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SICKLE HEMOGLOBIN GELATION—INHIBITION BY TRIS (HYDROXYMETHYL) AMINOMETHANE AND SUGARS*

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Abstract—Sickling is believed to be a consequence of intracellular gelation of deoxyhemoglobin S. One theoretical approach to the therapy of sickle cell anemia is direct interference with gel formation. Gelling, therefore, was studied in the presence and absence of inhibitors. In support of the theory that hydrophobic bonds are involved, it was found that lysolecithin and retinol, amphipaths, which interfere with hydrophobic interactions, inhibited gelation. Moreover, Tris (hydroxymethyl) aminomethane and sugars, agents which have been shown to interfere with hydrogen bond-mediated interactions, also inhibited gelation. Raising the osmolality of the solutions with NaCl interfered somewhat with gelation, but to a lesser degree than with Tris or sugars of equal osmolalities. The data suggest that several types of weak interactions are involved in the gelation phenomenon; hydrophobic, hydrogen and electrostatic.

HEMOGLOBIN S differs from normal hemoglobin only in that there is a substitution of a valine for a glutamic acid in the sixth position of the beta chains.[1] When maintained in the deoxygenated state, hemoglobin S molecules polymerize to form liquid crystals (tactoids).[2] If the hemoglobin concentration is sufficiently high, solutions of deoxy-Hb S will undergo gelation. It is often assumed that as a result of intracellular gelation the cellular architecture becomes distorted, forming a rigid sickled poikilocyte.[3] In the body, under conditions where deoxygenation is facilitated, cells assume the sickle form and some become irreversibly sickled cells.[4] Sickling, in turn, increases the viscosity of blood; this leads to sludging and further deoxygenation and a fall in pH. Since sickling is also enhanced at acid pH a "vicious cycle" is established and the clinical symptoms of vaso-occlusive crisis ensue. In addition, the mechanical fragility of sickled cells is markedly increased, leading to hemolytic manifestations.[3]

In the past, attempts at terminating or preventing vaso-occlusive crisis have mainly been directed at interfering with conditions which facilitate the sickling or the sludging of cells.[5] More recently, a new approach has been suggested: interference with intermolecular bonds holding together the polymerized molecules of deoxy-Hb S. Urea has been the agent most widely used, based on the suggestion that it interferes with inter-tetrameric hydrophobic bonds.[6] More recently, treatment with potassium

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cyanate has also been attempted.[7] Cyanate has been shown to carbamylate hemoglobin irreversibly at the amino terminal valines and consequently to inhibit gel formation. Bookchin and Nagel[8] have recently presented evidence that electrostatic bonds may play a role in the relation phenomenon. In the present study, we confirm their findings as well as present evidence that media which have been shown to constrain intermolecular hydrogen bonds also inhibit gel formation *in vitro*. This approach may indicate yet another line of attack.

MATERIALS AND METHODS

Blood was drawn from patients with sickle cell anemia using EDTA as an anticoagulant. The diagnosis was confirmed by standard sickle cell preparations with sodium metabisulfite, hemoglobin electrophoresis on cellulose acetate and acid agar gel, and family studies. Hb F content was determined by the method of Singer *et al.*[9] Red cells were separated from plasma by centrifugation at 500 *g* at 4°. The erythrocytes were washed three times with isotonic saline and the buffy coat was removed. The washed cells were lysed by the addition of an equal volume of water. Stroma were removed by centrifugation at 20,000 *g* for 15 min at 4°. The lysates were then centrifuged at 100,000 *g* for 3 hr at 4° to remove ribosomes. The lysates were concentrated by pressure dialysis at 4° to the desired hemoglobin concentration. Cell lysates were used within 1 week of collection and only if the methemoglobin concentration was less than 2 per cent of the total hemoglobin.

Prior to gelation, the hemoglobin solution was exposed to 100% oxygen for 15 min. Gelation was measured essentially by the method of Singer and Singer[10] at 37°. Experiments were performed in 10-ml Erlenmeyer flasks with a total of 0·5 ml of hemoglobin solution. Duplicate confirmatory experiments were performed also in 10-ml test tubes using 0·1 ml of solution, in order to conserve the amount of hemoglobin. Similar results were obtained with both volumes. The inhibitors were prepared at eleven times the final concentration desired and 1 vol. was added to 10 vol. of hemoglobin solution. Deoxygenation was achieved under a stream of water-equilibrated nitrogen in a rotary water-bath shaker at either pH 7·35 or 6·5 (adjusted with HCl). When the mixture began to become viscous, it was chilled and thereby liquified to achieve homogeneity. The end-point of gelation was the point at which no movement occurred when the flask was tipped and strongly tapped. At least three determinations were done for each mixture, such that three values for the minimum gelling concentration differed from the mean value by no more than 1·0 hemoglobin/100 ml. All hemoglobin samples were shown to liquify upon cooling in an ice-bath and to gel again upon warming, except for samples containing dimethylsulfoxide (DMSO). Since DMSO freezes at 4°, these samples were cooled to room temperature and oxygenated to disrupt the gel. The pH was measured at the beginning and end of the experiments with the hemoglobin in the oxygenated form. Hemoglobin and methomoglobin concentrations were determined on 0·1 ml of hemoglobin solution in a Beckman Kintrac VII spectrophometer by the method of Evelyn and Malloy.[11] In the confirmatory gelation experiments where only 0·1 ml of hemoglobin solution was used, aliquots of 20–50 μl were taken and appropriate corrections made for the smaller sample volumes. The sample was diluted in 10 ml of 0·167 M phosphate buffer at pH 6·6 and metheomoglobin converted to the cyanomethemoglobin with neutralized

KCN. Readings were taken at 635 mμ. To determine total hemoglobin, one drop of concentrated ammonium hydroxide was added to the sample in 10 ml of 0·0167 M phosphate buffer with cyanide. Two ml of this solution was added to 8 ml of 0·067 M phosphate buffer at pH 6·6 and converted to methemoglobin with one drop of 20% potassium ferricyanide (2 min). The methemoglobin was converted to cyanomethemoglobin with 10% KCN and read at 540 mμ against the blank.

Inhibition of gelation was studied by two methods. (1) The hemoglobin solutions in the flasks were initially approximately 1 g/100 ml below the minimum gelling concentration in water (as determined by previous experiments). These solutions were deoxygenated for 1 hr (with and without inhibitors) under water-equilibrated nitrogen and then concentrated under dry nitrogen. It was usually not possible to deoxygenate for more than 2 hr, as methemoglobin formation became considerable after this time period. (2) The hemoglobin solutions in the flasks were at a concentration above the minimum gelling concentration (30–40 g/100 ml). Deoxygenation under water-equilibrated nitrogen was then carried out for 1 hr. All inhibition experiments were compared to a simultaneous control flask with water as diluent, instead of an inhibitor solution, and with the hemoglobin solution at an identical concentration. With this method, little or no evaporation occurred.

Furthermore, all comparisons between the effects of various agents on the minimum gelling concentration were determined on each lysate individually. No comparisons were made between lysates from different patients. This was necessary, as there was considerable variation in the concentration of Hb F from one patient to the other (and therefore, the minimum gelling concentration). The range of Hb F in the lysates was 2–13·5 per cent.

Experiments were also performed with purified hemoglobin S solutions. Hemoglobin S was separated from the other hemoglobin components, as well as from other components of the lysate, by isoelectric focusing on LKB 8100 ampholine electrofocusing equipment for 65 hr with a live current of 2 W with the ampholine carrier ampholytes in a range of pH 6 to 8. Hb A migrates farther toward the anode than does Hb S. After collection from the column, the hemoglobins were dialyzed against two changes of 3 l. of deionized water for 16 hr and then concentrated by pressure dialysis. The identity of the hemoglobin was confirmed by hemoglobin electrophoresis on cellulose acetate.

RESULTS

Hb S solutions were obtained from erythrocytes drawn from five patients. The range between patients was 20–25 g/100 ml with water as the diluent, which is similar to that reported previously.[8,10] Each patient's determination, however, was such that three values for the minimum gelling concentration differed from the mean value by no more than 1·0 g/100 ml.

A representative experiment is shown in Table 1, using Hb S from a single patient. In this study, the hemoglobin was deoxygenated and concentrated under nitrogen for 2 hr. The results indicate that gelation of deoxy-Hb S was inhibited by a variety of agents (Tris(hydroxymethyl) aminomethane, sugars, lysolecithin and retinol), as well as by raising the osmolality of the suspending medium with sodium chloride. A comparison of the final osmolalities of the various solutions, however, demonstrated that the effect of Tris and sugars was greater than can be explained by osmolality.

TABLE 1. INHIBITION OF GELATION OF A DEOXY-Hb S SOLUTION*

Agent added	Final concn (M)	Minimum gelling concn (g/100 ml)
Water		22·3
Tris	0·3	>40
Sucrose	0·3	>40
Glucose	0·3	>40
Invert sugar	0·3	>40
Sodium chloride	0·15	29·7
Sodium chloride	0·27	33·5
Lysolecithin	0·001	31·0
Retinol	0·001	34·7
DMSO	10%	22·0

* Osmolalities of the final concentrations of the suspending media were measured in an Osmette precision osmometer. Osmolalities of Tris 0·3 M, and NaCl, 0·27 M, were 495 mOsm/kg; the sugar solutions and NaCl, 0·15 M, were 280 mOsm/kg. The hemoglobin concentration at the beginning of the experiment was 22 g/100 ml. In the tubes containing Tris and sugars, the experiment was terminated after 2 hr of deoxygenation and evaporation. Hemoglobin was not yet gelled at a hemoglobin concentration of 40–45 g/100 ml. Lysolecithin and retinol were suspended in 100% DMSO which, when added to the hemoglobin solutions, was at a final concentration of 10%.

In order to confirm the inhibitory effect of Tris and sugars on gelation, a portion of the same hemoglobin lysate was concentrated further by pressure dialysis to 35 g/100 ml. This solution was then deoxygenated under water-equilibrated nitrogen. Gelation did not occur in those flasks containing Tris and sugars (Table 2).

TABLE 2. INHIBITION OF GELATION OF A CONCENTRATED DEOXY-Hb S SOLUTION*

Agent added	Final concn (M)	Gelation
Water		+
Tris	0·3	−
Sucrose	0·3	−
Glucose	0·3	−
Invert sugar	0·3	−
Sodium chloride	0·15	+
Sodium chloride	0·27	+

* The hemoglobin concentration in each flask was 35 g/100 ml. Deoxygenation was achieved under a stream of water-equilibrated nitrogen for 1 hr at 37°. Gelation (+); no gelation (−).

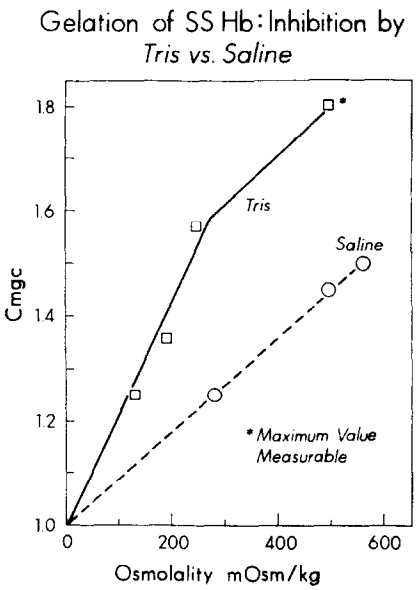

FIG. 1. Inhibition of gelation of Hb S suspended in Tris and saline. The osmolality given is of the suspending medium (Tris and saline). C_{mgc} represents a ratio of minimum gelling concentration experimental (Tris or saline) minimum gelling concentrations control (water). Minimum gelling concentrations were performed as described in Table 1.

The effect of raising the osmolality of the suspending medium with saline upon the minimum gelling concentration is shown in Fig. 1. The degree of inhibition is expressed as a ratio of minimum gelling concentration "experimental"/minimum gelling concentration control (water). This ratio we termed the C_{mgc}. The higher the C_{mgc}, therefore, the greater the degree of inhibition. While saline did inhibit gelation, Tris inhibited it to a much greater degree at any given osmolality. Since Tris is a potent buffer in the alkaline range, experiments were performed at pH 6·5, which is outside the buffer range of Tris, as well as at physiological pH 7·35. Similar results were obtained at both pH determinations.

In the experiments reported above, even though the hemoglobin solutions in the flasks containing Tris and sugars were not gelled, the experiments were terminated at a hemoglobin concentration of 40–45 g/100 ml. At this concentration, the hemoglobin was thick and the end-point became difficult to determine. Methemoglobin concentrations were also determined at the end of experiments. If there was any significant difference between flasks, the experiment was invalidated. Furthermore, if the per cent of methemoglobin was more than 4 per cent the experiment was repeated. Because of methemoglobin formation, deoxygenation and evaporation of water could not be carried out beyond a maximum of 2 hr.

To exclude further the possibility that the inhibition of gelation by Tris and sugars was due to an indirect effect upon minor hemoglobin components or interactions with other materials in the crude lysates, Hb S was purified by isoelectric focusing.

Figure 2 shows the separation of hemoglobins A and S from a patient with the sickle cell trait, with Hb A migrating further toward the anode than Hb S. After

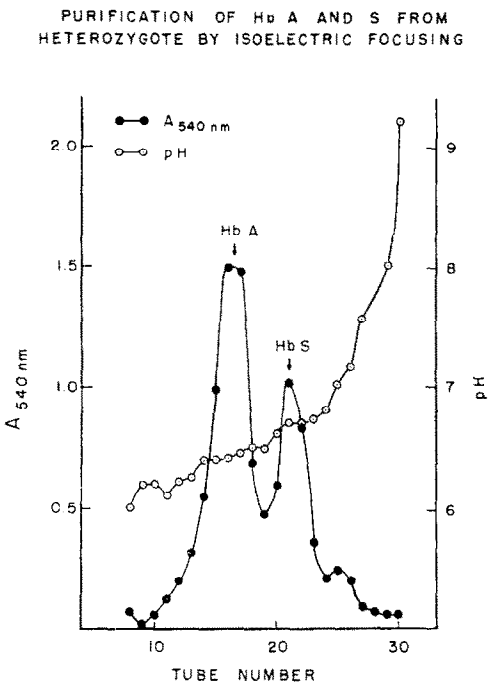

FIG. 2. Separation of hemoglobins A and S by isoelectric focusing. Hemoglobin A was collected from the column first, as it had migrated further towards the anode.

collection from the column, hemoglobin S was dialyzed against water and concentrated by pressure dialysis. Minimum gelling concentrations were then determined and comparisons made between Tris, glucose, hyperosmolar saline and water (Table 3). Similar inhibition was seen with the purified hemoglobin as with the crude lysate. The experiments were also terminated after a maximum of 2 hr of evaporation and deoxygenation, to avoid alterations in the hemoglobin solutions induced by prolonged

TABLE 3. INHIBITION OF GELATION OF PURIFIED DEOXY-Hb S BY TRIS AND GLUCOSE*

Agent added	Final osmolality (mOsm/kg)	Minimum gelling concn (g/100 ml)
Water		20·6
Saline	495	29·8
Tris	495	>33·0
Glucose	280	>33·0

* Deoxy-Hb S was purified by isoelectric focusing (Fig. 1). Minimum gelling concentration and osmolalities were determined as described in Table 1. The initial hemoglobin concentration was 16 g/100 ml.

incubation. The figure of 20·6 g/100 ml for the minimum gelling concentration of Hb S in water is slightly higher than that reported previously.[8] This might be explained either by less exhaustive dialysis in our experiments or by some contamination of the Hb S by either Hb A or methemoglobin. Unfortunately, we were unable to determine accurately the minimum gelling concentrations in the presence of inhibitors, as during the necessarily prolonged concentration procedures, methemoglobin 'formation was a problem. The lower minimum gelling concentration of the purified Hb S, as compared to the crude lysates, appears to result from dialysis against water.

DISCUSSION

The exact nature of the structure of the polymerized deoxy-Hb S in a gel has not yet been established. However, since the only abnormality in Hb S is the $\beta^{6\ Val}$, the primary intermolecular binding site should be between a site determined by the substituted amino acid (but not necessarily the $\beta^{6\ Val}$ itself) and a complementary site on a different hemoglobin molecule. Two bonds in Hb S alone, however, could not explain the tridimensional network indicated by gelation.[12,13] Some degree of secondary interactions between the polymers is required for gelation. Furthermore, the observations on how hemoglobins other than deoxy-Hb S interact in gelation suggest binding sites other than the primary $\beta^{6\ Val}$ determined site.[14,15]

Weak interactions appear to hold the polymerized deoxy-Hb S molecules together in a gel. One method of studying these intermolecular forces is investigation of the types of agents which inhibit gelation. In the present study, we have demonstrated inhibition of gelation in three types of media which have been reported to interfere with hydrophobic, electrostatic and hydrogen bonding, respectively.

The finding that lysolecithin and retinol, amphipaths which interfere with hydrophobic interactions,[16] raised the minimum gelling concentration tends to support the hypothesis that hydrophobic bonds are involved in interchain association.[17] Likewise, the inhibitory effect of hypersmolar sodium chloride tends to support the previous suggestion that electrostatic bonds are also involved.[8] Tris and sugars are agents which have been demonstrated to interfere with hydrogen bond-mediated reactions, such as that between urate or Silica crystals and biomembranes, while they have little effect in hydrophobic interactions.[18,19]

Consideration of the structure of these molecules indicates how they might interfere with hydrogen bonding. Tris contains an amino group (as NH_3^+) which (at these pH values) can form only ionic non-directional bonds, and three hydroxyl groups which can act as hydrogen donors and form hydrogen bonds. Sugars likewise possess vicinal hydroxyls which can function as hydrogen donors, as well as ring oxygens which can act as hydrogen acceptors. The hydrogen bond is an association between a covalently bound hydrogen atom, with some positive charge, and a negatively charged covalently bound acceptor atom. It is a weak cooperative bond with enethalpy between 2 and 10 kcal/mole. Efficient hydrogen bonding between adjacent molecules requires a high degree of directionality and complementarity between the reacting molecules.[20,21] The presence of small molecules, such as Tris and sugars, with the potential for hydrogen bond interactions, located between protein molecules, therefore, might be expected to constrain hydrogen bonding.

The inhibition by Tris and sugars of the gelation of deoxy-Hb S, therefore, might be evidence that hydrogen bonds are involved in the sickling phenomenon. An alternative explanation for any agent used, however, is that it alters the hemoglobin tetramer itself, thereby causing small conformational changes which modify the binding or complementary sites or oxygen affinity. The data available in this study do not allow one to distinguish between intermolecular and intramolecular interference and, therefore, interpretation remains speculative at present.

High concentrations of Tris and sugars were added to whole sickle blood, which was then deoxygenated. With Tris, there was protection against sickling, but the cells were sphered.[22] With sugars and hyperosmolar saline, considerable sickling still occurred. However, it has been previously demonstrated that hypertonic media cause an increase in sickling of intact cells,[23] This occurs as a consequence of cellular dehydration, resulting in an increased concentration of hemoglobin within the cells. Any interpretation of data using intact cells, then, must take into account the effect of agents upon the cell membrane, the final osmolality of the surrounding medium, and the uptake of the agent into the cell before conclusions may be drawn. The effect of Tris, or any agent which inhibits sickling of cells, therefore, might have nothing to do with the inhibition of gelation of deoxy-Hb S.

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REFERENCES

1. V. M. INGRAM, *Nature, Lond.* **180,** 326 (1957).
2. J. W. HARRIS, *Proc. Soc. exp. Biol. Med.* **7,** 197 (1950).
3. J. W. HARRIS, H. A. BREWSTER and T. H. NAM, *Archs. intern. Med.* **97,** 145 (1956).
4. J. F. BERTLES and P. F. A. MILNER, *J. clin. Invest.* **47,** 1731 (1968).
5. M. L. FREEDMAN, *Am. J. med. Sci.* **261,** 304 (1971).
6. R. M. NALBANDIAN, G. SHULTZ, J. M. LUSHER, J. W. ANDERSON and R. L. HENRY, *Am. J. med. Sci.* **261,** 309 (1971).
7. A. CERAMI and J. M. MANNING, *Proc. natn. Acad. Sci. U.S.A.* **68,** 1180 (1971).
8. R. M. BOOKCHIN and R. L. NAGEL, *Clin. Res.* **19,** 411 (1971).
9. K. SINGER, A. I. CHERNOFF and L. AINGER, *Blood* **6,** 413 (1951).
10. K. SINGER and L. SINGER, *Blood* **8,** 1008 (1953).
11. K. A. EVELYN and H. T. MALLOY, *J. biol. Chem.* **126,** 655 (1938).
12. J. D. FERRY, *Protein Gels* **4,** 1 (1948).
13. P. J. FLORY, *Principles of Polymer Chemistry*, p. 347. Cornell University, Ithaca, New York (1953).
14. R. M. BOOKCHIN and R. L. NAGEL, *J. molec. Biol.* **60,** 263 (1971).
15. S. CHARACHE and C. L. CONLEY, *Blood* **24,** 25 (1964).
16. G. SESSA and G. WEISSMANN, *J. Lipid Res.* **9,** 310 (1968).
17. M. MURAYAMA, *Science, N.Y.* **153,** 145 (1966).
18. G. WEISMANN and G. A. RITA and R. B. ZURIER, *J. clin. Invest.* **50,** 97a (1971).
19. G. WEISMANN and G. A. RITA, *Nature, Lond.*, in press.
20. L. PAULING, *The Nature of the Chemical Bond*, Cornell University, Ithaca, New York (1960).
21. J. D. WATSON, *Molecular Biology of the Gene*, p. 102. Benjamin, New York (1970).
22. M. L. FREEDMAN, B. D. GORMAN, W. CUNNINGHAM-RUNDLES and G. WEISSMANN, *American Society of Hematology Annual Meeting*, Dec. 5–7, Abstr. 312 (1971).
23. P. E. PERILLIE and F. H. EPSTEIN, *J. clin. Invest.* **4,** 570 (1963).