Table 1. Identification of 10935-1D virus by neutralizations tests.

Mouse serum, final dilution 1:2	10935-1D virus	
	Titer*	N.I.†
Normal	5.6*	
Dengue-1 immune	2.4	3.2
Dengue-2 immune	1.3	4.3
Dengue-4 immune	3.3	2.3

* The LD_{50}/0.02 ml (lethal dose, 50 percent effective) is expressed as reciprocal in $\log_{10}$ of virus titer. † N.I., neutralization index; the difference in reciprocal log units.

inoculated were signs of illness detected. Two that showed abnormality (slight ataxia, slight ruffling of hair, nose pawing, and slow circling movements) 11 and 13 days after inoculation were killed, and a suspension of brain from each was passed into two new litters. The second-passage mice likewise showed signs typical of dengue infection by day 10; some were paralyzed by day 11, and some were dead by day 16. At the time of the tenth mouse passage (the incubation period was then 5 days and the mortality was 100 percent by the 9th day after inoculation), a crude brain preparation was used as antigen in the complement-fixation test (*4*), and was identified as a strain of dengue type 2 virus. This strain was designated 10935-1D.

Application of the reverse experimental procedures to specimen 10939-1 taken from the patient in the acute phase of the disease resulted only in reisolation of dengue 2 virus, even though we attempted to isolate chikungunya virus. No evidence of dual infection was detected in the inoculated mice.

Table 2. Demonstration of neutralizing antibody to dengue type 2 and chikungunya viruses in three samples of patient's serum (10935-1, 23 October 1964; 10935-2, 4 November 1964; 10935-3, 12 November 1965).

Serum diluted 1:4	Dengue type 2 virus		Chikungunya virus	
	Titer*	N.I.†	Titer*	N.I.†
	Mouse			
Normal	5.3		6.9	
Dengue-2, immune	1.3	4.0		
Chikungunya, immune			<3.0	>3.9
	Patient			
10935-1	4.3	1.0	7.0	Nil
10935-2	<1.0	>4.3	3.7	3.2
10935-3	2.5	2.8	4.2	2.7

* The LD_{50}/0.02 ml (lethal dose, 50 percent effective) is expressed as reciprocal in $\log_{10}$ of virus titer. † N.I., neutralization index; the difference in reciprocal log units.

Evidently the chikungunya virus component of the 10935-1 serum specimen was neutralized by the addition of mouse antiserum to that virus (CMS), and the dengue virus component was thus able to express itself. The results of neutralization tests with 14th mouse-passage 10935-1D virus confirmed identification of this agent as a strain of dengue type 2 virus (Table 1). The tests were done in infant mice inoculated intracerebrally; virus was used in serial tenfold dilutions, with constant dilutions of mouse antiserums for dengue virus types 1, 2, and 4 (Vellore strains 82-1, 60-1, and 968-1R).

In other neutralization tests, serums from patient 10935 in the convalescent and postconvalescent state showed significant increase in antibody to both dengue 2 and chikungunya viruses as compared with serum obtained during the acute phase of the disease (Table 2). The amount of antibody to dengue 2 virus was determined in tests done in mice, with incorporation of CMS to neutralize the chikungunya virus present. The reverse procedure was used for determination of amount of antibody to chikungunya virus.

The isolation of two antigenically unrelated arboviruses from a single serum specimen from a patient in the acute phase of the disease supports the serologic evidence for simultaneous arbovirus infections encountered at Vellore and elsewhere. The unremarkable nature of this patient's illness and the normal development of antibody indicate that the host response was not appreciably altered by the dual infection. Whether or not man could support dual infection with dengue viruses of differing yet closely related antigenic types is not known. Presumably the viruses would compete for the same sites of replication. Though dengue and chikungunya illnesses may be characterized by signs and symptoms that are similar in many cases, the marked involvement of the joints in chikungunya, with prolonged arthralgia that may persist for several months, seems to be a differentiating feature (*3*). Further study may provide evidence of differing tropisms for chikungunya and the dengue viruses.

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3 July 1967

Oxygenation Properties of Snake Hemoglobin

Abstract. Natrix taxispilota *hemoglobin has a very high oxygen affinity which depends upon* pH *in an unusual manner. The oxygen affinity increases slightly upon protein dilution, but the* pK's *of the Bohr groups are unchanged. Oxidation promotes hemoglobin polymerization, which can be inhibited by prior treatment with iodoacetamide. Reaction with iodoacetamide also causes a slight increase in the oxygen affinity, no change in the* pK's *of the Bohr groups, and a drastic reduction in heme-heme interaction.*

Largely as a result of the work of Antonini, Wyman, and their co-workers, the oxygenation properties of human hemoglobin are becoming well defined (*1*). However, the oxygenation properties of few other vertebrate hemoglobins have been described. The available data support the idea that the underlying mechanisms controlling the oxygenation reaction of all vertebrate hemoglobins are the same (*2*). Because hemoglobin is thought to be closely adapted to the respiratory needs of each species, it is possible that broad correlations in both structure and function can be made for major taxonomic groups.

The oxygenation properties of hemoglobin from the water snake *Natrix taxispilota* are of interest for several reasons. Water snakes have habits much like those of aquatic turtles, and their hemoglobins may be similarly adapted (*3*). Essentially no data are available on the oxygenation properties of snake hemoglobins (*4*). Chiancone *et al.* (*5*) have reported that hemoglobins from lower vertebrates dissociate less readily

than those of higher vertebrates do. Since the dissociation of hemoglobin into subunits is thought to function in the mechanism of oxygenation (*6*), a comparison of the effect of dilution on the oxygenation properties of a variety of hemoglobins is of interest.

Blood from snakes (*Natrix taxispilota*) captured near Lake Waccamaw, Columbus County, North Carolina (*7*) was centrifuged, and the serum was removed. The cells were washed three times with cold 0.8 percent NaCl. Cells were lysed with cold glass-distilled water, and the stroma was removed by centrifugation. The resulting hemoglobin solutions were dialyzed for 24 hours against 0.2*M* NaCl and then 0.01*M* PO_4 buffers (*p*H 8.0). All buffer solutions were saturated with N_2. Oxygen equilibria were performed spectrophotometrically and calculated according to Wyman (*8*). Techniques of Sephadex chromatography (*9*), alkylation (*10*), and starch-gel electrophoresis (*11*) are described elsewhere. Methemoglobin concentrations were measured before and after all measurements of oxygen equilibrium (*12*).

The oxygenation properties of *Natrix taxispilota* hemoglobin are shown in Fig. 1. This hemoglobin is characterized by a very high oxygen affinity and unusual *p*H symmetry. The acid Bohr effect (*p*H dependence of the oxygen affinity) is larger than the alkaline Bohr effect. Compared to human hemoglobin

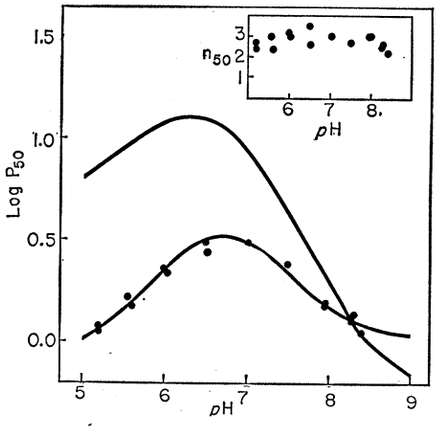

Fig. 1. Oxygenation properties of snake (*Natrix taxispilota*) hemoglobin (0.15 percent) at 20°C in 0.1*M* phosphate buffers. The logarithm of the value of half saturation (P_{50}) is plotted against the *p*H. The points are experimental; the smooth curve is theoretical. The theoretical curve for human hemoglobin under the same conditions has been included (*13*). Insert shows the heme-heme interaction values at 50 percent saturation as a function of *p*H.

Table 1. The *pK*'s and heats of oxygenation of snake (*Natrix taxispilota*) and human hemoglobins (*13*) at 20°C in 0.1*M* phosphate buffers (0.15 percent Hb) calculated from $\log P_{50} = C + \log \frac{(H^+ + K_1')\ (H^+ + K_2')}{(H^+ + K_1)\ (H^+ + K_2)}$, where K_1' and K_2' refer to deoxyhemoglobin and K_1 and K_2 refer to oxyhemoglobin, and C is a constant. This model assumes that two noninteracting ionizing groups are involved in the Bohr effect and that one becomes a weaker, and the other a stronger, acid upon oxygenation (*8*).

Hemoglobin	C	pK_2'	pK_2	pK_1'	pK_1	ΔpK_{acid}	$\Delta pK_{alk.}$	ΔH (kcal)
Human	.654	5.21	5.86	8.47	6.90	.65	1.57	−13.6
Snake	−.089	5.48	6.35	7.82	7.05	.88	.77	−15.5
Snake + iodoacetamide	−.217	5.48	6.35	7.82	7.05	.88	.77	

(*13*), the *pK* shifts of the snake hemoglobin are reduced in magnitude, and the *pK*'s are shifted toward neutral *p*H (Table 1). Heme-heme interaction values (Fig. 1) are very similar to those of human hemoglobin and unlike those of turtle hemoglobins, which are dependent on *p*H and perhaps on temperature (*3*).

The primary effect of dilution is to increase the oxygen affinity (Fig. 2). The magnitude of the increase is greater than that of human hemoglobin, but less than that of cat hemoglobin (*13*). Unlike cat hemoglobin there are no changes in the *pK* values, which would have indicated that the subunit dissociation perturbed ionized groups important in the oxygenation reaction.

Hemoglobins of various species are known to polymerize (*10, 14*). Although Svedberg (*15*) described the species distribution of many of these hemoglobin polymers, he reported polymers in only a single snake species. In turtles polymerization can occur in vivo (*16*) although it normally occurs in vitro after hemolysis (*10*). The addition of sulfhydryl reagents at the time of hemolysis prevents polymerization. In Fig. 3 are shown the chromatographic patterns of *Natrix taxispilota* oxyhemoglobin, methemoglobin, and methemoglobin which had been alkylated with iodoacetamide prior to its oxidation. No polymer was observed in the oxyhemoglobin sample. Upon oxidation at *p*H 8.00 approximately 26 percent of the hemoglobin polymerized. Alkylation of available sulfhydryl groups with iodoacetamide completely inhibited polymerization.

The oxygen affinity of *Natrix taxispilota* hemoglobin alkylated with iodoacetamide is increased (Fig. 4), but the *pK*'s are changed slightly if at all (Table 1). Human hemoglobin shows an increased oxygen affinity and change in the *pK*'s (*17*). Hemoglobin from at least one species of turtle whose hemoglobin polymerizes is unchanged when the unpolymerized form is reacted with iodoacetamide (*18*). Heme-heme interactions are unchanged in human and turtle hemoglobins, but snake hemoglobin shows very little heme-heme interaction after reaction with iodoacetamide. Although the methemoglobin concentration was slightly greater after alkylation, it still amounted to no more than 3 percent and could not account for these results.

Electrophoresis at *p*H 8.9 revealed at least one major and one minor hemoglobin in *Natrix taxispilota* hemolysates. The isoelectric point is high; hemoglobin moves only slightly from the point of insertion after 3 hours of electrophoresis at 25°C and 400 volts.

The sequences of amino acids in the alpha and beta chains of vertebrate hemoglobins are extremely variable (*19*). Few residues appear to be invariant. This implies that only a few regions of the molecule are critical for preservation of the basic functional

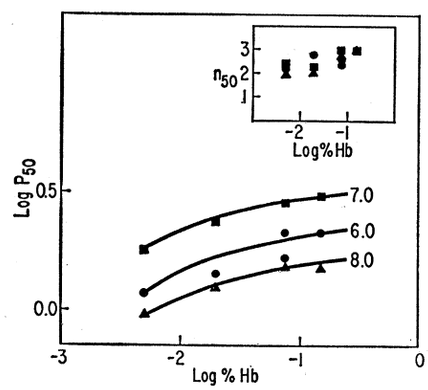

Fig. 2. The effect of dilution on the oxygenation properties of snake (*Natrix taxispilota*) hemoglobin at *p*H 6.0 (●), 7.0 (■), and 8.0 (▲). Heme-heme interaction values at 50 percent saturation as a function of protein concentration are shown in the insert.

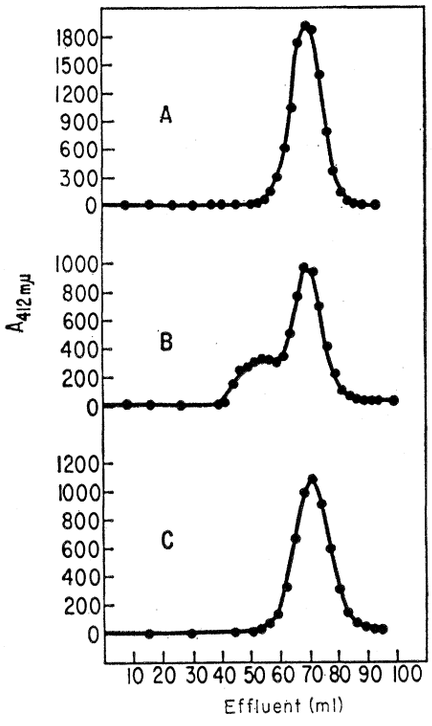

Fig. 3. Elution profiles of snake (*Natrix taxispilota*) hemoglobin applied to a Sephadex G-100 column (2 cm by 44 cm). (A) Oxyhemoglobin, (B) methemoglobin, and (C) methemoglobin which had been alkylated before its oxidation.

properties. Clearly, in this respect hemoglobin appears to differ from cytochrome *c*, which contains a greater percentage of invariant residues (*20*). I have recently proposed from a study of the structural and functional properties of primate hemoglobins that one of the key architectural features of the hemoglobin molecule is that it allows a wide degree of variation in functional properties (*21*). The data suggest that a large number of amino acid residues are able to influence the oxygenation properties. These residues act as modifiers of the physical properties of certain key residues. These key residues include those contributing the Bohr protons.

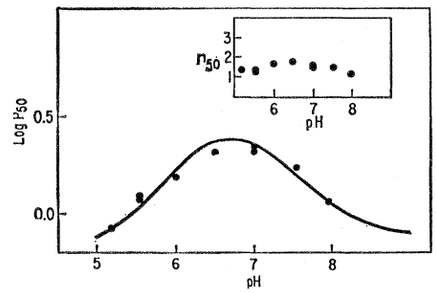

Fig. 4. Oxygenation properties of iodoacetamide-reacted snake (*Natrix taxispilota*) hemoglobin (0.15 percent) at 20°C in 0.1*M* phosphate buffers. The logarithm of the half saturation value (P_{50}) is plotted against the *p*H. The points are experimental; the smooth curve is theoretical. Insert shows the heme-heme interaction values at 50 percent saturation as a function of *p*H.

In its *pK* values and oxygen affinities, *Natrix taxispilota* hemoglobin is one of the most unusual described. Most hemoglobins have properties more similar to those of human hemoglobin (*2, 22*). Nevertheless, there is not sufficient evidence from the data to indicate any changes in the basic oxygenation properties such as a change in the Bohr residues. Other data (*23*) indicate that these peculiarities are probably characteristic of hemoglobins from many snake species. Snake hemoglobins appear to share characteristic functional properties which undoubtedly reflect characteristic structural features. Although water snakes and aquatic turtles appear to occupy similar habitats, the oxygenation properties of their hemoglobins do not appear to be very similarly adapted.

The supposition that many residue positions are able to modify the basic oxygenation properties helps to explain the wide variation in amino acid sequence and oxygenation properties encountered in vertebrate hemoglobins. Such variation naturally leads to a variety of derived properties (for example, salt effects, oxidation rates, polymerization, and alkylation effects). Unusual variation in some of these properties is characteristic of *Natrix taxispilota* hemoglobin. The sensitivity of key functional residues to a large number of modifying residue substitutions is good evidence for the strong selective value of a large portion of the entire molecule. Thus the selective value of an enzyme molecule is attributable not only to its catalytic capacity, but also to the flexibility (or inflexibility) of this capacity. Selection for flexibility (or inflexibility) may be an important force governing the size and shape of enzyme molecules. It may follow that smaller enzyme molecules should show less variation in functional properties (from species to species), and this can be supported to some extent. Unfortunately, the effects of subunit structure make it difficult to suggest exclusive rules. Until data on the structure and function of hemoglobins and other proteins from additional species become available, it is unlikely that we will understand clearly the intertwined evolution of structure and function under the selective pressure of adaptation.

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