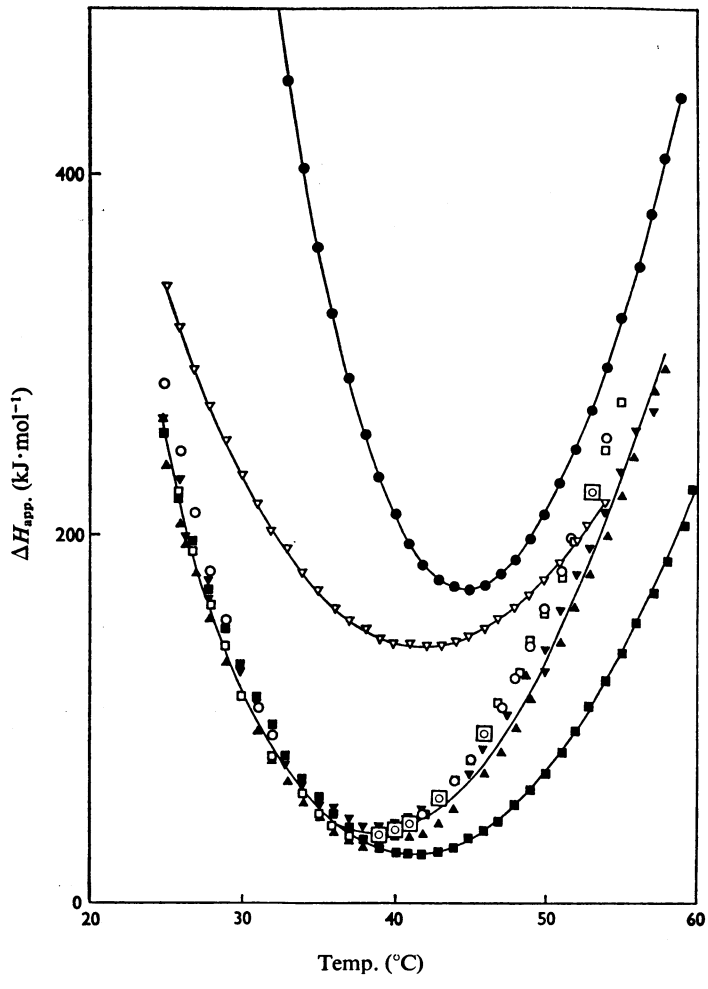


Fig. 5. *Enthalpy change ($\Delta H_{app.}$) calculated from the data in Figs. 3 and 4(a) for the denaturation of haemoglobins plotted against temperature (°C)*

The apparent equilibrium constant ($K_{app.}$) for the transition: native protein $\rightleftharpoons$ disordered protein was calculated from the data by using the relation $K_{app.} = (1-F_{obs.})/F_{obs.}$ (see the text), and a fifth-order polynomial fitted to the data relating the natural logarithm of $K_{app.}$ and the reciprocal of the absolute temperature ($1/T$). This polynomial was differentiated with respect to $1/T$ to give a measure of $\Delta H_{app.}$; for details see the text. ●, Haemoglobin A; ▽, haemoglobins C and S; ■, haemoglobin A_2; ○, haemoglobin C+1% haemoglobin A_2; □, haemoglobin C+6% haemoglobin A_2; ▼, haemoglobin C+17% haemoglobin A_2; ▲, haemoglobin C+20% haemoglobin A_2.

at any temperature (Tanford, 1968). If one assumes that the denaturation is not first-order but involves an intermediate as indicated by the fluorescence measurements:

$$\text{Native protein} \underset{K_1}{\rightleftharpoons} \text{X} \underset{K_2}{\rightleftharpoons} \text{denatured protein}$$

Then $K_{app.} = K_1 K_2$.

The calculation of the apparent equilibrium constants enables estimates of the apparent free energy, enthalpy and entropy changes associated with the transitions from native to disordered state to be calculated. Enthalpy changes ($\Delta H_{app.}$) were calculated from the Van't Hoff plot ($\ln K_{app.}$ against the reciprocal of the absolute temperature). This was accomplished by differentiation of polynomials of the fifth degree fitted to the data by regression analysis:

$$-R\frac{\partial \ln K_{app.}}{\partial(1/T)} = \Delta H_{app.}\ \text{J·mol}^{-1}$$

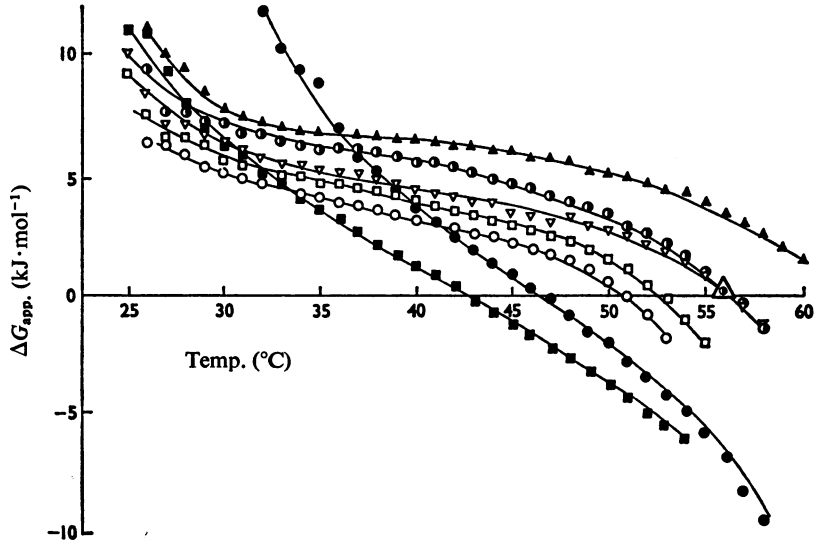


Fig. 6. *Free energy change ($\Delta G_{app.}$) calculated for the transition between native and disordered states of the proteins (see the text) for the data from Figs. 3 and 4(a) plotted against temperature (°C)*

The free energy changes calculated for the transitions at each temperature are much smaller than the enthalpy changes plotted in Fig. 5. For details of calculation of $\Delta G_{app.}$ see the text. ●, Haemoglobin A; ▲, haemoglobin A_2; ■, haemoglobins C and S; ○, haemoglobin C+1% haemoglobin A_2; □, haemoglobin C+6% haemoglobin A_2; ▽, haemoglobin C+17% haemoglobin A_2; ◑, haemoglobin C+20% haemoglobin A_2.

Enthalpy changes calculated in this manner are plotted against temperature in Fig. 5. They appear to be extremely high, and are positive for all the haemoglobins and mixtures in the range 20–60°C. The presence of haemoglobin A_2 in mixtures appears to lower the enthalpy changes associated with the apparent transition markedly (Fig. 5), indicating that there is indeed some interaction between haemoglobin C and haemoglobin A_2 in mixtures. No differences (within experimental error) could be detected among the curves obtained for mixtures of haemoglobin C and haemoglobin A_2, although the concentration of haemoglobin A_2 was in one instance only 1% of that of haemoglobin C.

Fig. 6 is a plot of the free energy changes against temperature ($\Delta G_{app.} = -RT\ln K_{app.}$ J·mol^{-1}). These changes are considerably smaller than those obtained for the changes in enthalpy. The curves indicate the considerable differences in the behaviour of the various haemoglobins and mixtures on heating, and the differences in 'melting' temperature ($\Delta G_{app.} = 0$). The entropy changes could be determined in two ways:

$$\Delta S_{app.} = (\Delta H_{app.} - \Delta G_{app.})/T \text{ J·mol}^{-1}\cdot{}^{\circ}\text{C}^{-1}$$
$$= -\partial\Delta G_{app.}/\partial T \text{ J·mol}^{-1}\cdot{}^{\circ}\text{C}^{-1}$$

Values for the second method were calculated in the same way as for $\Delta H_{app.}$, by differentiation of the fifth-order polynomial fitted for $\Delta G_{app.}$ and the absolute-temperature data from Fig. 6. They did not differ by more than 3% from those obtained from the first method. These entropy changes were again large and positive for all the haemoglobins and mixtures in the range 20–60°C.

The entropy and enthalpy changes associated with these transitions compensate each other, leaving the net free energy of the system small compared with the changes in $\Delta H_{app.}$ and $\Delta S_{app.}$ (Fig. 7). All the haemoglobins, although differing markedly in 'melting' temperature and enthalpy changes at a given temperature, appeared to give the same straight line when $\Delta H_{app.}$ was plotted against $\Delta S_{app.}$ (Fig. 7). Linear regression analysis of all the data, including values of $\Delta S_{app.}$ calculated in both ways, gave a straight line with correlation coefficient $r = 0.998$:

$$\Delta H_{app.} = 4.2(\pm 0.8) + 311(\pm 2)\Delta S_{app.} \text{ kJ·mol}^{-1}.$$

The compensation temperature (slope of the line) of 311±2°K (~37°C) found for all these haemoglobins is different from that reported by Lumry (1971) for similar compensation phenomena in the reactions of

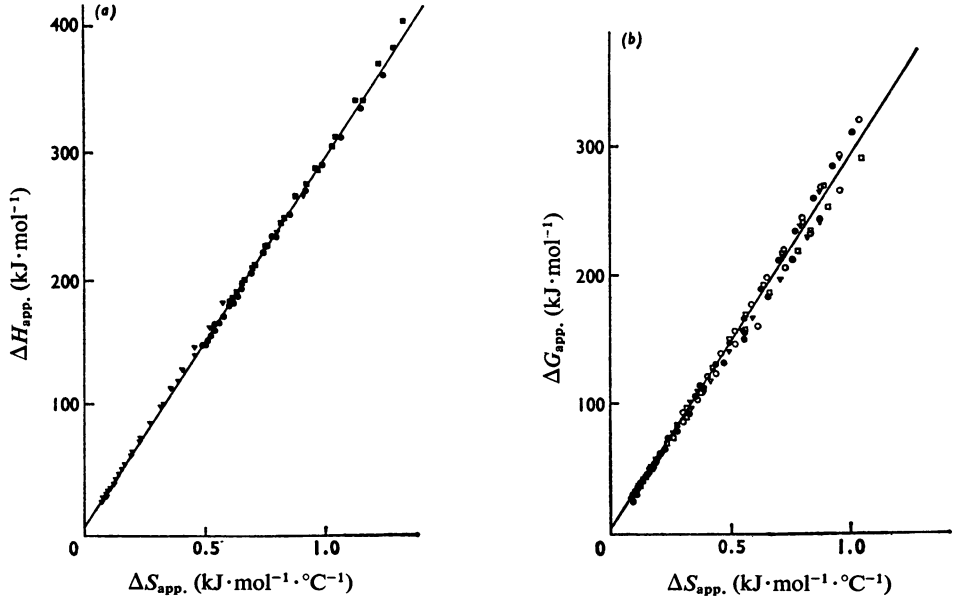


Fig. 7. *Enthalpy changes ($\Delta H_{app.}$) for the transition between native and disordered protein at any temperature (Fig. 5) plotted against the entropy changes for the same protein at the same temperatures, for all the haemoglobins and mixtures from the data in Figs. 3 and 4(a)*

All the changes observed appeared to lie on the same straight line (slope = 311±2°K), indicating compensation behaviour in the temperature range 20–60°C (293–333°K). For (*a*) ●, haemoglobin A; ■, haemoglobins S and C; ▼, haemoglobin A_2. For (*b*) ●, 1% haemoglobin A_2 in haemoglobin C; □, 6% haemoglobin A_2 in haemoglobin C; ▼, 17% haemoglobin A_2 in haemoglobin C; ○, 20% haemoglobin A_2 in haemoglobin C. The entropy changes, $\Delta S_{app.}$, were calculated in two ways; for details see the text.

haemoglobins and ligands, which lie between 270 and 290°K.

This linear correlation between the enthalpy and entropy changes for the different haemoglobins and mixtures would imply that the large variations observed might be associated with solvent–protein interactions. The smaller variations observed in the presence of haemoglobin A_2 suggests that haemoglobin molecules favour interaction with other protein molecules rather than with the solvent under these conditions.

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