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Viscosity and Gelation Studies in Deer Hemoglobins (39084)

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Deoxygenated human hemoglobin S (Hb-S) has a low solubility in phosphate buffers and will form aggregates. This low solubility is related to a change in viscosity of Hb-S upon deoxygenation when the hemoglobin assumes a semisolid gel-like state (1). This sol–gel formation of Hb-S liquefies reversibly when placed in ice water (2). The critical hemoglobin concentration (in g%) for gel formation is called the minimum gelling concentration (MGC) and is about 23 g% for deoxygenated Hb-S (3). This value changes in the presence of various hemoglobins; for example, a mixture of deoxy Hb-S with deoxy Hb-F has a high MGC of 41 g% and that of deoxy Hb-S and deoxy Hb-O-Arab has a low MGC of 20 g% (3, 4). Observations on the aggregation of deoxy Hb-S have led Murayama (5) to propose the "stacking hypothesis" suggesting that the endothermal aggregation involves hydrophobic interactions between adjacent tetramers with urea and cyanate being tested as possible antisickling agents to prevent the intermolecular interactions involved in the sickling process (6, 7). More recent studies by Finch *et al.* have given a detailed description of the "stacking" of the deoxy Hb-S molecules (8). Hemoglobin molecules are linked end-to-end at 62 Å intervals to form a filament. Six of these individual filaments form a fiber which aggregates with other fibers to form either square or hexagonally packed bundles.

The present study concerns similar sickling properties of certain hemoglobin types found in Virginia white-tailed deer (*Odocoileus virginianus*). As early as 1840, Gulliver reported the presence of peculiarly shaped red cells in deer blood (9), and *in vitro* studies have shown that this sickling occurs at high oxygen tensions and at a pH of 7.5 and higher (10, 11). Five major hemoglobin types have been found in deer and are designated Hb's II, III, IV, V, and VII, according to their electrophoretic mobilities (12). These hemoglobins differ in the β chains. Types II, III, and IV cause sickling; types V and VII do not and will prevent sickling in mixtures with types II, III or IV (10, 13).

Materials and methods. Blood was obtained from three Virginia white-tailed deer kept at the School of Veterinary Medicine, University of Georgia. Hematological data were obtained with the standard procedures (14). Oxygen equilibrium studies of whole blood were made with an IL 513 Digital pH Blood Gas Analyzer (Instrumentation Laboratory) equipped with an IL 180 CO-Oximeter, an IL 208 Gas Mixing Module and Oxygen Monitor and an IL 237 tonometer. The level of 2,3-DPG was determined by the method of Grisolia *et al.* (15). The effects of urea and potassium cyanate on sickling were observed microscopically. Urea (0–2.0 *M*) and potassium cyanate (0–0.2 *M*) were dissolved in Hematall Isotonic Diluent (Fisher Scientific Co.) and 0.1 ml of blood (from a sample that was oxygenated for 15 min in a tonometer) was added to 2 ml portions of various solutions. The mixtures were allowed to stand for 30 min at room temperature. A wet smear of the cells was prepared and the number of sickled cells was counted microscopically.

Viscosity studies were made on hemolysates prepared from washed red cells. The oxyhemoglobin solutions were dialyzed overnight against 0.05 *M* Tris–HCl buffers at pH 6.5 or 7.5 and concentrated *in vacuo* with a collodion bag apparatus (Schleichler and Schuell, Inc., Keene, New Hampshire). Measurements were made at 12°C and 37°C with a Cannon–Ubbelohde semimicro dilution viscometer (State College, Pennsylvania) with a flow time for distilled water of 21 s as described by Plese (16).

The MGC of oxyhemoglobins was determined by gradually concentrating a red cell hemolysate against 0.05 *M* Tris–HCl of

TABLE I. HEMATOLOGICAL AND FUNCTIONAL DATA

Deer number and Hb type	RBC count[a] 10^{12}/L	Hb g/dl	PCV	MCH pg	MCV fl	MCHC g/dl	P_{50}[b] mm Hg	Hill's constant n	2,3-DPG[b] μM/gm Hb
Deer D-533 (II)	15.3 (14.9–18.0)	16.7	47 (47–52)	10.9	30.7	35.5	26.5	3.2	0.33
Deer D-530 (III)	11.3 (11.0–15.0)	12.4	33 (30–38)	11.0	29.2	37.6	28.6	3.2	0.14
Deer D-532 (V)	17.8 (14.0–19.0)	16.5	46 (41–47)	9.3	25.8	35.9	29.7	3.2	0.44

[a] The figures in brackets are the ranges of five values determined over a 2-year period; the hemoglobin level was determined once.

[b] Values from our laboratory for normal human adults are P_{50}: 25.9–28.0 mm Hg (n = 15), n: 2.8–3.1 (n = 15); and 2,3-DPG: 15.4–17.1 μM/gm Hb (n = 15).

specific pH in a collodion bag apparatus *in vacuo* at room temperature (23–25°C). The concentrated sample (0.5 ml) was transferred to a small test tube and placed in ice (1°C). Gelation was assumed to be present when the sample did not move upon rotation or shaking of the test tube. Liquefaction of the gel was tested by placing the tube in a water bath at 37°C. The hemoglobin concentration of the liquefied sample was determined by the Drabkin's cyanferrihemoglobin procedure (17).

Results. Hematological data are shown in Table I. Deer D-530 (III) appeared slightly anemic at the time of testing, which was caused by a mild parasitic infection.

The effects of urea and cyanate on the sickling of red cells from deer D-533 (II) and D-530 (III) are shown in Fig. 1. The percentages of sickled cells decreased with increase in concentration of both urea and cyanate; the effect of these agents was about the same, but a much higher concentration of urea was required. No effect was observed in the round, biconcave, shape of the red cells from D-532 (V).

The flow time of the oxyhemoglobin solutions in the viscometer depended on both the hemoglobin concentration and the concentration of dialyzable solutes. When tested at 12 and 37°C and at concentrations varying between 12 and 37 g%, no significant differences in viscosity of the dialyzed hemoglobin solutions of types II, III and V were observed. At 12°C the viscosities of these hemoglobin types increased in the same ratio.

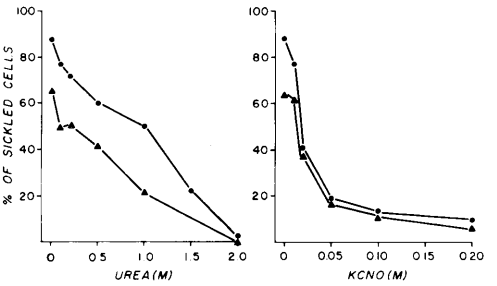

FIG. 1. Effect of various concentrations of urea and potassium cyanate on sickling of deer erythrocytes. Each value is the mean of three independent determinations. ▲——▲ deer D-533 (II); ●——● deer D-530 (III); no sickled cells were observed in deer D-532 (V).

A change in pH from 7.7 to 6.6 did not affect the viscosity.

Red cell hemolysates of D-533 (II) and D-530 (III) but not of D-532 (V) gelled readily at 1°C provided the pH was higher than 7.4, the hemoglobin was oxygenated, and the hemoglobin concentration was above a critical value (Fig. 2). Gel formation was reversed at higher temperatures through incubation at 37°C. Gelation of types II and III was prevented at pH 7.7 in the presence of equal amounts of type V.

Discussion. Hematological data show only slight variations between the three deer used in this study. Functional analyses indicate that the P_{50} values and Hill's constants are similar to the values of the normal human adult. The 2,3-DPG data agree with the observations made by Bunn *et al.* (18); thus,

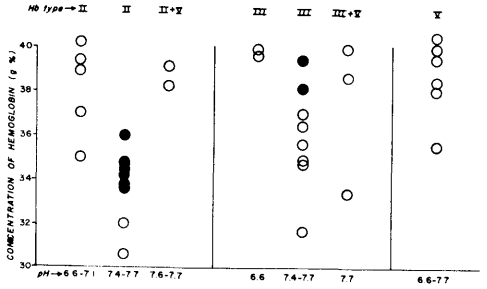

FIG. 2. Gelling of oxy Hbs II, III, and V at 1°C as a function of [Hb] and pH. Mixtures of Hbs II and V, and III and V were prepared in a 1:1 ratio. ● gel formation; ○ no gelation.

2,3-DPG does not play a significant role in regulating the oxygen affinity properties of hemoglobins of this animal species.

Both urea and cyanate prevent the *in vitro* sickling of deer red cells with hemoglobin types II or III. The concentrations of urea are considerably higher than those of cyanate to produce comparable effects, a difference which has also been observed for human erythrocytes containing Hb-S (6). Inhibition of sickling by urea indicates that hydrophobic interactions are likely involved in the sickling process of deer. Since cyanate reacts with the ϵ-NH_2 groups of lysyl residues and the α-NH_2 groups of the terminal amino acid residues of proteins to yield carbamyl derivatives (19), it can be assumed that some of these groups in the deer β polypeptide chains are also involved in the sickling process. Thus, some comparable mechanisms are involved in human sickling and in deer sickling, although the conditions for sickling to occur in human red cells with Hb-S are opposite to those in deer red cells with hemoglobin types II or III.

No difference in viscosity was observed when the oxygenated hemoglobin types II, III, and V were analyzed at temperatures of 12°C and above. However, the deer oxyhemoglobins are more viscous at lower temperatures (12°C) than at higher temperatures (37°C), which is in accordance with the general physical properties of proteins and is also true for human oxy Hb-A and oxy Hb-S (20). Thus, at temperatures of 12°C and above, viscosity alone cannot accout for the difference in sickling properties of types II, III and V. This is further substantiated by the lack of increase of viscosity when the experiments were conducted at an alkaline pH 7.7 that favors sickling. However, when the temperature was lowered to 1°C gelation occurred and viscosity measurements became inaccurate.

Data from analyses of minimum gelling concentrations at 1°C show characteristic differences between the hemoglobin types, and further indicate the importance of pH in the gelling process. The data show a MGC value at pH 7.4–7.7 of 33.5 g% for type II, 38 g% for type III, and of higher than 42 g% for type V; at pH 6.6–7.1, none of the hemoglobin types gelled at concentrations below 40 g%. These data are in accordance with *in vitro* observations indicating a sharp transition from the normal to the sickled shape of deer erythrocytes when the pH is increased from 7.0 to 7.5 (11). Equally interesting is the observation that mixtures of Hb-types II and V as well as III and V do not gel at alkaline pH, thus supporting the finding that type V is able to prevent sickling of types II and III *in situ*.

The differences in gelling properties of the three hemoglobin types are likely part of the characteristic chemical differences between the sickling (II and III) and the nonsickling (V) hemoglobin types. Data on the primary structures of the β chains will be published separately. However, it should be emphasized that the difference between the MGC of type II (33.5 g%) and type III (38 g%) is considerable, and that a higher MGC of type III (which presumably should result in a lesser tendency to sickle) does not explain a higher percentage of sickled cells in deer D-530 (III) than D-533 (II), as was observed. The absence of gel formation in the presence of type V may be the result of the formation of asymmetrical hybrids between types II and V or between types III and V that do not participate in intermolecular aggregations, or of a decrease in concentration of hemoglobin type II or III when type V is present, so that the required MGC is not reached.

The differences in gelling properties of the hemoglobin types may be correlated with the differences in "stacking" of hemoglobin molecules to form crystals. Crystal X-ray diffraction data show that the molecular

packing of deer hemoglobin types II and III are similar yet distinctly different (21). The contacts involved in filament direction of hemoglobin types II and III are much the same; but one of the two contacts (probably a β–β contact) perpendicular to the filament direction is different between types II and III, which leads to a more dense packing in type II and possibly a stronger intermolecular interaction between β chains. This higher density of packing means less water of solvation for type II and is consistent with a lower gelation concentration.

Summary. Sickling, viscosity and gelling properties of the red cells and the hemoglobins of three Virginia white-tailed deer homozygous for types II and III (the sickling types) and V (the nonsickling type), respectively, have been analyzed. The sickling of erythrocytes of deer with type II or III is inhibited by urea and cyanate at concentrations which are comparable to those used in *in vitro* studies of red cells from patients with sickle cell anemia. No differences were observed between the viscosities of the three deer hemoglobin types at temperatures of 12°C or above. High concentrations of deer hemoglobin types II and III gelled at 1°C and at pH values of 7.4–7.7; the minimum gelling concentration of type II was 33.5 g% and of type III was 38 g%. Gel formation was not observed at pH values between 6.7–7.1. Hemoglobin type V did not gel and prevented the formation of gels of type II and III in mixtures at pH 7.6–7.7.

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1. Harris, J. W., *Proc. Soc. Exp. Biol. Med.* **75,** 197 (1950).
2. Murayama, M., *J. Biol. Chem.* **228,** 231 (1957).
3. Bookchin, R. M., and Nagel, R. L., *J. Mol. Biol.* **60,** 263 (1971).
4. Milner, P. F., Miller, C., Grey, R., Seakins, M., De Jong, W. W., and Went, L. N., *New Engl. J. Med.* **283,** 1417 (1970).
5. Murayama, M., *Clin. Chem.* **14,** 578 (1967).
6. Cerami, A., and Manning, J. M., *Proc. Nat. Acad. Sci. USA* **68,** 1180 (1971).
7. Nalbandian, R. M., Henry, R. L., Barnhart, M. I., Nichols, B. M., Camp, F. R., Jr., and Wolf, P. L., *Amer. J. Pathol.* **64,** 405 (1971).
8. Finch, J. T., Perutz, M. F., Bertles, J. F., and Döbler, J., *Proc. Nat. Acad. Sci. USA* **70,** 718 (1973).
9. Gulliver, G., *Proc. Zool. Soc. London* **17,** 325 (1840).
10. Kitchen, H., Putnam, F. W., and Taylor, W. J., *Science* **144,** 1237 (1964).
11. Undritz, E., Betke, K., and Lehmann, H., *Nature London* **187,** 333 (1960).
12. Huisman, T. H. J., Dozy, A. M., and Blunt, M. H., *Arch. Biochem. Biophys.* **127,** 711 (1968).
13. Kitchen, H., Putnam, F. W., and Taylor, W. J., *Blood* **29,** 867 (1967).
14. Wintrobe, M. M., "Clinical Hematology," 7th ed. Kimpton, London (1974).
15. Grisolia, S., Moore, K., Luque, J., and Grady, H., *Anal. Biochem.* **31,** 235 (1969).
16. Plese, C. F., M. S. thesis, Medical College of Georgia (1972).
17. Jonxis, J. H. P., and Huisman, T. H. J., "Laboratory Manual on Abnormal Hemoglobins," Blackwell, Oxford (1968).
18. Bunn, H. F., Seal, U. S., and Scott, A. F., *Ann. N. Y. Acad. Sci.* **241,** 498 (1974).
19. Stark, G. R., "Methods of Enzymology," Vol. 11, p. 590. (1955).
20. Allison, A. C., *Biochem. J.*, **65,** 212 (1957).
21. Amma, E. L., personal communication.

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