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Demonstration of Lag Phase in the Sol-Gel Transformation of Deoxygenated S Hemoglobin Without Temperature Alteration[1] (38907)

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During the 25 years that the sol-gel transformation of deoxygenated sickle hemoglobin has been known (1), there has been an enormous increase in information about structure and behavior of the hemoglobin molecule and about the structure of the deoxyhemoglobin S gel (2–9). Numerous studies have in addition defined conditions, factors, and specific intermolecular interactions that influence formation of the gel (10–22). However, it is only within the past year that publications have appeared providing data concerning the *kinetics* of the gelation of deoxyhemoglobin S. By a variety of techniques, it has now been documented that in the sol to gel transformation there exists a preaggregation "lag phase" (no physical alteration detectable in the random molecular orientation in the solution) followed by rapid aggregate or polymer formation. The duration of this lag phase is greatly influenced by the concentration of deoxyhemoglobin S and markedly influenced by temperature. The techniques employed to define and evaluate gelation kinetics have been: laser light scattering (23), calorimetry, linear birefringence (24), viscometry by falling ball (25), apparent turbidity (26), viscometry by oscillating ball (27), and viscometry by cone and plate (28). To obtain a satisfactory zero point from which to measure the duration of the lag phase and evaluate the subsequent physical changes, a step is introduced whereby a hemoglobin S preparation, previously deoxygenated and gelled, is reliquified by chilling to around 2° (29), and the sol-gel transformation initiated by a "temperature jump". It has been assumed by all workers that the kinetics of the sol-gel transformation under these circumstances are the same as those that occur when gelation is induced by removing oxygen from an oxyhemoglobin S solution at constant temperature. Obviously the latter is more physiologically relevant to the *in vivo* sickling process. The present studies demonstrate that a lag period exists when gelation is induced by deoxygenation of hemoglobin S solutions at constant temperature (37.4°) and that the duration of the delay period is influenced by the concentration of deoxyhemoglobin S.

Materials and Methods. Blood samples were anticoagulated with appropriate volumes of citrate–phosphate–dextrose solution, U.S.P. (35 ml/250 ml). Hemolysates were prepared by a modification of the method of Drabkin (30) in which the cells were washed once with 0.9% and twice with 1.35% PO_4-buffered NaCl, pH 7.4, before lysis by an equal volume of water and 0.4 volumes of toluene. When required, hemoglobin solutions were concentrated by dialysis against Ficoll (Pharmacia). All the above procedures were carried out as close to 4° as practicable. The hemoglobin concentrations were determined by the Ferrihemoglobin cyanide method; fetal hemoglobin by the alkaline denaturation method of Singer *et al.* (31); and methemoglobin and sulfhemoglobin by the method of Evelyn and Malloy (32). The hemoglobin preparations were made from normal individuals, and from patients with known homozygous sickle cell anemia in whom fetal hemoglobin values lay between 3.6 and 7.0%. Only preparations containing less than 2% methemoglobin were used. Hemoglobin solutions were deoxygenated by gently shaking 4 ml in a 50 ml side-arm Erlenmeyer flask under a stream of hydrated gas (approximately 2 liters/min; 5% CO_2, 95% N_2) for 60 min. The pH of these preparations did not exceed 7.3–7.4. Transfer to

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the viscometer was made observing anaerobic precautions; the viscometer had previously been flushed with the gas mixture.

Rheologic properties of the hemoglobin solutions were studied employing the Schachman (33) modification of the capillary viscometer in a Fisher Isotemp Bath (±0.005°). Two different coiled capillary lengths were used: in one the flow-through time of double distilled, deionized water was 75 sec at 37.4° and in the longer, 272 sec were required.

Results. Capillary viscometer (Schachman modifications). Over a concentration range of 10–32 gm/100 ml (37.4°, pH 7.3–7.4), no differences could be demonstrated in the flow characteristics of oxyhemoglobins A and S and deoxyhemoglobin A expressed as time of flow (relative viscosity).* The viscosities are proportional to the hemoglobin concentrations with a constantly increasing slope that becomes very pronounced at about 17–18 g/100 ml (11, 25, 34 for comparable preparations see 11, Fig. 1). Below concentrations at which gelling could be induced, the behavior of the deoxyhemoglobin S preparations was indistinguishable from those above. To search for possible viscosity changes that might occur indicating aggregation stages intermediate in the sol-gel transition, the effects of step-wise increases in hemoglobin concentrations of 0.2 g/100 ml were examined beginning at a non-gelling concentration of 14.2 g/100 ml. At concentrations of 15 gm/100 ml, deoxyhemoglobin S preparations that were destined ultimately to gel would *initially* flow in the capillary viscometer yielding a rate (and relative viscosity) the same as that of equal concentrations of the oxyhemoglobin S preparation. If repeat flow-rate determinations were then made without removing the deoxy S preparation from the viscometer and without altering its temperature or oxygenation, cessation of flow and gelling would take place at some time during the second, third, or even fourth repeat of the flow-rate determination. Since at this hemoglobin concentration the flow time was about 10 min for the long coil viscometer, gelation of deoxyhemoglobin S had been delayed some 10–40 min after the deoxygenation procedure had been completed and with no further change in the status of the hemoglobin preparation other than flow through the capillary tube. If, following this delayed gelation and while observing anaerobic precautions, the preparation in the viscometer was chilled at 5°, flow resumed in the capillary; when returned to 37.4°, flow continued for a period but regelation ultimately occurred (incidentally confirming the maintenance of hemoglobin reactivity, anaerobic conditions, and deoxygenation of the S hemoglobin). The duration of the delay—here called lag phase—was roughly dependent upon the concentration of S hemoglobin; preparations of 16.8–15.8 g/100 ml would gel shortly after introduction into the viscometer or midway through the initial flow-rate determination; those at 15.8–15.0 g/100 ml would gel only during the second, third, or fourth repeat flow-rate determination (10–40 min).

Discussion. In agreement with the results of other investigators who have employed a variety of techniques (11, 25, 34, 35) the viscosities of preparations of oxyhemoglobins S and A, and deoxyhemoglobin A were indistinguishable when compared over a wide range of concentrations using the very sensitive extended capillary viscometer as modified by Schachman. Up to the concentration at which gelling occurred, deoxyhemoglobin S exhibited the same viscosity behavior: indeed, by this technique, the viscosity of preparations of deoxygenated hemoglobin S *within* the lower gelling concentration range become abnormally high only at the time of gelation. Thus, some concentrations of deoxyhemoglobin S that eventually gelled initially manifested a lag phase during which no alteration in viscosity occurred. It should be noted that in contrast to the other published studies, this lag phase in the gelation of deoxyhemoglobin S took place *without* alteration in temperature and without change in status of the hemoglobin preparation other than flow through

* Because the composition of the solvent for the hemoglobin is not defined, no attempt was made to convert the readings to specific viscosity or to extrapolate the reduced viscosity to obtain the intrinsic viscosity.

the capillary tube. From the results obtained during the studies of the effects of stepwise increase in concentration of deoxyhemoglobin S (0.2 g Hb/100 ml), it was evident that the duration of the lag phase was roughly dependent upon concentration. The above findings clearly indicate the existence of a time-dependent process preceding gel formation (lag phase) that was inversely proportional to a function of the hemoglobin concentration and that occurred *without* alteration in temperature, pH, or oxygen tension. It was not feasible to quantitate the duration of this lag phase since the deoxygenation procedure was such that the zero time of the lag phase was arbitrary. Moreover, the functional specifics of the capillary viscometer are such that it was not possible to characterize or quantitate changes in viscosity indicative of intermediate stages of hemoglobin aggregation and that occurred prior to the point of full gel formation (28). Because the estimates of the lag phase by this technique are of necessity semi-quantitative at best, no strict comparisons can be made with determinations of the lag phase made by the other techniques listed previously. However, 10–40 min for hemoglobin solutions of 15–16 g/100 ml are within the range of values reported.

Summary. 1). During the sol to gel transformation of deoxygenated sickle hemoglobin, a time-dependent process preceding gel formation (lag phase) was demonstrated that was inversely proportional to a function of the hemoglobin concentration and that occurred without alteration in temperature, pH, or oxygen tension.

2). As determined by the Schachman modification of the capillary viscometer, preparations of oxyhemoglobin S and A and deoxyhemoglobin A were indistinguishable when compared over a wide range of concentrations. Up to the concentration at which gelling occurred, deoxyhemoglobin S exhibited the same viscosity behavior. The viscosity of deoxygenated hemoglobin S within the lower gelling concentration range was normal during the lag phase and became abnormally high only at the time of gelation.

1. Harris, J. W., Proc. Soc. Exp. Biol. Med. **75**, 197 (1950).
2. Magdoff-Fairchild, B., Swerdlow, P. H., and Bertles, J. F., Nature (London) **239**, 217 (1972).
3. Arnone, A., Nature (London) **237**, 146 (1972).
4. Hofrichter, J., Hendricker, D. G., and Eaton, W. A., Proc. Nat. Acad. Sci. **70**, 3604 (1973).
5. Finch, J. T., Perutz, M. F., Bertles, J. F., and Dobler, J., Proc. Nat. Acad. Sci. **70**, 718 (1973).
6. Finch, J. T., and Perutz, M. F., Trans. Roy. Soc. Trop. Med. **68**, 79 (1974).
7. Edelstein, S. J., Telford, J. N., Crepeau, R. H., Proc. Nat. Acad. Sci. **70**, 1104 (1973).
8. White, J. G., Arch. Int. Med. **133**, 545 (1974).
9. Bertles, J. F., Arch. Int. Med. **133**, 538 (1974).
10. Singer, K., and Singer, L., Blood **8**, 1008 (1953).
11. Allison, A. C., Biochem. J. **65**, 212 (1957).
12. Bookchin, R. M., Nagel, R. L., and Ranney, H. M., J. Biol. Chem. **242**, 248 (1967).
13. Bookchin, R. M., and Nagel, R. L., J. Mol. Biol. **60**, 263 (1971).
14. Bookchin, R. M., and Nagel, R. L., J. Mol. Biol. **76**, 233 (1973).
15. Bookchin, R. M., and Nagel, R. L., Seminars Hematol. **11**, 577 (1974).
16. Paniker, N. V., Ben-Bassat, I., and Beutler, R., J. Lab. Clin. Med. **80**, 282 (1972).
17. Williams, R. C., Proc. Nat. Acad. Sci. **70**, 1506 (1973).
18. Briehl, R. W., and Ewert, S., J. Mol. Biol. **80**, 445 (1973).
19. Freedman, M. L., Weissmann, G., Gorman, B. D., and Cunningham-Rundles, W., Biochem. Pharmacol. **22**, 667 (1973).
20. Waterman, M. R., Yamaoka, K., Dahm, L., Taylor, J., and Cottam, G. L., Proc. Nat. Acad. Sci. **71**, 2222 (1974).
21. Benesch, R., Benesch, R. E., and Yung, S., Proc. Nat. Acad. Sci. **71**, 1504 (1974).
22. Yamaoka, K., Cottam, G. L., and Waterman, M. R., Biochem. Biophys. Res. Commun. **58**, 1058 (1974).
23. Wilson, W. W., Luzzana, M. R., Penniston, J. T., and Johnson, C. S., Proc. Nat. Acad. Sci. **71**, 1260 (1974).
24. Hofrichter, J., Ross, P. D., and Eaton, W. A., Proc. Nat. Acad. Sci. **71**, 4864 (1974).
25. Malfa, R., and Steinhardt, J., Biochem. Biophys. Commun. **59**, 887 (1974).
26. Moffat, K., and Gibson, Q. H., Biochem. Biophys. Res. Commun. **61**, 237 (1974).
27. Meadow, W. L., Crandall, E. D., Litt, M., and Forster, R. E., Fed. Proc. **34**, 355 (1975) (abstract 767).
28. Harris, J. W., and Bensusan, H. B., J. Lab. Clin. Med. in press, (1975).

29. Murayama, M., J. Biol. Chem. **228,** 231 (1957).
30. Drabkin, D. L., J. Biol. Chem. **164,** 703 (1946).
31. Singer, K., Chernoff, A. L., and Singer, L., Blood **6,** 413 (1951).
32. Evelyn, K. A., and Malloy, H. T., J. Bio. Chem. **126,** 655 (1938).
33. Schachman, H. K., *in* "Methods in Enzymology" (S. P. Colowick and M. O. Kaplan, eds.), Vol. IV, pp 99–100, Academic Press, New York (1957).
34. Chien, S., Usami, S., and Bertles, J. F., J. Clin. Invest. **49,** 623 (1970).
35. Minton, A. P., J. Mol. Biol. **75,** 559 (1973).

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