The Sickling Phenomenon and Heterogeneity of Deer Hemoglobin.* (29556)

AUSTIN S. WEISBERGER
(With the technical assistance of Wallace E. Wendt and E. Caesar Moss)
School of Medicine, Western Reserve University, and Department of Medicine, University Hospitals, Cleveland

Deer are the only animal species in addition to man which are known to have hemoglobin which can exhibit the sickling phenomenon. The sickle cells of the deer look remarkably like those found in man(1-3) and sickle tactoids have been demonstrated in free hemoglobin solutions(4). Unlike sickling in man, non-sickling in deer is a distinct rarity(5) and sickling of erythrocytes can be demonstrated in the majority of deer. The absence of a disease state in deer associated with sickling may be correlated with the observations that deer cells can be made to sickle only when exposed to high oxygen tension in the presence of an alkaline pH and that the sickling is readily reversed by low oxygenation and by lowering the pH. The requirement of such conditions for sickling suggests that it would tend to occur in the arterial system and that the process might be reversed in the capillaries and veins, thereby avoiding the pathologic processes which lead to difficulty in the human counterpart.

In previous studies, only a single component has been demonstrable by paper electrophoresis in all preparations of deer hemoglobin. The present report deals with a study of sickle hemoglobin in a small captive deer population in which an unusual degree of heterogeneity of hemoglobin was found in the animals examined. Four major hemoglobin components were demonstrated, one of which could be identified as the hemoglobin responsible for sickling. Although heterogeneity of hemoglobin has been demonstrated in a variety of animals(6,7), to our knowledge such heterogeneity has not been previously reported in deer.

Materials and methods. Blood samples were obtained from 7 deer maintained in captivity at the Cleveland Zoological Gardens. Five of the deer were North American White Tail deer *(Odocoileus virginianus)*, one was a Barasingha deer *(Cervus duvauceli)* and one was a Sika deer *(Cervus nippon nippon)* (Table I). Two of the White Tail deer (Nos. 4 and 5) were twin offspring born in captivity; the remainder were captured deer. Heparinized blood was obtained from the jugular veins of the animals after temporarily paralyzing the deer with succinylcholine injected with an air gun. Sickling tests were performed on oxygenated whole blood buffered with 0.1 M sodium phosphate, pH 8.9.

Hemoglobin solutions were prepared from washed erythrocytes by freezing and thawing 3 times, and the stroma was separated by centrifugation at 30,000 $\times$ *g* for 30 minutes at 4°C. The hemoglobin solutions were converted to carboxyhemoglobin and stored at —65°C for subsequent analysis.

Electrophoretic analysis of hemoglobins. Electrophoretic analyses of hemoglobin were performed on both starch and acrylamide gels. Starch gel electrophoresis (horizontal) was performed on hydrolyzed starch (Connaught) at 4°C using a boric acid buffer system, pH 8.6 and staining with amido-Schwartz black(9). Electrophoresis on 7.5%

TABLE I.

No.	Species	Age	Sex	Sickling	Number of HGBN components: Gel Electrophoresis	Number of HGBN components: DEAE Cellulose
1	White Tail	2 yr	♀	+	1	1
2	" "	?	♂	+	2	2
3	" "	?	♂	+	3	4
4 *	" "	6 mo	♀	+	3	3
5 *	" "	6 "	♂	+	3	3
6	Barasingha	6 yr	♀	+	1	1
7	Sika	5 "	♂	+	1	1

* Deer Nos. 4 and 5 were twin offspring of deer Nos. 1 and 3.
† See Fig. 1 and 2.

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acrylamide gels was performed by a modification of Hermans *et al*(10), in a vertical position with 0.15 M Tris-EDTA-borate buffer,† pH 8.9 at 4°C for 4 hours using 250 volts and 160 milliamperes.

Column chromatography. Separation of hemoglobin components by DEAE-cellulose‡ column chromatography was carried out by the methods of Huisman and Dozy(11). Hemoglobins were dialyzed for 24 hours against 0.005 M sodium phosphate buffer, pH 8.6 containing 10 mg per cent KCN. Fifteen mg of the dialyzed hemoglobin were placed on a column (45 × 0.9 cm) and eluted at 20°C by a gradient of decreasing pH and increasing NaCl concentration (Fig. 2). A 10 chamber variable gradient apparatus§ containing 100 ml of phosphate buffer with decreasing pH and increasing concentrations of NaCl in each chamber was used for elution and the absorbence of the eluate at 415 mμ was continuously monitored and recorded by an automatic UV Analyzer.‖ Constant flow rates were maintained with a micropump.¶

Peptide fingerprint analysis. Peptide analysis of the hemoglobins were carried out by the methods of Ingram(12) as modified by Baglioni(13) and as previously described(14). Analyses were performed on the whole hemoglobin, no attempt being made to determine the peptide fingerprints of the individual components. Ascending chromatography was carried out on all samples simultaneously after initial electrophoretic separation of the tryptic digests in pairs.

Results. Sickling of erythrocytes could be induced in all the animals examined by using oxygenated blood in the presence of 0.1 M sodium phosphate buffer, pH 8.9. As found by others, the sickling could be prevented or reverted by lowering the pH or by sealing the preparation so that oxygen tension was reduced.

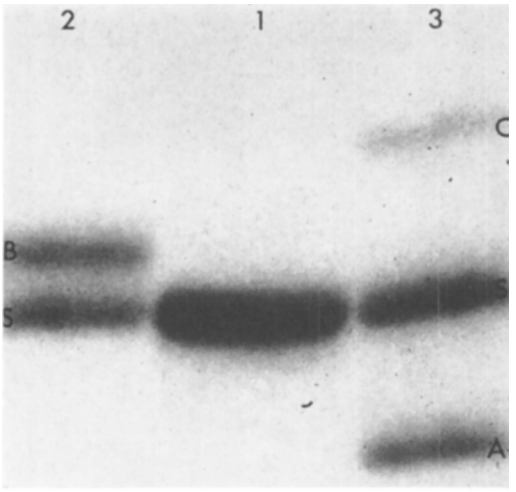

FIG. 1. Acrylamide gel electrophoresis of hemoglobin obtained from a different North American White Tail deer. The single component (S) present in deer No. 1 was demonstrable in all deer examined and was correlated with the presence of sickling in all animals examined. Three additional hemoglobin components (A, B and C) were present in these 3 deer.

Four separate hemoglobin components (A, B, C, S) were demonstrable by gel electrophoresis in the 5 White Tail deer studied (Table I, Fig. 1 and 2). The number of hemoglobin components demonstrable by column chromatography was in agreement with those found by gel electrophoresis except for deer No. 3 in which 3 components were found by electrophoresis and 4 components by column chromatography. Only a single hemoglobin component (S) was present in one of the White Tail deer as well as in the Barasingha and Sika deer. Accordingly, this hemoglobin component (S) was identified as that responsible for the sickling phenomenon. A component with the same electrophoretic mobility and with the same elution characteristics on column chromatography was identifiable in all of the deer examined.

The twin White Tail deer (Table I, Nos. 4 and 5) were offspring of parents with one hemoglobin component (S, Fig. 2, No. 1) and with 4 hemoglobin components (A, B, C, and S, Fig. 2, No. 3) respectively. Each of the twin deer had identical hemoglobins on electrophoretic and chromatographic

† 15.1 g 2-amino-2(hydroxymethyl) 1-3 propanediol, 1.95 g disodium ethylenediaminetetraacetate and 1.15 g of boric acid per liter.

‡ Diethylaminoethyl-Cellulose, Selectacel, Type 40, Schleicher & Schnell Co., Keene, N. H.

§ Block Autograd, Technicon Chromatography Corp., Chauncy, N. Y.

‖ Vanguard Model 1056, Vanguard Instrument Co., LaGrange, Ill.

¶ Buchler Instruments, Inc., Fort Lee, N. J.

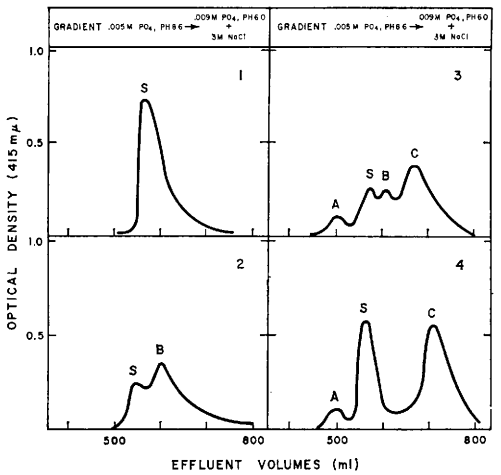

FIG. 2. Separation of hemoglobin components obtained from 4 different North American White Tail deer by DEAE cellulose column chromatography. Elution was carried out by establishing a gradient with decreased pH and increasing NaCl concentration using a 10 chamber variable gradient apparatus. Each chamber contained 100 ml of buffer as follows:

Chamber	PO_4 buffer	NaCl	pH
1	.005 M	.000	8.6
2	.009 M	.02 M	8.4
3	.009 M	.04 M	8.1
4	.009 M	.06 M	7.9
5	.009 M	.08 M	7.7
6	.009 M	.10 M	7.5
7	.009 M	.15 M	7.1
8	.009 M	.20 M	6.8
9	.009 M	.25 M	6.5
10	.009 M	.30 M	6.0

Four separate major hemoglobin components (A, B, C and S) were present in the 4 North American White Tail deer examined. Deer No. 4 was the offspring of deer Nos. 1 and 3 and inherited 3 of the 4 hemoglobin components present in the parents. The S component was present in all deer examined and could be correlated with the presence of sickling.

analyses consisting of 3 of the 4 hemoglobin components present in the parents (A, C & S, Fig. 2, No. 4).

Peptide fingerprint analyses of hemoglobin were performed on 3 of the White Tail deer. These deer had hemoglobins containing 1, 2 and 4 hemoglobin species respectively by column chromatography (Nos. 1, 2 and 3, Table I and Fig. 2). Distinctive differences in the peptide content of these hemoglobins were readily discernible. Variations in the number of peptides as well as in the position of the peptides were demonstrable (Fig. 3).

Peptide fingerprint analyses of Sika and Barasingha deer hemoglobin were also distinctly different from each other (Fig. 4) and could be readily distinguished from the fingerprints of White Tail deer. The differences were apparent even though the hemoglobin of the Barasingha and Sika deer as well as that of one of the White Tail deer contained a single hemoglobin component with similar electrophoretic and column chromatographic behavior.

Discussion. The number of components demonstrable in different hemoglobins varies with the techniques employed. Although only a single component has previously been described in deer hemoglobin when analyzed by paper electrophoresis, the techniques employed in the present study are known to be more sensitive than paper electrophoresis in demonstrating the presence of hemoglobin components. True heterogeneity must be distinguished from artifacts such as may occur with denaturation or modification during preparation and storage of hemoglobin samples. The demonstration of differences in peptide content by fingerprint analysis indicates that the heterogeneity observed is attributable to differences in globin synthesis and presumably amino acid sequences rather than to an induced heterogeneity during preparation of the hemoglobin samples. Although the mobility of the hemoglobin component responsible for sickling could be ascertained, the peptide (or peptides) controlling sickling was not identified in the present study.

There have been numerous reports on the presence of 2 or more hemoglobin components in different species of animals(5,6). In most instances all members of a species possess the several hemoglobin components and the animals are homozygous for the genes involved. When there is a variation in the number of hemoglobin phenotypes within a species, it is probable that a number of genetic loci on allelic genes are involved in the synthesis of different polypeptide chains. Such heterogeneity of hemoglobin within a species is uncommon and has previously been recorded only in man, monkeys, mice, cattle, sheep and goats(5,15). The degree of heterogeneity found in deer hemoglobin suggests

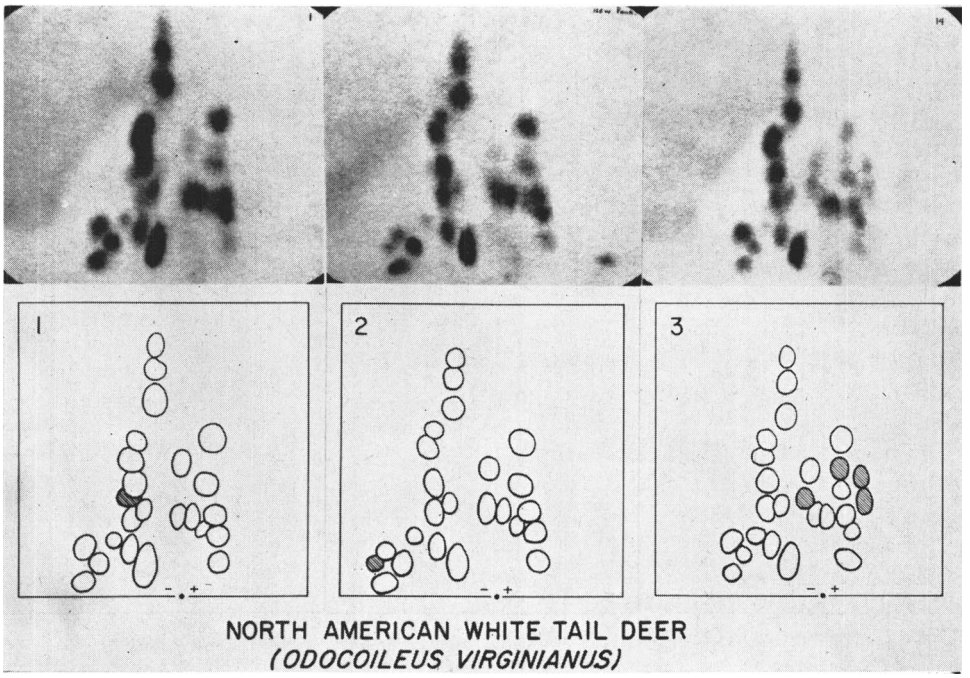

FIG. 3. Peptide fingerprints of hemoglobin obtained from 3 different North American White Tail deer. The peptides present in hemoglobin obtained from different animals were first separated by electrophoresis and then by ascending column chromatography. The differences in peptide content are apparent and are further demonstrated by cross-hatching in the tracings. These deer had hemoglobins with either 1, 2 or 3 different major components by electrophoresis (Table I).

that an unusual number of genetic loci may be involved in the synthesis of hemoglobin. The present studies do not, however, indicate the number of polypeptide chains involved since common subunits may be involved in different combinations to form the various hemoglobins.

Sickle cell disease in humans is of particular interest because the abnormal hemoglobins are the only system in human biochemical genetics where it can be shown that mutations produce chemical alterations in protein molecules. In many respects the hemoglobin components of the deer are similar to the inherited abnormalities of human hemoglobins and offer phenotypic "markers" which can be used for studies not possible in humans. A genetic basis for heterogeneity in the deer is indicated by segregation of some of the controlling factors in the twin offspring which inherited 3 of the 4 hemoglobin components present in their parents.

The biologic advantage conferred upon deer by having such unusual heterogeneity

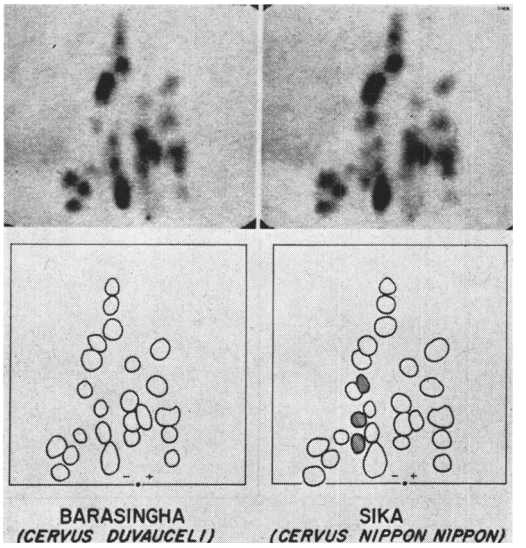

FIG. 4. Peptide fingerprints of hemoglobin obtained from Barasingha and Sika deer. The hemoglobin obtained from these deer contained a single major hemoglobin component both by gel electrophoresis and by column chromatography (Table I). However, peptide fingerprints of the 2 hemoglobins differ with each other and with those obtained from the North American White Tail deer with a single hemoglobin component.

of hemoglobin as well as sickling hemoglobin is not readily apparent. Neel(8) has suggested that persistence of genes in the course of evolutionary development should result from some positive contribution to fitness. Perutz *et al*(16), in discussing the biologic advantage of heterogeneity of hemoglobin in horses, has pointed out that the solubility of oxyhemoglobin in the horse is low and that by the phase rule, a saturated solution of 2 fractions should contain more protein than a saturated solution of either. The coexistence of various hemoglobin components in horses might therefore permit a higher concentration of hemoglobin without crystallization within the cell. The mean corpuscular hemoglobin concentration in all the deer examined was 36%. Since the blood values in deer with single hemoglobin components were essentially the same as those with several hemoglobin components, it seems unlikely that heterogeneity is necessary to prevent crystallization of hemoglobin within deer erythrocytes.

Summary. A marked degree of heterogeneity is demonstrable in deer hemoglobin. This heterogeneity appears to be on a genetic basis. A single hemoglobin component responsible for sickling of deer hemoglobin can be identified.

Addendum. Since this paper was prepared, similar electrophoretic results were reported by Kitchen, H., Putnam, F. W. and Taylor, W. J., in *Science,* 1964, v144, 1237.

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On the Anti-Inflammatory Activity of Aminophylline. (29557)

GORDON R. MCKINNEY AND PAUL M. LISH
Mead Johnson Research Center, Evansville, Ind.

Aminophylline, theophylline ethylene diamine, is one of the most effective bronchodilating agents available and it plays a very pertinent role in the management of the asthmatic patient(1). On theoretical grounds it would appear desirable that a bronchodilator possess, in addition to its smooth muscle-relaxing-property, a degree of anti-inflammatory or antiedemic activity to enable it to reduce airway obstruction due to engorged or swollen epithelial linings. In testing aminophylline in several laboratory models of inflammation, or local edema, it has become apparent that this xanthine derivative does indeed possess a significant degree of inhibitory activity in several models of increased capillary permeability.

Methods. Rat. Wistar rats of either sex, weighing 140-160 g each, were employed in these models of pedal edema which was measured plethysmographically by mercury displacement as described previously(2). Rats