Properties of Sickle-Cell Haemoglobin

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Hahn & Gillespie (1927) showed that susceptible erythrocytes are reversibly transformed into sickle cells when deprived of oxygen. Pauling, Itano, Singer & Wells (1949) found that the haemoglobin of patients with sickle-cell anaemia, who are homozygous for the sickle-cell gene, is different from normal adult haemoglobin: in buffers of pH 7.35, I 0.1, the normal molecule has three more negative electronic charges than the abnormal. Sherman (1940) had previously noticed that sickled cells are birefringent, and Pauling and his colleagues inferred that when sickle-cell haemoglobin molecules lose oxygen they combine with one another to form highly anisometric units which become partially aligned within the cell and have enough rigidity to distort it into the sickle shape. In keeping with this interpretation, Harris (1950) reported that stroma-free solutions of sickle-cell haemoglobin of concentration 10 g./100 ml. or higher show a marked increase in viscosity in the absence of oxygen. When such solutions were examined microscopically in the deoxygenated and highly viscous state, they were found to contain birefringent bodies in the shape of double circular cones. These showed all the properties of tactoids or nematic liquid crystals. Harris drew attention to the similarity in shape between these tactoids and sickle cells and concluded that sickle cells are haemoglobin tactoids 'thinly veiled and somewhat distorted by the cell membranes'. On the other hand, Perutz, Liquori & Eirich (1951), having demonstrated that sickle-cell haemoglobin has a much lower solubility than normal haemoglobin and both types of oxyhaemoglobin, suggested that the sickling phenomenon involves crystallization of the haemoglobin in susceptible cells.

The first part of the present investigation has been a more detailed analysis of the physicochemical changes occurring in sickle-cell haemoglobin solutions when they are deoxygenated. It has been possible to show that the conditions required for the formation of a liquid crystalline phase in stroma-free solutions are very similar to those required to induce sickling of susceptible cells.

The increased viscosity and birefringence of concentrated sickle-cell haemoglobin solutions suggests that when they lose oxygen the haemoglobin

molecules combine with one another to form highly anisometric polymers. To investigate the part played by hydrogen bonding in this process, the viscosity of sickle-cell haemoglobin has been measured in the presence of urea and of guanidinium ions, agents known to compete for hydrogen bonds. The effect of changes in hydrogen-ion concentration and in the ionic strength of the solvent has also been studied.

Sickling of susceptible erythrocytes is inhibited by Ag^+ , Hg^{2+} and As^{3+} ions, iodoacetamide, *o*-chloromercuribenzoate and *p*-chloromercuribenzoate, all of which combine with sulphhydryl groups (Thomas & Stetson, 1948; Ingbar & Kass, 1951; Allison, unpublished work). These findings suggested to Ingbar & Kass that sulphhydryl groups might be specifically concerned in sickling. They then investigated the sulphhydryl groups of normal adult and sickle-cell haemoglobin by amperometric titration with silver nitrate, and reported that the normal molecule has two, and that the abnormal molecule has three, available sulphhydryl groups. Intermediate values were obtained with specimens from sickle-cell trait carriers, which are mixtures of the two haemoglobin types. Ingram (1955), using a lower ammonia concentration in the silver nitrate titration, concluded that the normal adult haemoglobin molecule has four sulphhydryl groups as two pairs of closely neighbouring groups. In the present investigation the effect of various concentrations of agents combining with sulphhydryl groups on the viscosity of sickle-cell haemoglobin solutions has been observed, and an attempt has been made to define the way in which such agents inhibit sickling.

Itano & Neel (1950) described another abnormal haemoglobin type, haemoglobin C, which has two negative electronic charges less than sickle-cell haemoglobin in buffers of pH 7.35, I 0.10. The formation of haemoglobin C, normal adult haemoglobin and sickle-cell haemoglobin appears to be controlled by genes at the same locus (Ranney, 1954). Carriers of the sickle-cell trait have sickle-cell and normal adult haemoglobins in their erythrocytes, the former in somewhat greater concentration than the latter (Wells & Itano, 1951). Such cells do not become sickled at physiological oxygen tensions (Allison, 1956). Individuals who inherit both the sickle-cell

and haemoglobin C genes have in their erythrocytes sickle-cell haemoglobin and haemoglobin C in approximately equal proportions: these cells become sickled at relatively high partial pressures of oxygen (Allison, 1956), so that these persons are liable to develop a haemolytic disease known as haemoglobin C-sickle-cell anaemia, the clinical manifestations of which have been described by Smith & Conley (1954) and others. No satisfactory explanation for the much greater susceptibility to sickling of these cells as compared with the cells of sickle-cell trait carriers has been offered. Suggestive observations have, however, been presented by Singer & Singer (1953), who exposed sickle-cell haemolysates to a stream of carbon dioxide gas and recorded the minimum concentration of sickle-cell haemoglobin required for 'gelling'—the development of a solid phase. They reported that the presence of normal adult haemoglobin decreases the minimum concentration of sickle-cell haemoglobin required for gel formation and that haemoglobin C lowers this concentration still further; human foetal haemoglobin had no significant effect. No measurements of viscosity were made, and the pH, ionic strength and temperature of the solutions were not recorded. In attempts to assess the interaction of sickle-cell and other haemoglobin types more precisely, viscosity measurements have been made on a series of mixtures of different haemoglobin types. The results of experiments described below suggest that sickle-cell haemoglobin forms mixed aggregates with normal adult haemoglobin and, more efficiently, with haemoglobin C, and help to explain why subjects with the sickle-cell and haemoglobin C genes are liable to develop haemolysis.

MATERIAL AND METHODS

Blood samples were obtained by venepuncture from four normal adults, four subjects with sickle-cell anaemia (aged 5–18 years), two young adults with haemoglobin C-sickle-cell anaemia, one young adult with haemoglobin C disease (homozygous for the haemoglobin C gene, with all his haemoglobin of type C) and four young adults with the sickle-cell trait. In addition, two samples of umbilical cord blood from infants delivered after pregnancies of 32 and 36 weeks were used.

Preparation of haemoglobin. Concentrated stroma-free solutions of haemoglobin were prepared by washing erythrocytes three times in 0.9% NaCl solution, and lysing the packed cells by repeated freezing and thawing and separation of the stroma by centrifuging at 5000 g for 15 min. at 20°. Haemoglobin types were identified by electrophoresis on filter paper in an apparatus similar to that described by Smith & Conley (1954). The proportions of different haemoglobin types in mixed samples were estimated by scanning the paper strips in a densitometer. Replicate experiments gave comparable results and tests with mixtures of known composition were satisfactory, as shown by Larson & Ranney (1953) and Motulsky, Paul & Durrum (1954).

Foetal-haemoglobin components were estimated by the denaturation technique of Singer, Chernoff & Singer (1951). These groups of authors present evidence that these two methods of estimating the quantity of various haemoglobin components in mixtures give results which are accurate to $\pm 5\%$ in the range used, which is satisfactory for the present purpose. Foetal haemoglobin represented 84 and 79% of the total in the specimens of umbilical-cord blood and less than 5% in the other specimens. The haemoglobin mixtures used for analysis were natural (in sickle-cell trait carriers and subjects with haemoglobin C-sickle-cell anaemia) and artificially produced by mixing samples of pure haemoglobin types in various proportions. The viscosity measurements obtained with natural and artificial haemoglobin mixtures of the same composition by electrophoretic analysis were identical within the limits of experimental error.

Solutions of haemoglobin of various concentrations were made up in phosphate buffer, ionic strength I 0.01–1.0, pH 6.0–8.0, with K^+ ion as the only cation. The pH was measured with a glass electrode calibrated according to British Standard 1647: 1950. Unless otherwise stated, I was 0.15, pH was 7.35 and experiments were carried out at 37°. The oxyhaemoglobin concentration was measured after dilution in a Beckman spectrophotometer at 541 m μ . before and after each experiment. The oxyhaemoglobin solutions were deoxygenated by evacuation, care being taken to avoid bubbling, and equilibration with $N_2 + CO_2$ (95:5, v/v) for not less than 2 hr. at room temp. (18–19°). Deoxygenation was assumed to be complete when the absorption bands at 541 and 577 m μ . were completely replaced by a band at 555 m μ . Carboxyhaemoglobin was prepared by bubbling CO through haemoglobin solutions, cyanhaemoglobin by adding 6 moles of KCN to each mole of haemoglobin.

Viscosity measurements. These were made in a water bath either by the British Standard Capillary Viscometer (volume 2 ml., flow time 15 sec.) or a falling-ball method. The results with the two techniques were comparable, and the latter was adopted for most of the tests because of its greater convenience. By means of syringes with long needles oxyhaemoglobin specimens were placed in graduated-glass tubes of 10 cm. length and 2.5 mm. bore. Into each tube a steel ball was placed, the diameter of the ball being chosen so that the time of fall through 10 cm. of solvent was approximately 15 sec. at room temp. The tube was sealed with a silicone-treated rubber stopper, and the time taken for the ball to fall through 10 cm. of the solution was recorded at various temperatures. The specimens were then deoxygenated and cooled to 4°, at which temperature they were all fluid and could be placed in the viscometer tubes. The experiments were then repeated with the solvent only in the tubes.

Specific viscosity (η_{sp}) was then calculated from the equation:

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0},$$

where η is the viscosity of the solution and η_0 is the viscosity of the solvent alone.

EXPERIMENTAL

The viscosity of normal adult and sickle-cell oxyhaemoglobin, haemoglobin, carboxyhaemoglobin and cyanhaemoglobin solutions varying in concentration from 0.05 to 25 g./100 ml. was measured over the temperature range 0–40°. Concentrated solutions of sickle-cell haemoglobin

were examined under the polarizing microscope in preparations made by sealing the coverslip to the microscope slide with paraffin wax.

The viscosity of sickle-cell haemoglobin solutions was then measured in the presence of urea and guanidinium chloride. The solutions of sickle-cell haemoglobin contained 14.6 and 16.4 g. of haemoglobin/100 ml. and the concentrations of urea and guanidinium chloride were 0.01–1 M and 0.005–0.5 M respectively. The viscosity of concentrated sickle-cell haemoglobin solutions made up in a series of nine phosphate buffers (pH 6.0, 7.35 and 8.0, *I* 0.01; 0.1 and 1.0) was then measured at 37°. Known molar concentrations of AgNO₃, HgCl₂ and sodium *p*-chloromercuribenzoate were added to sickle-cell oxyhaemoglobin solutions, the concentrations of which were 14.6 and 16.4 g./100 ml. The haemoglobin specimens had been previously dialysed against a large volume of buffer for 24 hr. at 4° to eliminate glutathione. After 30 min. the viscosity of the oxyhaemoglobin solutions was measured; then the solutions were deoxygenated and the viscosity was measured again.

Finally, the viscosity of various oxyhaemoglobin and haemoglobin mixtures, both natural (from sickle-cell trait carriers and subjects with haemoglobin C-sickle-cell anaemia) and prepared artificially by mixing pure haemoglobin types in various proportions, was measured at 37°. The solutions were made up in phosphate buffer, *I* 0.15, pH 7.35. Two points were recorded for each mixture: the minimum concentration of sickle-cell haemoglobin required for the development of a uniformly liquid crystalline phase, and that required to produce an increment in viscosity on deoxygenation (i.e. the minimum concentration of sickle-cell haemoglobin at which aggregation of the haemoglobin

molecules may be assumed to take place). The recorded results refer to mixtures of sickle-cell (S) haemoglobin with foetal (F) and normal adult (A) haemoglobins and haemoglobin C. Mixtures without sickle-cell haemoglobin show no departure from the normal viscosity curve, either oxygenated or deoxygenated, so that the results have not been presented in detail.

RESULTS

Effect of concentration and temperature on the viscosity of normal adult and sickle-cell haemoglobin and oxyhaemoglobin solutions. The results of viscosity measurements of various haemoglobin solutions are shown graphically in Figs. 1 and 2. The specific

Fig. 1. Specific viscosity of haemoglobin solutions as a function of concentration. O, Normal adult haemoglobin; ●, normal adult oxyhaemoglobin; ●, sickle-cell haemoglobin; ●, sickle-cell oxyhaemoglobin; ▲, sickle-cell cyanhaemoglobin; ▼, sickle-cell carboxyhaemoglobin.

Fig. 2. Specific viscosity of sickle-cell oxyhaemoglobin (A) and haemoglobin (B) as a function of temperature. Curve (a), 12.0 g./100 ml.; (b), 14.6 g./100 ml.; (c) 16.4 g./100 ml. Normal adult haemoglobin and oxyhaemoglobin curves are similar to those of sickle-cell oxyhaemoglobin. Filled-in points and continuous lines are as the temperature is raised; open points and broken lines as it is lowered.

viscosity of normal adult haemoglobin solutions increases as the concentration rises; at any one concentration the viscosity falls as the temperature is raised from 0° to 40°. Normal adult oxyhaemoglobin and sickle-cell oxyhaemoglobin solutions give identical results, in agreement with the observations of Nakamura (1955) on dilute oxyhaemoglobin solutions. Sickle-cell haemoglobin solutions in concentrations up to 12 g./100 ml. have also the same viscosity as normal adult haemoglobin. Sickle-cell haemoglobin solutions of higher concentration, however, show a marked increase in viscosity. At 0° the specific viscosity of such solutions is significantly greater than that of corresponding normal adult haemoglobin solutions. The same order of difference is maintained up to 15°; above this temperature the specific viscosity of the concentrated sickle-cell haemoglobin solutions show a further sharp increase until at 20° transformation into a solid, gel-like mass takes place. When the solutions are cooled the sequence is reversed, the system showing marked hysteresis. The observation can be repeated several times on the same solution.

The viscosity of sickle-cell carboxyhaemoglobin and cyanhaemoglobin is indistinguishable from that of oxyhaemoglobin; and, as is well known, addition of carbon monoxide or cyanide to susceptible cells prevents sickling when they are exposed to atmospheres of nitrogen.

Formation of tactoids in sickle-cell haemoglobin solutions. The solid material obtained by deoxygenating concentrated sickle-cell haemoglobin solutions was found to be birefringent. With high concentrations of sickle-cell haemoglobin the birefringence was uniform, but with lower concentrations of pigment (12.5–16 g./100 ml.) or in partially oxygenated solutions, birefringent tactoids separated by isotropic material were observed. The tactoids had the form of spindles or double circular cones, 1–5 μ . long and 0.2–1 μ . wide, and were very similar to those described and figured by Harris (1950). When solutions containing tactoids were centrifuged at 4000 *g* for 30 min. at 20° two phases were separated. The concentration of haemoglobin in the upper, liquid phase was 12.6 g./100 ml., and that in the lower, solid phase was 16.5 g./100 ml.

Preliminary investigations showed that the phase rule is, in general, obeyed: raising the haemoglobin concentration between 12.6 and 16.5 g./100 ml. increases the size and number of tactoids visible in microscopical preparations and increases the volume of the solid phase at the expense of the liquid phase without significantly affecting the composition of either. However, this result is obtained only when oxyhaemoglobin solutions of various concentrations are deoxygenated at a constant temperature. A higher proportion of the solid

phase is obtained by diluting concentrated haemoglobin solutions to the same final concentration, presumably because of hysteresis. The system is profoundly affected by changes in temperature and by the presence of small amounts of oxygen.

Effect of urea and guanidinium chloride on the viscosity of sickle-cell haemoglobin solutions. From the results shown in Fig. 3 it seems that in the presence of relatively low concentrations of urea and guanidinium chloride (0.01 *M* and 0.005 *M* respectively) the increment in viscosity which takes place when sickle-cell haemoglobin solutions are deoxygenated is not affected. However, in the presence of higher concentrations of urea and guanidinium chloride (*M* and 0.5 *M* respectively) no viscosity increment at all was observed when the solutions were deoxygenated, and with intermediate concentrations (0.1 *M* urea and 0.05 *M* guanidinium chloride) the viscosity increment was present but reduced in magnitude.

Effect of H⁺-ion concentration and ionic strength on viscosity of sickle-cell haemoglobin solutions. The viscosity increment on deoxygenation was of the same order in all the buffers used, from which it appears that changes in H⁺-ion concentration and

Fig. 3. Specific viscosities. ●, Sickle-cell haemoglobin; ○, sickle-cell oxyhaemoglobin; ○●, sickle-cell haemoglobin (S) in the presence of 0.01 *M* urea; ○×, S in 0.1 *M* urea; ○+, S in 1 *M* urea; ■, S in 0.005 *M* guanidinium chloride; ▼, S in 0.05 *M* guanidinium chloride; ▲, S in 0.5 *M* guanidinium chloride.

ionic strength in the range tested do not significantly affect aggregation of the sickle-cell haemoglobin molecules. Field & O'Brien (1955) report that the sedimentation and diffusion of human carboxy-haemoglobin in 0.1M buffers are the same between pH 6 and 11 and with added sodium chloride concentrations of up to M.

Effect of agents combining with sulphhydryl groups on the viscosity of sickle-cell haemoglobin. From the results of the experiments shown in Fig. 4 it seems that the viscosity of the sickle-cell oxyhaemoglobin solutions is not affected by the presence of Ag^+ , Hg^{2+} and *p*-chloromercuribenzoate ions; when the concentration of these ions is high enough, however, no increment in viscosity takes place on deoxygenation. The critical concentration of Ag^+ ions (four per molecule of haemoglobin) is greater than that of Hg^{2+} or *p*-chloromercuribenzoate ions (two per molecule).

Effect of other haemoglobin types on the viscosity of sickle-cell haemoglobin solutions. The results of this series of experiments are summarized in Fig. 5. It is clear that the presence of foetal haemoglobin has no significant effect on the concentration of sickle-cell

haemoglobin required for the development of a solid phase or for a viscosity increment to occur on deoxygenation; the small effect observed could be due to the presence of minor components of normal adult haemoglobin in the foetal haemoglobin specimens used. In the presence of normal adult haemoglobin or haemoglobin C, however, the concentration of sickle-cell haemoglobin required for both these changes is diminished. Normal adult haemoglobin is nearly as effective as haemoglobin C in mixtures where the proportion of sickle-cell haemoglobin is about 75% or higher; when the proportion of normal adult haemoglobin or haemoglobin C is raised to above 25%, haemoglobin C is much more effective in producing viscosity increments than normal adult haemoglobin. Indeed, mixtures of equal quantities of sickle-cell haemoglobin and haemoglobin C show viscosity changes on deoxygenation nearly as great as those shown by pure sickle-cell haemoglobin solutions of the same total haemoglobin concentration. Raising the proportion of haemoglobin C above 50% seems to have very little, if any, additional effect.

DISCUSSION

There has been much speculation about the way in which deoxygenation of sickle-cell haemoglobin brings about the characteristic change in shape of

Fig. 4. Specific viscosities. ●, Sickie-cell haemoglobin; ○, sickle-cell oxyhaemoglobin; ●, sickle-cell haemoglobin (S) in the presence of 1 Ag^+ ion/mole; ●, S plus 2 Ag^+ ions/mole; ●, S plus 4 Ag^+ ions/mole; ▼, S plus 1 Hg^{2+} ion/mole; ▲, S plus 2 Hg^{2+} ions/mole; ◆, S plus *p*-chloromercuribenzoate (1 mole/mole); ■, S plus 2 moles of *p*-chloromercuribenzoate/mole.

Fig. 5. Concentration of sickle-cell haemoglobin (S) in mixtures with human foetal (F) and normal adult (A) haemoglobin and haemoglobin C required for the formation of a liquid crystalline phase (upper set of curves) and for a significant viscosity increment on deoxygenation (lower set of curves).

susceptible erythrocytes. In the paper announcing the discovery of sickle-cell haemoglobin, Pauling *et al.* (1949) proposed that there is a surface region on the globin of the sickle-cell haemoglobin molecule which is absent in the normal molecule and which has a configuration complementary to a different region of the surface of the haemoglobin molecule. The fact that sickling occurs only when the partial pressures of oxygen are low suggested to Pauling that the combining site is near the iron atom of one or more of the haems, and that when the iron atom is combined with either oxygen or carbon monoxide the complementary nature of the two structures is considerably diminished. When they lose oxygen, the sickle-cell haemoglobin molecules might be capable of interacting with one another at these sites sufficiently to cause at least a partial alignment of the molecules within the cell, so that the cell membrane becomes distorted to accommodate the now relatively rigid structure within its confines.

The development of complementary configuration in deoxygenated sickle-cell haemoglobin molecules might be due to the removal of sterically interfering oxygen or carbon monoxide molecules, as suggested by Pauling and his colleagues; or it might be due to the changes in molecular configuration which are known to accompany deoxygenation. These changes are reflected in the marked differences between the crystal forms of human adult haemoglobin and oxyhaemoglobin (Perutz & Weisz, 1947), and in the altered magnetic susceptibility of haemoglobin on deoxygenation (Pauling & Coryell, 1936), which suggests that the bonds between the haem prosthetic group and globin have changed from the essentially covalent to the essentially ionic type. This must, in turn, involve a secondary change in the electronic configuration of the globin molecule in the vicinity of the point of attachment of the haem groups, and the existence of such a change is made probable by the double-peaked fluctuation in the dielectric increment of haemoglobin in the course of oxygenation (Takashima, 1956).

Removal of oxygen from normal adult haemoglobin or haemoglobin C molecules, or sickle-cell haemoglobin molecules in dilute solutions, has no significant effect on their specific viscosity, which suggests that deoxygenation produces no large alteration in the hydration, partial specific volume or axial ratio of the molecules. The development of birefringence and increase in viscosity in concentrated solutions of sickle-cell haemoglobin when they are deoxygenated is probably due to aggregation of the nearly isometric molecules into highly anisometric polymers. Since this process of aggregation occurs only in concentrated solutions, is reversibly dependent on temperature and is easily reversed by addition of oxygen or carbon monoxide, it can be concluded that the intermolecular attrac-

tive forces are relatively weak. This interpretation is confirmed by the ease with which aggregation of the sickle-cell haemoglobin molecules can be prevented by the presence of relatively low concentrations of urea and guanidinium ions. This is indirect evidence that hydrogen bonding is involved in the process, and is probably reinforced by other low-energy bonds of the type existing between molecules of antigens and antibodies (Pauling, 1946). The aggregation of sickle-cell haemoglobin molecules is unaffected by changes in ionic strength and H^+ -ion concentration over a relatively wide range, from which it seems unlikely that salt linkages play an important part in the reaction.

In view of the dependence of the reaction on temperature, and the influence on it of urea and guanidinium ions, it is also very improbable that disulphide bonds are formed between the molecules. The prevention of sickling of susceptible erythrocytes, or aggregation of sickle-cell haemoglobin molecules, by agents combining with sulphhydryl groups is probably not a specific effect, as was concluded by Ingbar & Kass (1951). However, attachment by covalent bonds of molecules such as *p*-chloromercuribenzoate to sickle-cell haemoglobin molecules might well interfere sterically with the close apposition of the latter required for stable low-energy combination. This suggests that the potential combining sites on the surface of sickle-cell haemoglobin molecules are in the vicinity of the available sulphhydryl groups. Relevant in this connexion is the recent report of Riggs & Wolbach (1956) that the addition of two molecules of mersalyl, mercuric chloride and *p*-chloromercuribenzoate to each mole of horse haemoglobin greatly reduces the haem-haem interaction in oxygenation. From their results Riggs & Wolbach conclude that the two haems in each half-molecule of horse haemoglobin are situated on either side of a cluster of SH groups. The observations given in the present paper, showing that aggregation of sickle-cell haemoglobin molecules can be prevented by two moles of mercuric chloride or *p*-chloromercuribenzoate per mole of haemoglobin, as well as by attachment of oxygen, carbon monoxide or cyanide to haem groups, suggest that in human haemoglobin, also, the available sulphhydryl groups may be in the vicinity of the haem groups.

It has been noted that deoxygenation of concentrated sickle-cell haemoglobin solutions at low temperatures (0–15°) produces small, but consistent, increases in viscosity, whereas deoxygenation at higher temperatures produces large increases in viscosity. One interpretation of this finding is the formation of finite polymers at low temperatures and of high polymers when the temperature is increased above 15°. Another remarkable finding has been the effect of other haemoglobin types on the

aggregation of sickle-cell haemoglobin molecules. Foetal haemoglobin has no detectable effect on the process, from which it seems that mixed aggregates are not formed. This is to be expected, since normal human adult and foetal haemoglobins appear to have completely different configurations, whereas the close similarity in configuration of normal adult and sickle-cell oxyhaemoglobin molecules is emphasized by the identity of the X-ray diffraction patterns of most of their crystal forms (Perutz *et al.* 1951).

In the absence of sickle-cell haemoglobin no aggregation occurs at all. It is therefore inferred that the sickle-cell haemoglobin molecule is the only one that possesses the combining site when deoxygenated. This site is, however, complementary to the corresponding regions of normal adult haemoglobin and haemoglobin C, as well as other sickle-cell haemoglobin molecules. In polymers, then, at least every second molecule must be of the sickle-cell type. The fact that haemoglobin C is able to replace sickle-cell haemoglobin in mixtures where the proportion of haemoglobin C is as high as, but not higher than, 50 % suggests that mixed polymers may be of the type SCSCSC.... Normal adult haemoglobin, however, is capable of replacing sickle-cell haemoglobin in mixtures where the proportion of the former is not greater than about 25 %, from which it seems that the mixed polymers may be of the type SSSASSSA.... A possible explanation for this type of aggregation is offered below. The greater efficiency of formation of mixed aggregates with haemoglobin C than with normal adult haemoglobin explains the finding that erythrocytes from subjects with haemoglobin C-sickle-cell anaemia become sickled at higher partial pressures of oxygen than cells from carriers of the sickle-cell trait (Allison, 1956). This, in turn, explains the liability of the former but not the latter to develop haemolytic disease.

These two properties of sickle-cell haemoglobin—the formation of finite polymers at low temperatures and the greater efficiency of formation of mixed aggregates with haemoglobin C than with normal adult haemoglobin—can be explained by assuming that the asymmetric aggregates are helical and not linear. This type of aggregation of proteins has been discussed by Pauling (1953), who points out that if slightly asymmetric globular molecules combine with one another the resulting aggregates may be helical, and that these might easily form liquid crystals of the nematic type. He also notes that if the pitch of the helix is less than the width of the molecule in the direction of the axis of the helix, steric hindrance will prevent the formation of a fibril; the process of aggregation resulting from the combination of complementary surfaces would then result in the formation of finite polymers. This process might well occur in sickle-cell haemoglobin

solutions at low temperatures, whereas complete fibrils are formed at higher temperatures. In the course of formation of mixed aggregates of sickle-cell and normal adult haemoglobin, steric hindrance might prevent the attachment of a molecule of the latter to every sickle-cell haemoglobin molecule in the helix, so that an SSSASSSA... pattern of combination, and not an SASASA... pattern, is produced. Further observations on the lateral dimensions of the aggregates should establish whether they are, in fact, helices.

However the anisometric aggregates are formed, it seems clear that they attract one another into a parallel orientation to produce a liquid crystalline phase. When the sickle-cell haemoglobin concentration is between 12.6 and 16.5 g./100 ml., spindle-shaped, birefringent, liquid crystalline masses (tactoids) are separated by haemoglobin molecules in a state of low order. These are very similar to tactoids seen in other systems, e.g. the tobacco-mosaic virus (Bernal & Fankuchen, 1941). When the sickle-cell haemoglobin concentration is above 16.5 g./100 ml. the entire haemoglobin mass is in the liquid crystalline phase. It may be noted that sickled cells are completely liquid crystalline masses, separated by cell membranes, not tactoids separated by haemoglobin in a state of low order, as supposed by Harris (1950) and others. It has been shown by Allison (1956) that the conditions required for sickling of susceptible erythrocytes are very similar to those required for the formation of a liquid crystalline phase in sickle-cell haemoglobin solutions *in vitro*. Hence there is little doubt that the two processes are the same, and that the erythrocyte membrane and stroma play little direct part in the sickling phenomenon.

SUMMARY

1. The specific viscosity of sickle-cell haemoglobin has been measured under a variety of conditions. Sickle-cell oxyhaemoglobin shows the same type of viscosity behaviour as normal adult oxyhaemoglobin and haemoglobin. The viscosity rises steadily as the concentration is increased from 0.5 to 25 g./100 ml. and falls at any one concentration as the temperature is increased from 0° to 40°. When the concentration of sickle-cell haemoglobin is raised above 12 g./100 ml. the viscosity increases very much more rapidly than that of normal adult haemoglobin, until at 16 g./100 ml. a completely rigid, birefringent, liquid crystalline phase is formed. When the concentration of the solution is between these two critical points, spindle-shaped liquid crystalline masses or tactoids, separated by haemoglobin in a state of low order, are observed.

2. When concentrated sickle-cell oxyhaemoglobin solutions are deoxygenated at low temper-

atures (0–15°) the viscosity increment is small, from which it is concluded that finite polymers are formed. When the temperature is raised the usual liquid crystalline phase develops, and this disappears when the solution is cooled.

3. The increment in viscosity usually observed when concentrated sickle-cell oxyhaemoglobin solutions are deoxygenated does not occur in the presence of M urea or 0.5M guanidinium chloride, but is unaffected by changes in pH from 6.0 to 8.0 or changes in the ionic strength of the solvent from 0.01 to 1.0. It is inferred that hydrogen bonding plays an important part in aggregation of sickle-cell haemoglobin molecules, and formation of salt linkages only a minor part.

4. Sickling of susceptible cells, and aggregation of sickle-cell haemoglobin molecules, do not take place in the presence of Ag^+ , Hg^{2+} and *p*-chloromercuribenzoate ions in concentrations of four, two and two respectively per molecule of haemoglobin. It is concluded that these compounds form covalent bonds with exposed sulphhydryl groups in the vicinity of the complementary sites where sickle-cell haemoglobin molecules normally combine with one another (probably close to the haem groups), so that by steric hindrance the combination is prevented.

5. Measurements of the viscosity of haemoglobin mixtures suggest that sickle-cell haemoglobin forms mixed aggregates with normal adult haemoglobin and haemoglobin C, but not with human foetal haemoglobin. It seems that haemoglobin C can replace more molecules of sickle-cell haemoglobin in the mixed aggregates than can normal adult haemoglobin. This finding, and the formation of finite sickle-cell haemoglobin polymers at low temperatures, can be explained by postulating a helical rather than a linear process of aggregation of sickle-cell haemoglobin molecules when they are deoxygenated.

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REFERENCES

- Allison, A. C. (1956). *Clin. Sci.* **15**, 497.
 Bernal, J. D. & Fankuchen, I. (1941). *J. gen. Physiol.* **25**, 111.
 Field, E. O. & O'Brien, J. R. P. (1955). *Biochem. J.* **60**, 656.
 Hahn, E. V. & Gillespie, E. B. (1927). *Arch. intern. Med.* **39**, 232.
 Harris, J. W. (1950). *Proc. Soc. exp. Biol., N.Y.*, **75**, 197.
 Ingbar, S. H. & Kass, E. H. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 74.
 Ingram, V. M. (1955). *Biochem. J.* **59**, 653.
 Itano, H. A. & Neel, J. V. (1950). *Proc. nat. Acad. Sci., Wash.*, **36**, 613.
 Larson, D. K. & Ranney, H. M. (1953). *J. clin. Invest.* **32**, 1070.
 Motulsky, A. G., Paul, M. H. & Durrum, E. L. (1954). *Blood*, **9**, 897.
 Nakamura, T. (1955). *J. Biochem., Tokyo*, **42**, 745.
 Pauling, L. (1946). In *The Specificity of Serological Reactions*. By Landsteiner, K. Harvard University Press.
 Pauling, L. (1953). *Disc. Faraday Soc.* **13**, 170.
 Pauling, L. & Coryell, C. D. (1936). *Proc. nat. Acad. Sci., Wash.*, **22**, 210.
 Pauling, L., Itano, H. A., Singer, S. J. & Wells, I. C. (1949). *Science*, **110**, 543.
 Perutz, M. F., Liguori, A. M. & Eirich, F. (1951). *Nature, Lond.*, **167**, 929.
 Perutz, M. F. & Weisz, O. (1947). *Nature, Lond.*, **160**, 786.
 Ranney, H. M. (1954). *J. clin. Invest.* **33**, 1634.
 Riggs, A. F. & Wolbach, R. A. (1956). *J. gen. Physiol.* **39**, 585.
 Sherman, I. J. (1940). *Johns Hopk. Hosp. Bull.* **67**, 309.
 Singer, K., Chernoff, A. I. & Singer, L. (1951). *Blood*, **6**, 429.
 Singer, K. & Singer, L. (1953). *Blood*, **8**, 1008.
 Smith, E. W. & Conley, C. L. (1954). *Johns Hopk. Hosp. Bull.* **94**, 289.
 Takashima, S. (1956). *J. Amer. chem. Soc.* **78**, 541.
 Thomas, L. & Stetson, C. A. J. (1948). *Johns Hopk. Hosp. Bull.* **83**, 176.
 Wells, I. C. & Itano, H. A. (1951). *J. biol. Chem.* **188**, 55.