Hemoglobin Polymorphism in White-Tailed Deer: Subunit Basis

By Hyram Kitchen, Frank W. Putnam and W. Jape Taylor

The sickling of erythrocytes under in vitro laboratory conditions has been demonstrated in most species of deer. This remarkable distortion of the erythrocytes from the biconcave disc shape to such bizarre forms as holly leaf, crescent and oat shapes in several species of deer was first described by Gulliver in 1840. Seventy years later Herrick independently observed the existence of similarly shaped erythrocytes associated with a severe anemia in man.

In contrast to the racial and geographical prevalence of the sickled erythrocyte associated with sickle cell anemia in man, the sickling phenomenon occurs in most species of the deer which represent a variety of ecological and geographical areas of the world. Sickling in the deer is an in vitro phenomenon which occurs under high oxygen tension and elevated pH and has no apparent pathological consequences. However, the occurrence of polymorphic hemoglobins and their relationship to the sickling or nonsickling of erythrocytes has been reported only in white-tailed deer (Odocoileus virginianus).

Although the predominant number of deer have erythrocytes which have the propensity to sickle, this ability to assume reversibly bizarre erythrocyte forms cannot be attributed to the presence of a particular hemoglobin common to all white-tailed deer. In white-tailed deer the various polymorphic hemoglobins occur so frequently in the population that it is difficult to identify individual components as "normal" or "variant."

Hemoglobin heterogeneity has been reported in many mammalian species other than man. However, in most cases only two or three hemoglobins are found within a species. The white-tailed deer, having seven adult and two fetal hemoglobins, is a marked exception. This study will establish that the hemoglobin heterogeneity of white-tailed deer is due to structural differences in the protein moieties and describes the subunit basis for these polymorphic hemoglobins.

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This work was supported in part by United States Public Health Service grants H-5004, H-02966-09 and HE-271-HE-5493. Dr. Kitchen is the recipient of a Research Career Development Award (1-K3-AM 31,811-01) from the National Institutes of Health.

First submitted Oct. 14, 1966; accepted for publication Jan. 9, 1967.

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MATERIALS AND METHODS

Deer. The majority of the present herd of approximately 70 penned deer are Florida white-tailed deer (Odocoileus virginianus), representing most geographical and ecological areas of the state. Other white-tailed deer from Georgia, Maryland and Texas were obtained through the cooperation of the Southeastern Cooperative Wildlife Disease Study. Deer trapped from the wild formed the nucleus of the research animals and represent a random sample. Subsequently, offspring from selected breedings have been raised in captivity.

Collection of Samples. The deer are housed in a 2-acre site, surrounded by a 12-foot fence, within a mile of the laboratory. This proximity to the laboratory has assured the safe handling of collected samples, as well as the opportunity to study animals repeatedly during various seasons and times of the year. Using disposable syringes and 19-gauge needles, blood samples were obtained from the jugular vein of restrained or immobilized animals. Samples were placed into heparinized tubes or BD vacutainers containing EDTA and were kept on ice until reaching the laboratory. Samples from more than 600 field specimens were obtained as a result of hunting and trapping operations, and these samples were refrigerated until arrival at the laboratory.

Production and Screening of Sickled Deer Erythrocytes. The examination and classification of sickled deer erythrocytes was performed on whole blood and buffered blood samples (isotonic potassium phosphate buffer, pH 7.6). Samples were oxygenated for 20 minutes in a tonometer with 100 per cent oxygen at room temperature. The production of sickled erythrocytes by these conditions can be shown to be reversible and dependent upon controlled conditions. A gas mixture of 95 per cent N₂ and 5 per cent CO₂ through the blood in a tonometer restores the cells to the normal biconcave discoid shape. 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.

Preparation of Hemoglobin. Red blood cells were washed three times in 1.2 per cent saline, hemolyzed with distilled water and frozen. Samples were thawed and frozen repeatedly to ensure complete hemolysis. The hemoglobin solutions were separated from ghosts and incompletely hemolyzed red blood cells by centrifugation at 40,000 g for 30 minutes. Samples were stored in the carbon monoxide form in the freezer at -10 C.

Electrophoresis. Starch gel electrophoresis and agar gel electrophoresis were performed as previously described. Individual hemoglobins for routine identification were quantitated following electrophoresis on starch block according to the method of Kunkel et al. The α and β chains were separated for qualitative examination by electrophoresis in 6 M urea starch gel, pH 8.1, following the method of Chernoff and Pettit. High voltage electrophoresis was done in a routine manner at pH 6.5, following the technic described by Putnam and Easley. Specific color reactions for peptides containing histidine, tyrosine, arginine and tryptophan, as well as ninhydrin staining, were performed following the methods described by Easley.

Isolation of α and β Chains. Separation of the α and β chains by column chromatography was performed as described by Wilson and Smith, modified by placing 0.025 M mercaptoethanol in the starting and limiting buffers.

RESULTS

Seven adult deer hemoglobins and two fetal hemoglobins have been identified by their differences in electrophoretic behavior, which can be demonstrated best in starch gel electrophoresis, pH 8.6, using a discontinuous buffer system. The electrophoretically distinguishable hemoglobins have been arbitrarily identified by Roman numerals according to their increasing mobility toward the anode during starch gel electrophoresis, pH 8.6. For example, I = slowest adult hemoglobin, VII = fastest adult hemo-
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globin, F₁ = slower fetal hemoglobin, and F₂ = faster fetal hemoglobin. Hemoglobin samples from nine different adult deer and one fetus are shown in Figure 1 and illustrate all the polymorphic hemoglobins so far identified. However, not all the naturally occurring mixtures of polymorphic hemoglobin* that have been observed in this study are demonstrated. A summary of the naturally occurring mixtures that have been demonstrated in penned and wild deer is given in Table 1. The frequency of occurrence of these polymorphic hemoglobins is listed, and the various hemoglobins have been associated with reversible changes in red blood cell morphology observed during light microscopy under controlled laboratory conditions.

The varieties of morphologic forms seen under in vitro laboratory screening conditions are the traditional crescent or holly leaf form, the matchstick form, the burr or star form, and the biconcave disc form (Fig. 1). The crescent or holly leaf form seen in most white-tailed deer is associated with hemoglobins I-III and hemoglobin III. In deer whose cells transformed to a crystalline "matchstick" appearance after sickling initially in the crescent form, hemoglobin II has been found singly or combined as hemoglobins I-II, I-II-III or II-IVs. In a smaller percentage of deer, the cells will not sickle, but instead become burr shaped. This aberrant form of red blood cells has been associated with hemoglobin IVs. Deer whose erythrocytes do not become sickle shaped or which do not show other morphologic aberrations under controlled laboratory conditions have hemoglobin mixtures III-V, II-V, III-VII or F₁, F₂. Table 1 summarizes the relationship of morphology to the various polymorphic hemoglobins. It is apparent that erythrocyte morphology can be related to specific hemoglobins. Three hemoglobins (I, II and III) have been associated with the sickling propensity of deer erythrocytes under in vitro conditions, and two hemoglobins (V and VII) have been found to preclude sickling. In total, seven adult deer hemoglobins have been identified with the number in a given animal varying from one to three. Seventy-nine per cent of the deer studied had more than one hemoglobin. Although a variety of combinations of the varying hemoglobins were demonstrated by electrophoretic studies, not all the possible combinations have been observed. Non-sickling and the occurrence of deer with only a single hemoglobin are rarely observed.

The variety of red blood cell forms seen initially stimulated further studies of the chemical and physical properties of several hemoglobins with very close electrophoretic behavior. Although a striking difference in electrophoretic mobilities readily identifies F₁, F₂, I, II, V, VII and IVs (Fig. 1), the close electrophoretic mobilities of II, III and IVs make it difficult to distinguish

*Because of the degree of hemoglobin polymorphism, the authors refer to "naturally occurring mixtures of hemoglobins" to identify the various combinations of hemoglobin present in an individual animal. Naturally occurring hemoglobin mixtures reflect the genetic hemoglobin phenotype of the individual animal. The term "hemoglobin" is used in reference to the occurrence of a single hemoglobin found in an animal, whereas "hemoglobins" or "hemoglobin mixture" then refers to the occurrence of more than a single hemoglobin in an individual animal.
Table 1.—Classification of Penned* White-Tailed Deer According to
Hemoglobin Type and Red Blood Cell Morphology

<table>
<thead>
<tr>
<th>Frequency Per Cent</th>
<th>Electrophoretic Hemoglobin Type Determined at pH 8.6 and pH 6.0</th>
<th>Red Blood Cell Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>I-III</td>
<td>Sickling, crescent, holly leaf</td>
</tr>
<tr>
<td>19</td>
<td>II-III</td>
<td>Matchstick</td>
</tr>
<tr>
<td>11</td>
<td>III</td>
<td>Sickling, crescent, holly leaf</td>
</tr>
<tr>
<td>6</td>
<td>III-V</td>
<td>Normal biconcave discoid shape; no sickling</td>
</tr>
<tr>
<td>6</td>
<td>IV_b</td>
<td>Burr, &quot;thorny apple&quot;</td>
</tr>
<tr>
<td>5</td>
<td>I-II-III</td>
<td>Matchstick</td>
</tr>
<tr>
<td>4</td>
<td>II</td>
<td>Matchstick</td>
</tr>
<tr>
<td>2</td>
<td>II-V</td>
<td>Normal biconcave discoid shape; no sickling</td>
</tr>
<tr>
<td>3</td>
<td>I-II</td>
<td>Matchstick</td>
</tr>
<tr>
<td>†</td>
<td>III-VII</td>
<td>Nonsickling</td>
</tr>
<tr>
<td>†</td>
<td>II-IV_b</td>
<td>Matchstick</td>
</tr>
</tbody>
</table>

*Over 150 penned deer studied.
†Only observed from a blood sample taken from a wild deer.

Panama white-tailed deer, *Odocoileus chiriquensis virginianus*, is a subspecies of the North American deer, *Odocoileus virginianus*, and is presented here because of the interesting finding of hemoglobin II, a hemoglobin common to the North American species. A second hemoglobin, IV_b, not found so far in North American white-tailed deer, was also found in this species.

these individual hemoglobins when studied by starch gel electrophoresis at pH 8.6. Evidence establishing a structural difference between these electrophoretically similar hemoglobins is essential in light of the unusual degree of hemoglobin polymorphism.

Chemical and physical differences can be demonstrated by a difference in behavior at pH 6.0 on agar gel electrophoresis or by a difference in the characteristics of the soluble tryptic peptides of subunits (α- and β-like chains) of the corresponding hemoglobin types.

As illustrated in Figure 2A, position 6, the broad hemoglobin band seen at pH 8.6 contains hemoglobin mixtures II-III. However, in agar electrophoresis at pH 6.0, the electrophoretic separation of these two hemoglobins is quite evident (Fig. 2B). Hemoglobin III and IV_b have identical electrophoretic behavior at pH 8.6 and at pH 6.0.

The remarkable heterogeneity of deer hemoglobins makes it imperative to ascertain which subunits are structurally different because amino acid substitutions in either the α or the β chains, or both, could account for polymorphic hemoglobins within this species. The electrophoretic separation of the dissociated α and β chains in alkaline urea starch gel, pH 8.1, is shown in Figure 3 to illustrate the electrophoretic behavior of the numerous polypeptide chains. Under these conditions, the α chain migrates toward the cathode (−), and the β or γ chains migrate toward the anode (+). Designation of α or β chains is dependent upon the comparison of peptide maps of the isolated polypeptide chains. That is, the characteristic peptide maps of the individual α or β chains are quite analogous to those for the respective α or β polypeptide chains of human hemoglobins.
Each of the α and β chains has been designated with an arabic number which corresponds directly to the Roman numeral designation for the hemoglobin type with which it was first associated. For example α3 was first associated with hemoglobin III, which is by far the most frequent hemoglobin type.

An α component (α3) is common to most deer hemoglobins. A second α (α1) of faster mobility is a component of hemoglobins F1 and I, but not of the other hemoglobins. A single γ chain is seen in fetal hemoglobins F1 and F2. The comparison of the electrophoretic migrations of the subunits of the isolated F1 and F2 was made to identify the number of α- and γ-like chains in these fetal hemoglobins (Table 2). Three β chains (βα, β1, and β4) with nearly identical electrophoretic mobility account for the structural differences between hemoglobins II, III, and IVα, respectively. A fast β chain component (β3) has been associated with hemoglobin types III-V and II-V. Of still faster mobility is the β chain associated with hemoglobin VII (β7). The presence of hemoglobin V or VII precludes the sickling of the erythrocytes. By referring
To Table 1, one can see that aberrant cell forms are found in all adult hemoglobins that do not have one of these two β chains (βS or βT); hence, the failure to sickle or to form matchsticks or burrs is associated with the β chain type or, in the case of the fetal hemoglobins, with the γ chain type.

A comparison of the one-dimensional electrophoresis of tryptic peptides of the isolated α and β chains has given further evidence for structural differences of all of the nine electrophoretically distinguishable hemoglobins. This comparison of the one-dimensional electrophoretic patterns of the α and β chains of hemoglobin II, III and IVα is particularly important because of their close electrophoretic mobilities. The α chains of the corresponding hemoglobin are identical when compared by one-dimensional electrophoresis of the tryptic digest of the respective α chains.

The digests of the respective β chains of deer hemoglobins II, III and IVα were run side by side at pH 6.4 on one-dimensional electrophoresis. Several separate applications of such samples were made so that strips made under identical conditions could be compared by several specific staining reactions. The results of the one-dimensional electrophoresis stained with ninhydrin are shown in Figure 4. Differences in the number and position of the ninhydrin positive spots are apparent; 15 spots are present in the β chain of hemoglobin III, while 18 are noted in the β chain of hemoglobin II and 16 in the β chain of hemoglobin IVα. Further differences in specific staining reactions, especially spots which are positive for histidine and tyrosine (Pauly) and negative for the tyrosine (α-nitroso-β-naphthol) reaction, are also shown in Figure 4 in schematic form. This one-dimensional technic combined with specific staining reactions illustrates that hemoglobins II, III and IVα, which have

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**Fig. 2.**—A: Vertical starch gel electrophoresis of deer hemoglobins at pH 8.6. B: Agar gel electrophoresis of deer hemoglobins in 1 per cent agar, pH 6.0. Hemoglobin samples in A are identical to those in B.
Fig. 3.—6 M urea starch gel electrophoresis, pH 8.1, demonstrating the electrophoretic migration of the various α, β- and γ-like polypeptide subunits of deer hemoglobins.

similar electrophoretic behavior at pH 8.6, have indeed structurally different β chains.

Conclusions

In this study of over 700 wild and penned Florida white-tailed deer, a variety of aberrant erythrocyte forms was noted and related to polymorphic hemoglobins. Seven different adult and two fetal hemoglobins have been found in white-tailed deer, the number in a given animal varying from one to three. Sickling is not caused by the presence of a particular hemoglobin common to all white-tailed deer whose erythrocytes exhibit this phenomenon, as is the case for sickle cell anemia in man. However, sickling does not occur if either one of two kinds of hemoglobin is present, either hemoglobin V or hemoglobin VII. Hemoglobin IV, not associated with sickling, is responsible for the burr shaped erythrocytes.

Indirect evidence indicates that hemoglobin heterogeneity is present in all erythrocytes of deer with polymorphic hemoglobins; that is, every cell from an individual deer contains all of the kinds of hemoglobin found in the blood of that deer. This conclusion is based on the observation that none of the erythrocytes sickle from deer that have either V or VII, either of which preclude sickling, even when these are present with hemoglobins which sickle when in other combinations. The conclusion that hemoglobin heterogeneity exists within each cell is in accord with the finding that in the human heterozygote both hemoglobins S and A are present in all cells.

Proof for two kinds of polypeptide structural subunits (α and β chains) was provided by the dissociation of deer hemoglobin in 6 M urea starch gel electrophoresis. This system also gave evidence for the existence of more than one kind of α chain and more than one kind of β chain among the
Table 2.—Polypeptide Chains* Present in the Electrophoretic Types of Deer Hemoglobin and Their Components

<table>
<thead>
<tr>
<th>Electrophoretic Hemoglobin Types</th>
<th>α₁</th>
<th>α₂</th>
<th>γ</th>
<th>β₁</th>
<th>β₂</th>
<th>β₃</th>
<th>β₄</th>
<th>Hemoglobin Components</th>
<th>α₁</th>
<th>α₂</th>
<th>γ</th>
<th>β₁</th>
<th>β₂</th>
<th>β₃</th>
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<tr>
<td>F₁,F₂</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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<td></td>
<td></td>
<td>F₁</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
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<td>a₁α₁γ₂</td>
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<tr>
<td>F₂</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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<td>F₂</td>
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<td></td>
<td></td>
<td></td>
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<td>I-II</td>
<td>+</td>
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<td>I</td>
<td>+</td>
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<td>a₁β₁β₂ or a₁β₁β₃</td>
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<td>IV₅</td>
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<td>III-V</td>
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<td>IV₆</td>
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<td>IV₇</td>
<td>+</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>a₇β₇β₇</td>
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*Polypeptide chains have been classified in accordance with electrophoretic mobility.
†In the case of hemoglobin I-II-III, both II and III occur as major components; and two minor hemoglobin components I, having nearly identical electrophoretic behavior, would be expected. Therefore, the subunit structure of hemoglobin I is dependent upon whether it occurs in combination with II or III as the major component. Proof of this is dependent upon the isolation of two I components with further structural studies.
polymorphic deer hemoglobins. Among the nine electrophoretically distinguishable deer hemoglobins, two different α chains, six distinct β chains and one γ chain have been identified. From the study of the results of 6 M urea starch gel electrophoresis and one-dimensional electrophoresis of the isolated α, β or γ chains, the types of α and β chains present in individual hemoglobins can be deduced; and thus the shorthand formulas can be written. Table 2 lists the kinds of α, β and γ chains found in each of the nine hemoglobins examined. Although not all of the possible tetrameric combinations of α and β chains or α and γ chains were found, the total number of hemoglobin polypeptide chains identified is unique and unexpected in light of the limited number of structurally different polypeptide chains which account for the multiple and polymorphic hemoglobins of animals thus far studied.

Differences in the morphology of erythrocytes associated with hemoglobins II, III and IV₄ suggested that these hemoglobins, whose α and β chains were virtually identical on 6 M urea starch gel electrophoresis, were structurally different. A side-by-side comparison of the one-dimensional electrophoretic patterns of the α and β chains of hemoglobins II, III and IV₄ confirmed that the structural differences occurred in the β chains, and that the α chains were identical. This method was also used in the laboratory to verify the structural differences and similarities of all the α, β and γ chains. A great number of differences in the number of ninhydrin positive spots and the reactions of these numerous spots to specific staining reaction for amino acids were noted. These findings suggest that a mere single amino acid substitution...
does not account for the remarkable degree of hemoglobin heterogeneity in white-tailed deer.

SUMMARY

A variety of aberrant erythrocyte forms have been related to seven adult and two fetal hemoglobins in white-tailed deer. While sickling of the erythrocyte was not associated with a single hemoglobin type, it was precluded by hemoglobin V or VII, even when in combination with other hemoglobin types normally associated with sickling. The subunit basis of the hemoglobin polymorphism was presented. Two kinds of α subunits, six kinds of β subunits and one γ subunit were related to the whole hemoglobin molecule. The heterogeneity of the deer hemoglobins was based upon a variety of combinations of these numerous polypeptide chains. It was concluded from the results of limited structural studies that there were multiple peptide differences upon comparison of three non-α polypeptide chains.

SUMMARIO IN INTERLINGUA

Un varietate de aberrante formas erythrocytic esseva relationate a septe hemoglobinas adulte et duo fetal in cervos albicaudate. Ben que le falciformation del erythrocytos non esseva associate con un tipo individual de hemoglobina, illo esseva excludite per hemoglobina V o hemoglobina VII, mesmo in combination con altere typos de hemoglobina normalmente associate con falciformation. Le base subunitari del polymorphismo de hemoglobina es presentate. Duo generes de subunitates α, sex generes de subunitates β, e un subunitate γ esseva relationate al molecula total de hemoglobina. Le heterogeneitate del hemoglobinas de cervo esseva basate in un varietate de combinationes de numerose catenas polypeptidic. Es concludite ab le resultatos del restringite studios structural que multiple differentias peptidic esseva presente, a judicar per un comparation de tres catenas polypeptidic non-α.

ACKNOWLEDGMENTS

The authors are indebted to Beatrice P. Godwin and Sarah L. Martin for technical assistance. We acknowledge the cooperation of the Agricultural Experiment Station, Department of Veterinary Science, University of Florida, in the establishment of deer pens. The collection of blood samples and animals over this 5-year period was made possible through the permission and cooperation of the Florida Game and Fresh Water Fish Commission. Many samples were possible through the cooperation of the Dallas, Texas; Bronx, New York; and Jacksonville, Florida zoos.

REFERENCES

6. Kitchen, H.: Handling and restraint in
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deer and other wild animals. Lab. Anim. Dig. 2:2-7, 1966.


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