typing phages is a relatively stable characteristic. It would also seem that white, phage insensitive strains are relatively unstable and tend to lose the ability to produce coagulase.

No attempt was made to correlate phage sensitivity with potential pathogenicity, although from the relationship to pigmentation and coagulase production some degree of association does exist. Further evaluation would be required before phage sensitivity could replace the commonly accepted criteria for pathogenicity.

A phage sensitivity test, employing a drop of pooled undiluted phages would be a simple, economical and expedient procedure for routine testing of staphylococci, but at present seems limited to use as an adjunct to phage typing procedures. By itself it may serve as an index of typability since all typable strains were sensitive to the pooled phage with but one exception (lytic pattern 52 44A 81). The combination of phages in the pool was selected empirically; other combinations may serve equally well.

Summary. 1) A high correlation was observed between pigmentation, bound coagulase and sensitivity to typing phages in staphylococcal strains freshly isolated from routine clinical hospital specimens. 2) A simple method for determining phage sensitivity using a pool of 3 undiluted typing phages is described and possible applications are discussed.

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Isolation and Characterization of Two Hemoglobins Found in the Turtle, *Pseudemys scripta elegans.* (23579)

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Evidence has accumulated which indicates that a correlation exists between the taxonomic relationships of animals and the chemical and physiological properties of their hemoglobins(1). With such evidence as a background, an attempt was made to correlate paper electrophoretic migration of hemoglobins with phylogeny in Amphibia and Reptilia(2). Of the reptilian hemoglobins studied those of turtles were found to be especially interesting. Hemolysates of turtles included in families Chelydridae and Kinosternidae have one hemoglobin; those included in Emydidae, Trionychidae and Testudinidae have at least 2 hemoglobins. When the 2 hemoglobins of such turtles were fractionated by means of paper electrophoresis a trail of pigment marked the area on the paper strip between the slow and fast migrating hemoglobins. This trailing suggested that an interaction was occurring between the 2 hemoglobins. Because of these interesting properties, we decided to investigate somewhat more thoroughly the hemoglobins of one of the species in which we found 2 hemoglobins, the red eared slider turtle, *Pseudemys scripta elegans.*

Materials and methods. Turtles used in this study were captured in the New Orleans area. In captivity they were kept in con-

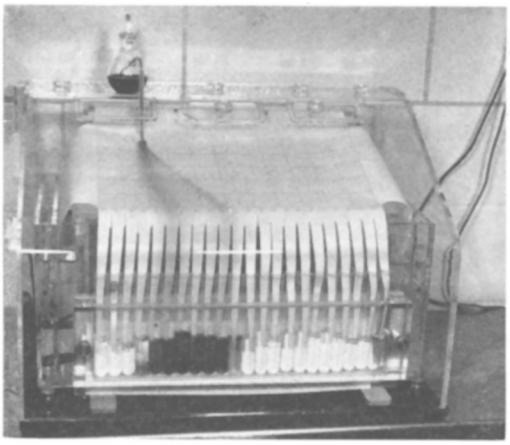

FIG. 1. Continuous flow electrophoresis. Band to right is fast Hb; band to left is slow Hb.

crete tanks located in a constant temperature room (26 to 29°C). Both a pool of water and a dry area were provided in the tanks, which were irradiated periodically with ultraviolet light. Turtles were fed rabbit and rat meat. Blood was obtained by cardiac puncture of unanesthetized turtles. The animals were turned ventral side up and a ¼ inch hole was drilled at the junction of the pectoral and abdominal laminae at the midline of the plastron. Care was taken to break through only the epidermis and bony shell so as not to damage any internal structures. A 20 gauge needle, 2½ inches long, was used to penetrate the heart. Ten to 20 ml of blood were drawn. Blood was mixed with 2 or 3 drops of a 1% heparin solution to prevent clotting. The plastral hole was closed with wax and the animal returned to the tank. Blood was processed in a refrigerated centrifuge (3 to 5°C). After centrifugation plasma was removed and erythrocytes were washed 3 times with 0.9% NaCl and hemolyzed in approximately 3 volumes of distilled water, added in a number of aliquots. The hemoglobin solution obtained was used in subsequent preparative procedures. This solution contained from 5 to 7% hemoglobin and henceforth will be designated as the hemolysate. Attempts were made to fractionate the hemolysate into its constituent hemoglobins by classical technics of salting out with ammonium sulfate or with phosphate buffers(3). These attempts were unsuccessful. Isolations were successfully carried out with a Karler-Misco* continuous-flow electrophoretic unit in either borate or barbital buffers of ionic strength 0.01 or 0.05. Separation was less successful in phosphate buffer of pH 7.4 and ionic strength 0.05. Fig. 1 demonstrates the appearance of the curtain during fractionation. The band farthest to the right (anode) is due to a hemoglobin which migrates very rapidly in a buffer of pH 8.6. For the purposes of description this hemoglobin will be designated subsequently as Fast Hb. The other component will be designated as Slow Hb. Since relatively long periods of time were required to isolate and characterize these hemoglobins precautions were observed to prevent protein denaturation. Continuous flow electrophoresis was performed in a cold room maintained at 3 to 5°C. In early preparations hemoglobins were handled as their oxygenated derivatives. Later the carbon monoxide derivative was formed before fractionation as a further precaution against denaturation or oxidation to methemoglobin. Analytical paper *electrophoresis* was carried out on an LKB unit.† A diethylbarbiturate buffer, pH 8.6, ionic strength 0.05, containing 15% glycerol by volume, was used. Runs were carried out for 17 hours at room temperature (26 to 28°C), 170 v, 4 ma. In some instances, paper strips obtained in the electrophoresis of hemolysates were stained with bromophenol blue and were scanned at 590 mμ in a Beckman DU spectrophotometer. Graphs of optical densities against distance migrated were plotted. The relative concentrations of the 2 hemoglobins of the hemolysate were estimated from the area beneath the resulting curves. Fast Hb approximated ⅓ of the total hemoglobin of the hemolysate. Moving boundary electrophoresis of dialyzed hemoglobins was carried out in a Perkin-Elmer, Model 38, Apparatus‡ operating at approximately 100 v. Specific conductance of buffered protein solutions was calculated from measurements of their specific resistance with a Leeds and Northrup bridge. Oxygen

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† Ivan Sorvall Inc., Norwalk, Conn.

‡ The Perkin-Elmer Corp., Norwalk, Conn.

TABLE I. Properties of *Pseudemys* Hemoglobins.

	Fe:O_2	Absorption maxima (mμ)						pI*	Alk. denat. (min.)†	Sed. const. (Svedbergs)
	HbO_2	Hb	HbO_2		HbCO			HbCO	HbO_2	HbCO
Fast Hb	1:1	555	576	541	568	538		5.7	4	4.6
Slow Hb	"	"	"	"	"	"		7.2	120	9.0
Hb A	"	"	"	"	"	537		6.8(3)	3	4.3 to 5.3(3)

* Isoelectric pH. † Time necessary for 50% denaturation.

capacity was measured either with a Van Slyke manometric apparatus(4) or with a Scholander syringe pipette(5). The method of Drabkin(6), slightly modified, was used to measure iron content. Sedimentation rates were determined in a Spinco Model E ultracentrifuge, accelerating at about 175,000 g (rotor temperature 23°C). To determine the alkali stability of turtle hemoglobins, an oxyhemoglobin solution of known concentration was added to a large volume of 0.1 N NaOH. The resulting solution had a pH of about 13. The rate of disappearance of the absorption band of oxyhemoglobin in the region of 540 mμ was followed in a Klett-Summerson colorimeter. *Absorption spectra* were determined with a Beckman DU spectrophotometer. Positions of absorption bands and observations of band shifts were observed with a Bausch and Lomb hand spectroscope. All absorption spectra were determined at room temperature (26 to 28°C) on solutions of the pigments in diethylbarbiturate buffer of pH 8.6, and ionic strength 0.05. Reduced hemoglobin was prepared by adding a trace of dithionite to oxyhemoglobin. Carbon monoxide hemoglobin was prepared by passing washed CO over a thin layer of hemoglobin solution. In all absorption spectra studies, solutions of human Hb A or its derivatives, dissolved in the diethylbarbiturate buffer, were used as reference standards. With the exception of studies on CO derivatives, all preparations of Hb A were purified previously by means of continuous flow electrophoresis. A simple hemolysate of human erythrocytes acted as the reference standard in CO hemoglobin studies.

Results. Preparations of Fast Hb and Slow Hb were routinely tested for homogeneity by means of paper electrophoresis. Only hemoglobins which migrated as single, sharp zones were considered to be homogeneous. Most such preparations also migrated as sharp boundaries in the moving boundary apparatus. Although electrophoretic criteria were used routinely to establish homogeneity of preparations, hemoglobins behaved as homogenous compounds in the ultracentrifuge. Constants which characterize these hemoglobins are assembled in Table I. Both turtle hemoglobins were found to be active oxygen carriers. Oxygen could be removed from both Slow HbO_2 and Fast HbO_2 either by adding traces of sodium dithionite or by evacuation. Deoxygenation of the oxyhemoglobins was accompanied by a shift in their absorption bands in the visible to the single band characteristic of reduced Hb. The absorption band of the reduced hemoglobins was replaced by the two bands of oxyhemoglobin upon reequilibration of the hemoglobins with oxygen. Absorption spectra between 460 and 680 mμ of Slow and Fast Hbs and their oxygenated derivatives were identical to the spectra of Hb A and HbO_2 A, within the resolution of the Beckman DU spectrophotometer. The specific extinction of these oxy and reduced hemoglobins, calculated at wave lengths of maximum and minimum absorption, were within 5% of those of Hb A. The agreement between the extinction coefficients was even closer for both preparations of Slow Hb and for one of the 2 preparations of Fast Hb on which absorption spectra measurements were carried out. The relatively low specific extinction of the first preparation of Fast Hb was interpreted as indicating that an oxidation of a fraction of that preparation to alkaline methemoglobin had occurred. In subsequent fractionations, the hemolysate was handled in the form of CO derivative to minimize oxidation. Positions of maxima in the visible region of

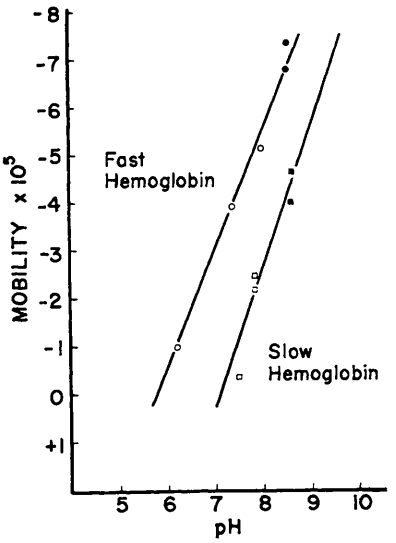

FIG. 2. Electrophoretic mobility of hemoglobins of *Pseudemys scripta elegans* in 0.1 molar ionic strength buffers. Open circles and open squares represent determinations of mobilities in phosphate buffers; closed circles and squares represent mobilities in diethylbarbiturate buffers. Protein concentrations were approximately 1%.

the electromagnetic spectrum of the CO derivatives of an hemolysate from *Pseudemys* and of Slow HbCO and Fast HbCO were identical with each other and very nearly identical with those of HbCO A.

To determine the isoelectric points of Slow HbCO and Fast HbCO, electrophoretic mobilities of 1% hemoglobin solutions were measured in buffers of varying pH and of constant ionic strength. Phosphate buffers were utilized except in the region of pH 8.6. A diethylbarbiturate buffer was used in this region. Electrophoretic mobilities obtained are plotted against pH in Fig. 2. Because of instability of the hemoglobins in acid, data are lacking in that region of the curve. Hemoglobins differ in their stability in strongly alkaline solutions. In buffers of pH 12 or above Fast HbO_2 denatures almost as rapidly as human HbO_2 A. Slow HbO_2 is very stable in alkali.

Five ultracentrifugal analyses were carried out upon samples which contained 0.2% hemoglobin. Hemolysates centrifuged both in pH 7.4 and pH 8.6 buffers resolved into 2 components, one of which sedimented approximately twice as rapidly as the other. The lighter component included about 1/3 of the total hemoglobin present in the hemolysate. Uncorrected sedimentation rates of pure samples of Slow and Fast Hbs indicated that this light component was Fast Hb and that the heavy component included Slow Hb. *In vitro* mixtures of Slow and Fast Hbs resolved into 2 components upon ultracentrifugation.

As stated previously, turtle hemoglobins appear to interact(2). In a further test of this phenomenon, 1:1 mixtures of Slow and Fast Hbs were subjected to moving boundary electrophoresis. In the ascending limb of the electrophoretic cell such mixtures resolved in the form of 2 rounded peaks; in the descending limb of the cell little or no resolution of the mixture occurred, and the pattern appeared as a single, broad, rounded peak. Although exact mobility measurements of Slow HbCO and Fast HbCO in mixtures were difficult to obtain because of this poor resolution, at pH 7.4 the mobility of the Slow component of this mixture was found to be greater than the mobility of pure Slow HbCO (-2.0×10^{-5} $cm^2/v/sec$ as compared to -0.4×10^{-5} $cm^2/v/sec$ in the ascending limb). Mobility of the Fast component was found to be less than the mobility of pure Fast HbCO (-3.6×10^{-5} $cm^2/v/sec$ as compared to -3.9×10^{-5} $cm^2/v/sec$ in the ascending limb). Measurements in diethylbarbiturate buffer indicated the same directions of mobility alterations. Moving boundary electrophoresis of a mixture of Fast HbCO and human HbCO A in a buffer of pH 8.6 indicated that interaction did not occur between these hemoglobins. Both resolved readily as sharp boundaries with no alterations in mobility. On paper, they separated as distinct spots which migrated at the same rate as adjacent samples of pure Fast Hb and pure Hb A.

Discussion. Both Fast and Slow turtle Hbs are homogeneous and active oxygen carriers. As would be expected, the absorption spectra in the visible region of these hemoglobins and of their CO and O_2 derivatives are very nearly identical to the spectra of other hemoglobins. They differ from each other and from human Hb A in isoelectric

point and in resistance to alkali. In addition, Slow Hb apparently consists of molecules of approximately twice the size of both Fast Hb and human Hb A. Svedberg and Hedenius (7) have previously reported the presence of 2 components with such differences in sedimentation constant in hemolysates of turtle blood.

Recently a pigment was found in the blood of a "desert tortoise"(8) which has the absorption spectra of a myoglobin. Such a pigment, if present in the blood of *Pseudemys*, apparently is not found in the quantities present in the tortoise blood (15 to 20% of the total circulating hemoproteins). CO derivatives of neither the unfractionated hemolysate nor of the fractions obtained by means of continuous flow electrophoresis had absorption spectra similar to the absorption spectra of CO myoglobin. Korzhuev and Kruglova reported that the oxygenated derivative of this circulatory myoglobin has absorption maxima at 582 and 543 mμ. The HbO_2 derivatives of Slow and Fast turtle Hb have absorption maxima at 576 and 541 mμ.

It is often very dangerous in comparative biochemistry to base generalizations upon observations on only a few species. An example of such a false generalization is the commonly held view that only hemoglobins of invertebrates have relatively acid isoelectric points(9). Lemberg and Legge(10) cite Svedberg and Pedersen as giving an example of a fish hemoglobin with an isoelectric point of 5.75. The presence of a hemoglobin in *Pseudemys* with an isoelectric point of 5.7 is another vertebrate for which the generalization does not hold. Data given in other work(2) also indicate that the presence in vertebrates of hemoglobins of acid isoelectric point is more common than generally realized.

Slow and Fast Hbs behave as if they interact in mixtures. The interaction was identified by the presence of a number of peculiar physicochemical properties of such mixtures. Evidence indicates that this interaction may be of a somewhat specific nature. Data obtained in physicochemical studies of turtle hemolysates are very difficult to interpret. Part of this difficulty is certainly due to the interaction which occurs between the hemoglobin components of the hemolysate.

Summary. The 2 hemoglobins of the turtle, *Pseudemys scripta elegans*, were prepared by means of a continuous flow electrophoresis. Both hemoglobins bound oxygen in a molar ratio of 1:1 with respect to iron. They have identical absorption spectra in the visible region but differ in isoelectric point, resistance to alkali, and in sedimentation constant. Physiochemical evidence is presented which indicates that these hemoglobins interact in mixtures.

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