though our experimental conditions are not strictly comparable with those of the earlier workers (*15, 16*) since we use a much higher ratio of nucleic acid to solvent, our results are consistent with their conclusion that either poly(A + U) or poly(A + 2U) can be the predominant complex at cation concentrations below 0.1*M* and a temperature of 25°C. In our experiments, the predominant species clearly depends on the ratio of polyA to polyU with the poly(A + 2U) complex being favored when the ratio is low.

A "melting" curve for double-stranded calf thymus DNA based on PMR intensities of spectral regions 1 and 2 is shown in Fig. 2*d*. Again the intensities are expressed as fractions of intensities expected if all the protons of region 1 or 2 contributed to the intensity, now taking into consideration the base composition of the DNA (*17*).

Thus, it appears that intensities of PMR spectra can be used in the way optical-density measurements are used to elucidate secondary structure and "melting" of synthetic and natural nucleic acids. The PMR technique presently requires higher concentrations of nucleic acids than the optical-density technique but has the compensating advantage of permitting absolute determination of the degree of strand complexing (optical density changes give a relative measure of changes in secondary structure). In addition, observation of behavior of individual PMR lines can give detailed insight into changes in molecular structure and motion during complex formation or "melting." For example, as a polyA-polyU complex is "melted," sharp resonances characteristic of single-stranded polyA and polyU emerge from the broad background resonance of the complex. The resonances do not gradually sharpen as the complex is "melted," which would be expected if during "melting" there existed a fast equilibrium between the portions that are and are not "melted" within individual complexes. If the latter were true, the resonances would be expected to narrow gradually over the "melting" range. This result may be taken as further evidence for the all-or-nothing, cooperative nature of "melting" in the polyA-polyU complexes. On the other hand, individual resonances of the DNA spectra exhibit a considerable decrease in width as well as an increase of integrated intensity as the temperature is increased through the "melting" range. This NMR "melting" behavior of DNA suggests the existence of discrete structural regions in individual DNA double strands that exhibit a range of "melting" temperatures. This tentative interpretation is consistent with the nonuniformity of strengths of base pair interactions over the length of DNA double strands (guanine-cytosine as opposed to adenine-thymine interactions).

C. C. McDonald
W. D. Phillips
Central Research Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware*
Sheldon Penman
Department of Biology, Massachusetts Institute of Technology, Cambridge

References and Notes

1. Abbreviations: NMR, nuclear magnetic resonance; PMR, proton magnetic resonance; polyA, polyadenylic acid; polyU, polyuridylic acid; polyC, polycytidylic acid; polyI, polyinosinic acid; DNA, deoxyribonucleic acid; poly(A + U), double-stranded helical complex of polyA and polyU; and poly(A + 2U), triple-stranded helical complex of polyA and polyU.
2. O. Jardetzky, *Nature* **197**, 183 (1963); L. C. Allen and L. F. Johnson, *J. Am. Chem. Soc.* **85**, 2668 (1963).
3. Potassium salts of the ribonucleic acids, polyA, polyU, polyC, and polyI were obtained from Miles Chemical Co. Deoxyribonucleic acid (type 1, sodium salt) extracted from calf thymus was obtained from Sigma Chemical Co. These nucleic acids were used without further purification. The following instruments were used: HR-60 NMR spectrometer, Varian Associates, Palo Alto, Calif.; 400-channel computer of average transients, "Mnemotron CAT 400," Mnemotron Corp., South Main St., Pearl River, N.Y.
4. E. G. Richards, C. P. Flessel, J. R. Fresco, *Biopolymers* **1**, 431 (1963).
5. Determined from sedimentation analyses by L. L. Anderson.
6. C. D. Jardetzky and O. Jardetzky, *J. Am. Chem. Soc.* **82**, 222 (1960); S. Matsuura and T. Goto, *Tetrahedron Letters*, No. 22, 1499 (1963).
7. F. A. Bovey and G. V. D. Tiers, *J. Polymer Sci.* **44**, 173 (1960).
8. J. A. Pople, *J. Chem. Phys.* **24**, 1111 (1956); C. E. Johnson and F. A. Bovey, *ibid.* **29**, 1012 (1958).
9. R. J. Abraham, P. A. Burbidge, A. H. Jackson, G. W. Kenner, *Proc. Chem. Soc.* **1961**, 134 (1963).
10. Since this study was completed, temperature dependent shifts in the PMR spectra of polyA have been reported by O. Jardetzky [*Biopolymers Symp.* **1**, 501 (1964)].
11. G. E. Pake, *Solid State Phys.* **2**, 1 (1956); R. E. Richards, in *Determination of Structures of Organic Molecules*, F. C. Nachod and W. D. Phillips, Eds. (Academic Press, New York, 1962), pp. 537–562.
12. F. A. Bovey, G. V. D. Tiers, G. Filipovich, *J. Polymer Sci.* **38**, 73 (1959).
13. R. C. Warner, *Federation Proc.* **15**, 379 (1956); A. Rich and D. R. Davies, *J. Am. Chem. Soc.* **78**, 3548 (1956).
14. R. F. Steiner and R. F. Beers, *Polynucleotides* (Elsevier, New York, 1961), p. 223.
15. J. R. Fresco, in *Informational Macromolecules*, H. J. Vogel, V. Bryson, J. O. Lampen, Eds. (Academic Press, New York, 1963), pp. 121–142.
16. G. Felsenfeld and A. Rich, *Biochim. Biophys. Acta* **26**, 457 (1957).
17. E. Chargaff and R. Lipshitz, *J. Am. Chem. Soc.* **75**, 3658 (1953).

* Contribution No. 939.

26 February 1964

Hemoglobin Polymorphism: Its Relation to Sickling of Erythrocytes in White-Tailed Deer

Abstract. *Hemoglobins with different electrophoretic mobilities are associated with bizarre shapes seen during ordinary light microscopy of erythrocytes from the white-tailed deer,* Odocoileus virginianus; *nonsickling is contingent upon a specific hemoglobin. Sickling in vitro, which is produced to a maximum degree in the presence of oxygen and elevated* p*H, is not associated with hematological abnormalities or disease despite marked differences in physical characteristics of sickled and nonsickled cells.*

Although the sickling of deer erythrocytes was reported more than a century ago by Gulliver (*1*), the phenomenon has not previously been related to the presence of variant hemoglobins. Both Gulliver and Mandel described peculiar shapes of certain mammalian erythrocytes, especially in the families *Camelidae* and *Cervidae*, 70 years before the discovery of sickling in the human erythrocyte. Various bizarre shapes were found for different animals, but the red cells of all deer which were examined became sickle-shaped regardless of species.

The discovery of an abnormal hemoglobin in sickle cell anemia by Pauling *et al.* (*2*) prompted numerous investigations which have revealed many genetically controlled abnormal hemoglobins in man. Some of these have been directly associated with morphological changes in the red blood cell. As a consequence, investigators of the sickled and crescent-shaped erythrocytes in several species of deer have attempted to incriminate hemoglobin in the sickling phenomenon, but they have offered no evidence for the occurrence of more than a single hemoglobin within each species (*3*). In previous reports, the red blood cells of all the

Table 1. The relation between polymorphic hemoglobins and aberrant forms of erythrocytes in Florida white-tailed deer. Forty-seven penned deer, whose blood has been repeatedly studied and characterized, are represented.

Frequency (%)	Hemoglobin type	Sickling shape*
53.4	I, III	"Crescent," "Holly leaf"
25.5	I, II, III	"Matchstick"
2.1	III	"Crescent," "Holly leaf"
8.5	I, II	"Matchstick"
4.2	II	"Matchstick"
4.2	III, V	"No sickling"
2.1	II, V	"No sickling"

* The screening of deer blood and classification of morphological characteristics was done under controlled conditions which would produce maximum sickling of whole blood. Whole blood was oxygenated for 20 minutes in a tonometer with 100 percent oxygen at room temperature within 1 hour after collection.

deer examined exhibited some degree of sickling under appropriate conditions.

The purpose of this communication is to report the existence of multiple hemoglobins in white-tailed deer and to relate these hemoglobins to specific changes in morphology of deer erythrocytes. During the past 3 years a herd of 47 captive Florida white-tailed deer has been established. Most of the deer have erythrocytes which will sickle and maintain the crescent or holly-leafed appearance (Fig. 1) similar to human sickle cells. In some deer the cells transform to a matchstick appearance (Fig. 2) after having sickled initially. These aberrant forms can be shown to be reversible and to depend upon the conditions of study. Only three animals in this herd have red blood cells that do not become sickle-shaped or show other morphological aberrations under controlled laboratory conditions (*4*). The existence of polymorphic hemoglobins in this small population of captive deer accords with findings in over 600 field samples obtained during hunting and trapping (*5*) (Table 1).

In this species multiple hemoglobins (designated I, II, III, and V) can be demonstrated most easily by starch gel electrophoresis at *p*H 8.6 in the discontinuous buffer system (*6*) (Fig. 3), and by agar gel electrophoresis at *p*H 6.0 (*7*) (Fig. 4). The electrophoretic mobilities of II and III at *p*H 8.6 are very close, and these hemoglobins can be best distinguished by comparing mobilities at *p*H 6.0, as shown in Fig. 4. For the preparation of samples for electrophoresis in the carbon monoxide form, accepted methods were followed (*8*). Figure 3 shows typical electrophoretic patterns obtained for adult deer and for a fetal sample as seen on starch gel at *p*H 8.6, stained with Buffalo Black; specific staining with dianisidine indicates that the separated proteins are hemoglobins. For mature animals the pattern is constant and characteristic for the individual. Much evidence indicates that the bands designated I, II, III, and V represent chromatographically separable polymorphic forms of deer hemoglobin. The frequency of occurrence has been examined in 47 specimens (Table 1). In the majority of the deer, the hemoglobin patterns exhibit components I and III (see deer 63-1074 in Fig. 3). A few animals have only a single component, either II or III.

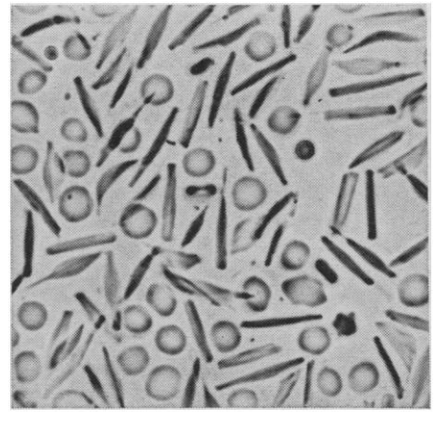

Fig. 1 (left). Sickled erythrocytes from deer: typical crescent and holly-leaf forms. Suspended in isotonic potassium phosphate buffer, *p*H 7.6, saturated with 100 percent oxygen (× 1000). Fig. 2 (right). Sickled erythrocytes, matchstick form. Suspended in isotonic potassium phosphate buffer, *p*H 7.6, saturated with 100 percent oxygen (× 1000).

The different electrophoretic patterns of the deer hemoglobins have been related to the several morphological forms that the erythrocyte assumes under appropriate conditions. If hemoglobins I and III, or III alone are present, the red blood cells sickle and maintain this characteristic shape. If hemoglobin II is present, either alone or in combination with I or III, the cells gradually assume a matchstick appearance after passing through a crescent stage.

The erythrocytes of the few deer in which hemoglobin V is present do not form sickle cells but maintain the normal biconcave discoid appearance under the same conditions that induce sickling in other deer erythrocytes. In three deer with erythrocytes that will not sickle, hemoglobin V represents 25 to 28 percent of the total hemoglobin; thus it constitutes a minor component. However, the presence of hemoglobin V precludes the ability of erythrocytes to sickle even when associated with II or III as major components, both of which readily sickle in other combinations.

The existence of two electrophoretically distinguishable fetal hemoglobins (F_1 and F_2) which are not seen in the adult deer was established by a study of 60 samples of blood from fetuses after 50 to 170 days' gestation. The fetal hemoglobins are not readily separated from the adult forms I and II by electrophoresis (Figs. 3 and 4). However, the fetal erythrocytes will not assume abnormal forms even though the erythrocytes of the does from which these fetuses were taken all assume sickle or matchstick shapes. At birth, only 3 to 5 percent of the erythrocytes can be induced to form sickle-shaped cells. This corresponds to the amount of adult hemoglobin present at fawning (5 to 7 percent) as quantitated by starch block electrophoresis according to the method of Kunkel *et al.* (*9*). The development of adult hemoglobins electrophoretically different from the fetal forms has been observed during the growth of several fawns. The increase in the percentage of cells sickling in whole blood parallels the relative increase in adult hemoglobin during the development of the fawn.

Tiselius, starch block, and paper electrophoresis, as well as column chromatography and alkaline denaturation, have further confirmed the heterogeneity of deer hemoglobins. Hemoglobins

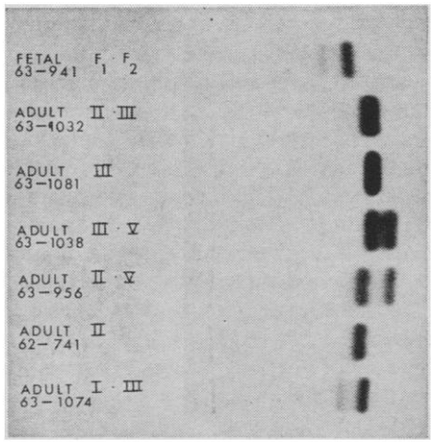

Fig. 3. Vertical starch gel electrophoresis of deer hemoglobins. Ethylenediaminetetraacetic acid-tris-borate buffer, 0.06*M* and *p*H 9.0, used in starch. Barbital buffer 0.12*M*, *p*H 8.6, used in electrode vessels (10 v/cm, 2 hours at 10°C).

II, III, and V have been isolated chromatographically from natural mixtures and migrate with their characteristic rates in starch gel electrophoresis. Absorption spectrum ratios at 280 mμ/540 mμ have given evidence for the absence of nonhemoglobin components. By ultracentrifugal analysis, the presence of a single peak with a sedimentation coefficient of 4.2*S* has been demonstrated; this is the usual value for mammalian hemoglobins. This excludes the possibility of a dimer form which could produce an apparent heterogeneity in starch gel electrophoresis.

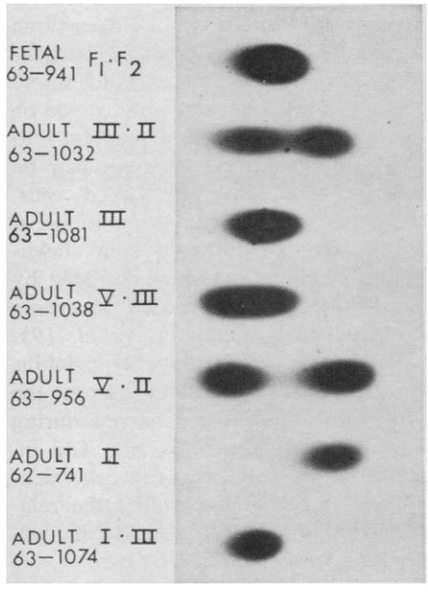

Fig. 4. Agar gel electrophoresis of deer hemoglobins in 1 percent agar, sodium citrate-citric acid buffer, *p*H 6.0 and 0.03*M* (15 v/cm, 1½ hours at 10°C). Samples identical with those in Fig. 3.

In addition to characterizing the hemoglobins of the deer, this study has correlated the hematological and physical characteristics of deer erythrocytes with the sickling phenomenon. Maximum sickling of deer erythrocytes in vitro is induced by complete saturation with oxygen or carbon monoxide in whole blood, or in buffers with a *p*H greater than 7.6. Partial association with oxygen or carbon monoxide is required since sickling will not occur in the presence of 100 percent carbon dioxide, nitrogen, or helium regardless of *p*H. These conditions are outside the usual physiological range, which may explain why no difference has been noted in the hematological values for deer whose erythrocytes will sickle or will not sickle. Hematological values are characterized by packed red cell volumes of 50 to 58 percent with hemoglobin concentrations of 17 to 20 g percent and red blood cell counts of 17 to 23 million per cubic millimeter. Aside from the smaller size (3.5 to 4.5 μ diameter in the discoid form), sickled deer erythrocytes appear identical to human cells in the sickled form when studied by ordinary light microscopy or with the electron microscope (*10*). Many physical characteristics, such as viscosity and mechanical fragility and the ability to form reversible gels, further document the similarity of the sickling phenomenon of deer with that seen in human sickle cell disease.

Although many unexplored facets of deer hemoglobins remain, the following facts have been established. (i) Sickling occurs in vitro and is attributable to the specific hemoglobin within the deer erythrocytes. This conclusion is supported by the observation that the differences in the bizarre shapes of erythrocytes produced during studies in vitro of the sickling phenomenon in deer can be related to biochemically distinguishable hemoglobins (II and III). The presence of an electrophoretically different deer hemoglobin (V) precludes the formation of sickle-shaped erythrocytes in white-tailed deer. Further support for the conclusion comes from the finding that sickling in the red blood cells of newborn fawns increases as the percentage of adult hemoglobin components increases. (ii) The sickling phenomenon of the red blood cells of white-tailed deer is a reversible process dependent upon the percentage of oxygen saturation and the *p*H of the whole blood or buffer. (iii) Aside from the hemoglobin type, no obvious hematological or clinical differences exist between deer whose erythrocytes will or will not sickle.

Breeding experiments thus far suggest that the polymorphic forms of deer hemoglobins are under genetic control. Peptide mapping studies of the purified hemoglobins are now under way to ascertain if the several components differ from each other in a single peptide as is the case for normal human hemoglobin and all its abnormal forms so far examined.

HYRAM KITCHEN
FRANK W. PUTNAM
W. JAPE TAYLOR
Departments of Medicine and
Biochemistry, University of Florida College of Medicine, Gainesville

References and Notes

1. O. Gulliver, *Proc. Zool. Soc. London* **17**, 325 (1840).
2. L. Pauling, H. A. Itano, S. J. Singer, I. C. Wells, *Science* **110**, 543 (1949).
3. J. H. Moon, *Am. J. Physiol.* **199**, 190 (1960); P. M. DeTraverse, M. L. Coquelet, L. C. Brumpt, *Compt. Rend. Soc. Biol.* **150**, 1107 (1956); L. C. Brumpt, P. M. DeTraverse, M. L. Coquelet, *ibid.*, p. 292.
3. The examination and classification of sickled deer erythrocytes is performed on whole blood and buffered blood samples (isotonic potassium phosphate buffer, *p*H 7.6) as described in Table 1. The production of sickled erythrocytes by these conditions can be shown to be reversible and dependent upon controlled conditions. The reversal to the normal biconcave discoid shape can be demonstrated by passing a gas consisting of 95 percent N_2 and 5 percent CO_2 through the blood in a tonometer. Demonstration of reversibility and examination within the shortest possible time are considered important since aberrant forms, some of which become sickle shaped, can be demonstrated in other species. Abnormal forms can be demonstrated in red blood cells held in prolonged storage, placed in hypertonic solutions, or stained with super-vital stains. We have produced such abnormal shapes in the erythrocytes of normal raccoons, hamsters, and squirrels, but these sickled forms are not dependent on changes in O_2 tension or *p*H, and in most cases are irreversible.
4. The collection of samples and animals over a 3-year period has beeen made possible through the permission and cooperation of the Florida Game and Fresh Water Fish Commission.
5. O. Smithies, *Biochem. J.* **71**, 585 (1959).
6. A. R. Robinson, M. Robson, A. P. Harrison, W. W. Zuelyen, *J. Lab. Clin. Med.* **50**, 745 (1957).
7. D. L. Drabkin, *J. Biol. Chem.* **164**, 703 (1946).
8. H. G. Kunkel, R. Ceppellini, U. Müller-Eberhard, J. Wolf, *J. Clin. Invest.* **34**, 1615 (1957).
9. W. R. Pritchard, T. D. Malewitz, H. Kitchen, *Exptl. Mol. Pathol.* **2**, 173 (1963).
10. We are indebted to Beatrice P. Godwin for technical assistance. We acknowledge the cooperation of the Agricultural Experimental Station, Department of Veterinary Science, in the establishment of deer pens, and the Jacksonville City Zoo in the collection of samples. We thank John Refour for help with the management of the deer herd. This work was supported in part by U.S. Public Health Service grants H-5004 and H-02966.

26 February 1964