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AN ELECTROPHORETIC COMPARISON OF
HEMOGLOBINS IN THE BAT FAMILY VESPERTILI-
ONIDAE.

University of Arizona, Ph.D., 1969
Zoology

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April 17, 1969

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>METHODS AND MATERIALS</td>
<td>7</td>
</tr>
<tr>
<td>Collection of Animals and Preparation of Blood Samples</td>
<td>7</td>
</tr>
<tr>
<td>Procedure for Comparing Unfractionated Hemoglobins</td>
<td>8</td>
</tr>
<tr>
<td>Continuous Vertical Acrylamide Gel Electrophoresis</td>
<td>8</td>
</tr>
<tr>
<td>Quantitation of Multiple Hemoglobins</td>
<td>12</td>
</tr>
<tr>
<td>Procedure for Comparing Hemoglobin Peptides</td>
<td>13</td>
</tr>
<tr>
<td>Purification of Hemoglobins and Preparation of Peptides</td>
<td>13</td>
</tr>
<tr>
<td>Peptide Mapping</td>
<td>17</td>
</tr>
<tr>
<td>Color Reactions to Distinguish Specific Peptides</td>
<td>18</td>
</tr>
<tr>
<td>RESULTS</td>
<td>20</td>
</tr>
<tr>
<td>Electropherograms of Bat Hemoglobins</td>
<td>20</td>
</tr>
<tr>
<td>Hemoglobin Peptide Maps</td>
<td>32</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>42</td>
</tr>
<tr>
<td>Some Possible Physiological Functions of Multiple Hemoglobins in Bats</td>
<td>42</td>
</tr>
<tr>
<td>Chiropteran Hemoglobins and Phylogeny</td>
<td>46</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>51</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>54</td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>62</td>
</tr>
</tbody>
</table>
## LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cross-section of the EC470 Gel Electrophoresis Cell in the vertical or running position.</td>
<td>9</td>
</tr>
<tr>
<td>2. Electropherograms of hemoglobins from seven vespertilionid species compared with human hemoglobin F.</td>
<td>21</td>
</tr>
<tr>
<td>3. Electropherograms of hemoglobins from six vespertilionid species compared with human hemoglobins A, C, and S.</td>
<td>22</td>
</tr>
<tr>
<td>4. Electropherograms of hemoglobins from five vespertilionid species compared with human hemoglobins A, C, F, and S.</td>
<td>23</td>
</tr>
<tr>
<td>5. Composite diagram of electropherograms of hemoglobin bands for 23 species of vespertilionid bats.</td>
<td>24</td>
</tr>
<tr>
<td>7. Relative concentrations of hemoglobins for vespertilionid species found to possess multiple hemoglobins.</td>
<td>29</td>
</tr>
<tr>
<td>8. Composite diagram of all hemoglobin bands found in five families of bats.</td>
<td>31</td>
</tr>
<tr>
<td>9. Human hemoglobin A tryptic peptide map.</td>
<td>34</td>
</tr>
<tr>
<td>10. Tryptic peptide map for vespertilionid bats belonging to hemoglobin Group I (one hemoglobin, $R_a$ value .45).</td>
<td>35</td>
</tr>
<tr>
<td>11. Tryptic peptide map for the vespertilionid bat, <em>Nycticeius humeralis</em>, belonging to hemoglobin Group II (one hemoglobin, $R_a$ value .66).</td>
<td>36</td>
</tr>
</tbody>
</table>
12. Tryptic peptide map for vespertilionid bats belonging to hemoglobin Group III (two hemoglobins, $R_a$ values, .45 and .66). ... 37

13. Tryptic peptide map for the vespertilionid bat, *Pipistrellus hesperus*, belonging to hemoglobin Group IV (three hemoglobins, $R_a$ values .29, .45, and .66). ... 38
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Specific staining amino acid residues in tryptic peptides of vespertilionid hemoglobins.</td>
<td>41</td>
</tr>
</tbody>
</table>
**ABSTRACT**

Electrophoretic properties of bat hemoglobins were investigated for their possible use in biochemical taxonomy. Emphasis was placed on members of the family Vespertilionidae with hemoglobins from 23 species electrophoretically isolated and compared. Hemoglobins from members of four other bat families were also investigated: Emballonuridae (1 species), Molossidae (1 species), Natalidae (1 species), and Phyllostomatidae (8 species).

Analysis of hemoglobin numbers and electrophoretic mobilities revealed no population or geographic variation. In the cases where more than one species was examined in a given genus, variations in hemoglobin numbers were found in three of the five genera.

Analysis of electrophoretic mobilities allowed separation of the 23 species of vespertilionids into four distinct groupings: Group I contains 16 species whose one hemoglobin shows an $R_a$ value (electrophoretic mobility in relation to the migration of human hemoglobin A) of .45; Group II contains one species with one hemoglobin that has an $R_a$ value of .66; Group III has six species with two hemoglobins which have $R_a$ values of .45 and .66; and Group IV with one species with $R_a$ values for three
hemoglobins of .29, .45, and .66. Peptide mapping studies of representative hemoglobins from these four vespertilionid hemoglobin groupings showed electrophoretically homogeneous hemoglobins to be, probably, the same in primary structure.

Possible physiological advantages of multiple hemoglobins in bats were discussed. Some correlation was shown between presence of multiple hemoglobins associated with bats that migrate and/or have a wide altitudinal range.

In comparing all hemoglobins found among the five families of bats investigated in this study some relationships can be seen. On the bases of similarities of hemoglobin numbers and electrophoretic properties the following families are considered more closely related: Emballonuridae-Natalidae, Molossidae-Vespertilionidae, and Phyllostomatidae an intermediate between the two groupings.

Results from this study imply that hemoglobins may be considered as a phylogenetic conservative character in bats. Their use in understanding chiropteran phylogeny appears to lie at the family level.
INTRODUCTION

This study was conducted to investigate some of the physico-chemical properties of bat hemoglobin in an attempt to clarify certain aspects of chiropteran phylogeny. One of the major tools in the study of mammalian phylogeny has been the fossil record, but in many instances this record is incomplete. For bats the fossil record is virtually lacking, existing only from middle Eocene, at which time specialization (morphological and physiological) within the order appears to have been as great as that of modern bats. Because of the inadequate fossil record, it has been impossible to determine whether evolutionary lines present in the Eocene persisted, giving rise to modern families and genera or whether most became extinct and living families and genera resulted from a later radiation of only one or a few Eocene lines (Baker 1967a). With such a scanty fossil record chiropteran taxonomists have attempted to reconstruct bat phylogeny, relying exclusively on the living forms for clues to the past.

Taxonomy was once limited in zoology to the use of direct measurements of preserved morphological characters to delineate categories as a basis for classification, but
has advanced in recent years to include many other criteria. Such criteria as serum proteins, enzyme patterns, karyology, and serological differences are being used for classification in addition to the more conventional measurements. As with the conventional tools the new methods of understanding phylogeny have a double function: (1) they have a diagnostic aspect as indicators of difference (emphasis on differentiating properties which is useful in the lower taxonomic categories); and (2) they function as indicators of relationship (this property makes them especially useful in the study of the higher categories). (Mayr, Linsley, and Usinger 1953, p. 105). Thus, as one reviews the literature one character examined may be phylogenetically conservative and therefore, useful in understanding relations among phyla, classes, and orders. Others may add to the understanding of relationships at the lower taxonomic levels. Quite often the same character may be conservative in one group but not so in another. Karyology when applied to chiropteran classification appears to be valuable only at the family level and above (Baker 1967b, Baker and Patton 1967), but in rodent studies (Patton 1969) it has proved valuable even at the subspecific level.

Proteins also have been shown to be conservative in some instances but not in others. Probably the best documented case of a conservative protein used as a taxonomic
character is cytochrome $c$, an electron carrier which plays a central role in cellular respiration and is present in essentially all eukaryotic organisms, both animal and plant (Keilin 1966). All cytochromes $c$ react readily with cytochrome oxidase preparations from mammalian sources, can effectively substitute one for another in the terminal oxidation chain of nearly intact mitochondria, and have similarities in primary structure (Nolan and Margoliash 1968). To date the primary structures of cytochromes $c$ from 23 vertebrate, 4 invertebrate, 3 fungal, and one higher plant have been established, all with little variation in protein amino acid sequences. These findings permit the conclusion that all eukaryotic cytochromes $c$ are in fact ancestrally homologous. Correlations of protein structure and phylogeny have therefore, been limited to comparisons of the differences between amino acid sequences in cytochromes $c$. Minimal mutation distances, a statistical method for the construction of phylogenetic trees from comparative protein structures, has been applied to the primary structures for the cytochromes $c$ of 20 species (Fitch and Margoliash 1967). The resultant phylogenetic tree agreed remarkably with classical concepts of phylogeny. This character when applied at lower taxons was found to be virtually worthless as a taxonomic tool, but proved quite
valuable in strengthening some of the higher categories of classification.

Another protein which taxonomists have investigated is hemoglobin and, as with cytochromes c, the similarities of hemoglobins thus far studied may indicate that it is another phylogenetically conservative character. In fact, the basic structure (metalloporphyrin plus protein) is the same for hemoglobin and cytochrome c suggesting that both have common ancestry. One interesting difference reported for hemoglobin and not for cytochromes c has been the finding of multiple hemoglobins which have variations in electrophoretic mobilities. Many investigators have compared hemoglobin electrophoretic properties and phylogeny. Dunlap, Johnson, and Farner (1956) studied 21 species of birds and showed that the differences in their hemoglobins apparently correlated with taxonomic kinship; Marchlewksa-Koj (1963) and Guttman (1967) noted that amphibian hemoglobin heterogeneity may serve as complementary data for taxonomy. Comparable investigations of fish hemoglobins have been conducted by Hashimoto and Matsuura (1959, 1960) and Smith (1963) and of mammalian hemoglobins by Blumberg, Allison and Garry (1960), Foreman (1960, 1964, 1966, 1968), Rasmussen, Jensen, and Koehn (1968), Ewy and Wojcik (1961), Buettner-Janusch (1963), Kitchen, et al. (1968), Horvath, et al. (1968) and others.
In 1966, electrophoretically distinct hemoglobins of bats were first reported. Manwell and Kerst (1966) using starch-gel electrophoresis compared hemoglobin, lactate dehydrogenase, and esterases of bats and investigated the possibility of using these proteins for biochemical taxonomy. Species included in the study were *Eptesicus fuscus*, *Myotis keenii*, *Myotis lucifugus*, and *Pipistrellus subflavus*. They found minor differences among the hemoglobins and esterases. One polymorphism of the $\mathbf{H}$ chain of lactate dehydrogenase occurred in *Myotis keenii* and *Myotis lucifugus* and a different one in *Eptesicus fuscus* and these findings suggested that lactate dehydrogenase could possibly serve as a taxonomic character at the generic level. Mitchell (1966), the only other investigator to compare hemoglobins in bats, used five species (*Myotis grisescens*, *Myotis lucifugus*, *Pipistrellus subflavus*, *Plecotus townsendi*, and *Eptesicus fuscus*) and the technique of cellulose acetate electrophoresis. Two electrophoretically different hemoglobins were found in *Pipistrellus subflavus* and *Plecotus townsendi*, whereas hemoglobins of *Eptesicus fuscus*, *Myotis grisescens*, and *Myotis lucifugus* were found to be homogeneous. This report of multiple hemoglobins occurring in bats suggested the present study: comparing the hemoglobins of members of the bat family Vespertilionidae.
The common bats of North America belong to the family Vespertilionidae with 12 genera and 41 species presently recognized for this continent (Hall and Kelson 1959). Though the family has been extensively studied, relationships between some of its members have been difficult to determine. This is especially true for the genus Myotis the taxonomy of which is constantly undergoing revision (Baker 1967b, Druecker 1966, Findley and Jones 1967, Harris and Findley 1962). This genus is currently under worldwide study by Dr. James S. Findley, University of New Mexico. In the studies reported here hemoglobins of vespertilionid species, especially Myotis, were selected for comparison. Aspects investigated were electrophoretic mobilities of hemoglobins of 23 North American species, hemoglobin peptide maps, and the possibility of using hemoglobins as biochemical characters in the study of bat taxonomy.
MATERIALS AND METHODS

Collection of Animals and Preparation of Blood Samples

All specimens used in this study are listed in Appendix I. These specimens were collected from day roosts (mines, caves, abandoned buildings, and bridges) or by the use of mist nets (Nippon Kenmo Co., Kuwana, Japan) placed over streams, ponds, or other bodies of water.

Blood samples were drawn by heart puncture of live bats, within 24 hours after capture, using a 0.5 cc tuberculin syringe wetted with heparin solution (NK Heparin, Medical Chemical Corp., Los Angeles, California). Blood was mixed with physiological saline (0.85% NaCl) and the erythrocytes were separated by centrifugation at 2000 rpm. This procedure was repeated three times. The packed cells were then suspended in an equal volume of 0.06 M barbital buffer, pH 8.6 (10.3 g/l Na barbital, 1.4 g/l barbital in CO₂-free H₂O). Cells were lysed by freezing and storing at -20 F. Upon thawing, cell debris was removed by centrifugation at 7000 rpm for 15 min. The resulting supernatant (hemoglobin suspended in barbital buffer) was used for electrophoretic separation studies.

The method used in this study to determine hemoglobin concentrations was that developed by Hycel, Inc. (Houston, Texas) and followed the procedure described by Poor (1960).
Hemoglobin was converted to cyanmethemoglobin by mixing 0.02 ml hemoglobin solution with 5.0 ml of Hycel Cyanmethemoglobin Reagent. The concentration, expressed in gram percent, was determined colorimetrically at 540 μm in a Bausch and Lomb Spectronic "20" colorimeter against a standard curve based on appropriate dilutions of the Hycel Cyanmethemoglobin Standard. Each bat hemoglobin sample was diluted with cyanmethemoglobin reagent to make a 40-50 gram percent solution.

Procedure for Comparing Unfractionated Hemoglobins

Continuous Vertical Acrylamide Gel Electrophoresis

Electrophoretic separation of cyanmethemoglobins was carried out using the EC470 Vertical Gel Electrophoresis Cell (E-C Apparatus Corp., Philadelphia, Pa.) following the procedures described in the E-C Technical Bulletin 141. The EC470 electrophoresis cell consists of two electrode chambers (Figure 1). On each side of the gel chamber is a cooling plate (the inner and outer cooling plates). Stock solutions and the gel were prepared as follows:

Solution a: (7% acrylamide gel)

Cyanogum-41 (purchased from E-C Corp.)...... 14.0 gm
Tris-Na₂EDTA-Borate buffer (pH 8.4) ......200 ml
TMED (N,N,N',N'-Tetramethylethlenediamme). 0.2 gm
Add 0.2 gm ammonium persulfate (AP) catalyst just before using
Figure 1: Cross-section of the EC470 Gel Electrophoresis Cell in the vertical or running position. Captions are: a. upper buffer chamber; b. lower buffer chamber; c. overflow; d. upper electrode; e. lower electrode; f. inner coding plate; g. outer cooling plate; h. gel; and i. slots.
Solution b: (Tris-Na$_2$EDTA-Borate buffer, pH 8.4)
  Tris (hydroxymethylaminomethane) ............... 43.1 gm
  Na$_2$EDTA (Disodium (Ethylenedinitrilo) tetraacetate 3.7 gm
  Boric acid (crystalline)........................... 22.0 gm
  H$_2$O to ........................................ 4.0 liters

Solution c: (40% sucrose solution)
  sucrose ................................. 40.0 gm
  Tris-Na$_2$EDTA-Borate buffer (pH 8.4) to .......... 100. ml

After mixing, the catalyzed gel (Solution a) was poured into the 3 mm wide space between the two cooling plates of the electrophoresis cell (Figure 1). An eight-place slot former was placed in the gel between the cooling plates, and the acrylamide was allowed to polymerize with the cell resting in a horizontal position (about 15 min). After polymerization the cell was placed in the running or vertical position, the slot former was removed, and the cooling plates were connected to a circulating water coolant system (0°C). Cooling of the gel was necessary to prevent temperature increase due to its resistance to electron flow.

The upper electrode compartment was filled with Tris-Na$_2$EDTA-Borate buffer, pH 8.4, (Solution b) and allowed to overflow until the electrode in the lower compartment was completely covered. The electrodes were connected to a constant voltage power supply (E-C power supply EC454) and the system was prerun for 30 min at 400V. Prerunning
allowed traces of the catalyst which remain in the gel to dissipate throughout the buffer in the two electrode chambers. Following the prerun, both electricity and cooling were shut off before introducing the hemoglobin samples.

Fifteen microliters of 40-50 gram percent cyanmethemoglobin samples were mixed with 10 microliters of 40% sucrose solution (Solution c) to increase the density of the samples. The samples were then introduced by micropipette into the gel slots and allowed to settle for 15 min. before proceeding with the electrophoresis. The 15 microliter sample of 40-50 gram percent cyanmethemoglobin was found, after repeated testing, to yield the best electrophoretic separation and banding. Proteins which differ only slightly in their electrophoretic mobilities will not be separated if their concentrations are excessively high. Inversely, the final position in the gel of proteins present in very low concentrations cannot be determined with accuracy.

Electrophoretic separation of the hemoglobins was carried out at a constant voltage of 400V, at 0°C, for 2 hours by which time the more rapidly migrating fraction had moved about 50 cm from the origin. Increasing the time of electrophoresis to 4 hours gave neither a visible increase in the number of hemoglobins present nor any improvement in resolution. Hyland human hemoglobin electrophoresis controls A, C, F, and S (Hyland Laboratories, Los Angeles, Calif.)
were subjected to electrophoresis simultaneously with bat hemoglobin samples and served as reference standards.

Visible hemoglobin bands were developed in the gel using a peroxidase stain as described in the E-C Corp. Technical Bulletin 141. Thus far, only two mammalian proteins with peroxidase activity have been reported in the literature. They are hemoglobins and haptoglobins and the latter, if present in bat plasma, was discarded in the initial saline washings of the erythrocytes. Two hundred mg 3,3'-dimethoxybenzidine was completely dissolved in 5 ml glacial acetic acid and then diluted to 100 ml with distilled water. Immediately prior to use, 0.1 ml 30% hydrogen peroxide was added. The gel was incubated in this solution for 20 min in the dark at which time color development of hemoglobin bands was complete.

Quantitation of Multiple Hemoglobins

The relative concentrations of the multiple hemoglobin fractions were obtained by spectrophotometrically measuring the amount of dye bound to each. Stained bands were cut from the gel and each was thoroughly macerated in 4.0 ml glacial acetic acid. The mixture was then centrifuged to remove the gel. Absorption peaks for dimethoxybenzidine bound to hemoglobin in glacial acetic acid were found to be at 300 μμ and 405 μμ using the Beckman DB Scanning Spectrophotometer. For quantitation the optical
density of the eluted dye was read on a Beckman DU-2 Spectrophotometer at 405 \textmu m. In the cases in which there were two hemoglobin fractions in one sample the optical density of the two was taken as 100% hemoglobin present. Individual hemoglobin fractions were then represented as a percentage of this total.

**Procedure for Comparing Hemoglobin Peptides**

**Purification of Hemoglobins and Preparation of Peptides**

**Column chromatography.** The initial preparation of hemoglobin samples for peptide mapping was the same as used for the vertical acrylamide gel electrophoresis. The hemoglobin was suspended in barbital buffer and then dialyzed against 0.05 M Tris-HCl, pH 8.6, to remove traces of salt left from the initial washings with saline. Following dialysis, hemoglobin samples were isolated from other erythrocytic proteins (aldolase, catalase, and lactate dehydrogenase) by anion exchange column chromatography on DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) according to the method described by Leyko and Gondko (1963).

**Discontinuous Vertical Acrylamide Gel Electrophoresis.** The purity of the hemoglobin eluted from the DEAE-Sephadex column was ascertained by discontinuous gel electrophoresis and this was carried out according to the method described earlier (Figure 1). The discontinuous technique is a
system in which low voltage electrophoresis accomplishes an initial separation of proteins in a large-pore spacer gel. Definitive separation is then carried out by high voltage electrophoresis in a smaller-pore running gel. In the spacer gel each component of a protein mixture is concentrated into a separate thin layer and these layers are stacked closely in the order of their mobilities before entering the running gel for separation. Concentrating in this way allows for better identification of any proteins or contaminants which may be present in very small quantities.

Stock solutions and gels were prepared as follows:

Solution a: (running buffer, pH 8.9)
Tris (hydroxymethylaminomethane) .................. 46 gm
conc. HCl .................................. 4 ml
H₂O to .................................... 1 liter

Solution b: (spacer gel, pH 6.7)
Tris ........................................... 7.5 gm
conc. HCl ................................... 4 ml
H₂O to ..................................... 1 liter

Solution c: (electrode buffer, pH 8.3)
Tris ........................................... 1.2 gm
Glycine ....................................... 5.8 gm
H₂O to ..................................... 2 liters

Solution d: (plug and running gel solution, 7.0%)  
Cyanogum-41 .................................. 14 gm
solution a (running buffer, pH 8.9) .......... 200 ml
TMED (N, N, N',N'-tetramethylethylenediamine) 0.2 ml
Ammonium persulfate ............................ 0.2 gm

Solution e: (spacer gel solution, 4.0%)
Cyanogum-41 .................................. 4 gm
solution b (spacer buffer, pH 6.7) .......... 100 ml
TMED ....................................... 0.1 ml
Ammonium persulfate ............................ 0.1 gm
All the solutions were stored in the refrigerator (3°C). Ammonium persulfate catalyst was added to the gels just prior to use.

The assembled EC470 electrophoresis cell was placed at a 45° angle and the plug gel solution (Solution d) was mixed and poured into the gel chamber to a level of 12 cm from the top of the inner cooling plate. The top surface of the gel was layered with 5 ml of distilled water and the gel was allowed to polymerize at room temperature. The function of the plug gel was to serve as support for the pouring of the running gel and was not actually involved in electrophoretic separation. After polymerization the distilled water was removed, and the top of the plug gel was rinsed with running gel solution. Next the cell was placed in a vertical position (Figure 1) and, after mixing, the running gel (Solution d) was poured between the cooling plates to a level 2.5 cm from the top of the inner cooling plate. The top surface of the running gel was layered with 5 ml of distilled water and allowed to stand until fully polymerized. After removal of the distilled water and rinsing with spacer gel solution, the spacer gel (Solution e) was mixed and poured with the cell in a horizontal position. The slot former was then inserted and the spacer gel was allowed to polymerize. Tris-glycine buffer (Solution c) was poured into the upper electrode chamber and allowed
to overflow until the electrode in the lower chamber was completely covered with buffer.

Lyophilized hemoglobin samples were made to 4 mg per ml in 0.1M Tris-HCl, pH 6.7. One hundred microliters of this solution was placed in each gel slot. Applying 200V for 30 min, (0°C), allowed the proteins to "stack" in the spacer gel. Thereafter, 400V was maintained for 1½ hrs by which time the proteins had moved 60-70 cm into the running gel. Protein bands were made visible by staining with a general protein stain, Amido Black 10B. The stain was an 0.2% Amido Black 10B solution made by dissolving 2 gm of the dye in 100 ml of 7.5% glacial acetic acid. Destaining was accomplished by exhaustively washing the gel slab with 5% glacial acetic acid.

Tryptic Digestion. Following purification by column chromatography, hemoglobin samples were dialyzed against distilled water to remove the salts used to elute them from the column. Tryptic digestion was carried out following the procedure described by Chernoff and Liu (1961). Following digestion, the mixture was lyophilized to remove the volatile (NH₄)₂CO₃ buffer and to concentrate the resulting peptides.
Peptide Mapping

**Descending Chromatography.** Since only 80% of the hemoglobin molecule is susceptible to tryptic digestion (the heme group remains immune to the action of trypsin and constitutes an insoluble resistant core), sufficient 0.02M NH$_4$HCO$_3$ was added to yield a concentration of 1 mg of the peptides resulting from the tryptic digestion per 25 microliters of solution. The insoluble core was removed by centrifugation and the clear supernatant was then utilized for descending chromatography.

From 100 to 125 microliters (approximately 4⅝ to 5 mg) of solution was applied to one corner of an 18½ by 22½ inch sheet of Whatman No. 3MM filter paper in 10 microliter aliquots. The spot was dried between applications by a stream of cold air to keep its area within the range of 1 to 1½ cm in diameter. Descending chromatography in n-butanol:acetic acid:H$_2$O (4:1:5) was carried out in a Chromatocab chromatography cabinet, Model A125 (Research Specialities Co., Berkeley, California) for 22 hours. Fresh solvent was prepared by mixing the ingredients in a separatory funnel, discarding the aqueous phase after settling for 2 hours, and filtering the solvent free of water droplets which remained. The spotted peptide papers were permitted to equilibrate overnight in the cabinet in
an atmosphere saturated with the solvent before chromatography was begun. Finally, the paper was dried under a hood overnight before proceeding with high voltage electrophoresis.

**High Voltage Electrophoresis.** Electrophoresis was carried out in an E-800-213 electrophoresis tank (Savant Instruments, Inc., Hickman, N.Y.) in a formic acid: acetic acid buffer (formic acid 21 ml; glacial acetic acid 80 ml; H₂O 900 ml, pH 1.9). In preparation for electrophoresis the dried paper was moistened with electrophoretic buffer (formic acid, glacial, acetic acid, H₂O, pH 1.9) in the following manner. The paper was draped over two rods so that the original point of peptide application and the final positions of the peptides following descending chromatography both lay between the two rods. Then the paper between the rods was moistened on both sides of the peptide line so that the peptides were washed into a sharp line by the advancing buffer fronts. High voltage electrophoretic separation of the peptides was carried out for 1½ hrs at 2000V. Following electrophoretic separation, the paper was allowed to dry overnight.

**Color Reactions to Distinguish Specific Peptides**

Following two-dimensional separation, the peptides were either made visible with ninhydrin or by means of a
combination of specific color reactions for certain amino acids occurring in the peptides. The use of specific color reactions makes it possible to distinguish peptides which only show up slightly following ninhydrin treatment, and to distinguish the peptides in the large central mass which remain closely together during separation. The color reactions used have been described by Easley (1965) and are: Sakaguchi for arginine, Pauly for histidine, Platinic iodide for methionine, Ehrlich for tryptophan, and the tyrosine reaction.

Purified arginine, histidine, methionine, tryptophan, and tyrosine (Sigma Chemical Co., St. Louis, Mo.) were developed with their respective color tests to serve as standards. Peptide maps were made of human hemoglobin A (Hyland Laboratories, Los Angeles, Calif.) to serve as reference material. Peptide numbering was patterned after that proposed by Chernoff and Liu (1961) for human hemoglobin A.
RESULTS

Electropherograms of Bat Hemoglobins

Human hemoglobin electrophoretic standards A, C, F, and S were examined by the vertical acrylamide electrophoresis technique to serve as standards in comparing the electrophoretic mobilities of the bat hemoglobins. Electropherograms prepared as outlined in the materials and methods section are presented in Figures 2, 3, and 4 for sixteen of the twenty-three vespertilionid species examined. The diagrammatic representation shown in Figure 5 gives a composite of the four hemoglobin patterns found in those species investigated. In comparing the actual electropherograms (Figures 2, 3, and 4) one can see slight electrophoretic variations among hemoglobins represented as homogenous in Figure 5. These slight variations in mobilities may be caused by the following: inconsistencies in gel polymerization, slight variations in gel or protein concentration, differences in length of time for electrophoresis or heating as a result of the voltage used. Electrophoretic separation of mixed samples for species reported as having homogeneous hemoglobins is presented in Figure 6. No electrophoretic separation was found for the mixed samples. Increasing the time for electrophoresis
Figure 2: Electropherograms of hemoglobins from seven vespertilionid species compared with human hemoglobin F. From left to right: (1) Rhogeesa parvula; (2) Antrozous pallidus; (3) human hemoglobin F.; (4) Myotis fortidens; (5) Pipistrellus hesperus (6) Pipistrellus subflavus; (7) Myotis thysanodes and (8) Myotis sodalis.
Figure 3: Electropherograms of hemoglobins from six vespertilionid species compared with human hemoglobins A, C, and S. From left to right: (1) Myotis lucifugus; (2) Lasiurus cinereus; (3) human hemoglobins A and S; A is the bottom band; (7) Plecotus phyllotis; and (8) Plecotus townsendi.
Figure 4: Electropherograms of hemoglobins from five vespertilionid species compared with human hemoglobins A, C, F, and S. From left to right: (1) Lasionycteris noctivagans; (2) Plecotus phyllostis; (3) Lasiurus ega; (4) human hemoglobin F; (5) Nycticeus humeralis; (6) human hemoglobins A and C, A is the bottom band; (7) Lasiurus borealis; and (8) human hemoglobins A and S, A is the bottom band.
Figure 5: Composite diagram of electropherograms of hemoglobin bands for 23 species of vespertilionid bats. Bands are shown in the order of their relative mobilities from left to right from the origin (0). Average $R_a$ values are given in relation to the migration of human hemoglobin A and are explained in the text. Figures in parentheses represent the number of species showing this hemoglobin pattern. The bottom line gives mobilities for human hemoglobins C ($R_a$ value .61), S ($R_a$ value .78), F ($R_a$ value .91), and A. Groups I, II, III, and IV are explained in the text.
Figure 6: Electropherograms of mixed hemoglobins from vespertilionid species compared with human hemoglobins A, C, F, and S. From left to right: (1) Myotis sodalis and Myotis volans; (2) Myotis californicus and Myotis griseocens; (3) human hemoglobins A and S, A is the bottom band; (4) Myotis subulatus and Myotis velifer; (5) human hemoglobin F; (6) Lasiurus cinereus and Lasiurus ega; (7) Lasiurus borealis and Lasiurus cinereus; and (8) human hemoglobins A and C, A is the bottom band.
two-fold (4 hrs) still produced no separation of the mixed hemoglobins.

Much criticism has been directed toward some of the interpretations of electrophoretic results which have appeared in the literature. In some cases (e.g. serum proteins) such factors as age, diet, sex, and season have effected the electrophoretic mobilities as well as the apparent numbers of proteins present (Ashton 1957, Kaplan 1960). Intrapopulation variations have also been reported for hemoglobins (Glueckson-Waelsch, Ranney, and Sisken 1957, Popp and St. Amand 1960, Rasmussen, Jensen, and Koehn 1968). In this study no intraspecific variations of hemoglobin numbers or mobilities were obtained except in one instance. A gravid Eptesicus fuscus from one sample demonstrated two hemoglobin fractions. For all other Eptesicus fuscus investigated in this study only one hemoglobin was found. The finding of one hemoglobin for this species is in agreement with the literature (Manwell and Kerst 1966, Mitchell 1966). This one exception is interpreted as a mixing of the maternal and fetal hemoglobins or possibly the tetrameric hemoglobin molecule has dissociated into dimers. Unfortunately no fetal hemoglobin samples were taken for study. In comparing young—of the year with adults, the numbers and mobilities of hemoglobins were identical. The same results were also obtained when comparing samples from hibernating specimens with
samples from active summer specimens of *Eptesicus fuscus*, *Myotis lucifugus*, *Myotis sodalis*, *Pipistrellus subflavus*, and *Plecotus townsendi*.

Electrophoretic values given in Figure 5 are based on mean $R_a$ values (relative to migration of human hemoglobin A). For all vespertilionid species studied three hemoglobin bands were observed in four combinations. The following grouping based on number of hemoglobins present and $R_a$ values divide the species examined into the four hemoglobin patterns.

Group I: one hemoglobin ($R_a$ value .45)

- *Antrozous pallidus*
- *Eptesicus fuscus*
- *Myotis californicus*
- *Myotis fortidens*
- *Myotis grisescens*
- *Myotis lucifugus*
- *Myotis sodalis*
- *Myotis subulatus*
- *Myotis thysanodes*
- *Myotis velifer*
- *Myotis volans*
- *Myotis yumanensis*
- *Pizonyx vivesi*
- *Plecotus phyllostis*
- *Rhogeesa parvula*
Group II: one hemoglobin \((R_a\ value = .66)\)

*Nycticeus humeralis*

Group III: two hemoglobins \((R_a\ values = .45\ and\ .66)\)

*Lasionycteris noctivagans*

*Lasiurus borealis*

*Lasiurus cinereus*

*Lasiurus ega*

*Pipistrellus subflavus*

*Plecotus townsendi*

Group IV: three hemoglobins \((R_a\ values = .29, .45,\ and\ .66)\)

*Pipistrellus hesperus*

Of the twenty-three species of vespertilionids studied, seven species (Figure 5, Groups III and IV) had multiple hemoglobins. Relative quantitations for these hemoglobins were obtained following the procedure given in the materials and methods section. Presented in Figure 7 are averages for the relative distribution of each hemoglobin fraction. Relative hemoglobin concentrations for the seven species are (slower migrating hemoglobin: faster migrating hemoglobin): *Lasiurus borealis* and *Lasiurus cinereus* (50%:50%); *Lasiurus ega* (67%:33%); *Lasionycteris noctivagans* (60%:40%); *Plecotus townsendi* (55%:45%); *Pipistrellus subflavus* (58%:42%). *Pipistrellus hesperus*, the only species with three hemoglobins, had concentrations of 28%:30%:42%. The slower migrating hemoglobin was the
Figure 7: Relative concentrations of hemoglobins for vespertilionid species found to possess multiple hemoglobins (see text).
more common hemoglobin in four of the species examined while the other two species had equal concentrations of both hemoglobin types. In Pipistrellus hesperus the fastest migrating hemoglobin was the major portion of the total complement.

Presented in Figure 8 is a composite of all hemoglobins found in members of five families of bats. Hemoglobins from members of other families were separated electrophoretically to be used as comparative material for vespertilionid hemoglobin patterns. Explanation of Figure 8 is as follows:

Vespertilionidae
In the twenty-three species examined three hemoglobins were found (R_a values .29, .45, and .66). Groupings for different hemoglobin patterns are presented in Figure 5 and the text.

Molossidae
In one species examined, Tadarida brasiliensis, two hemoglobins were found (R_a values .45 and .66).

Phyllostomatidae
In eight species examined two hemoglobins were found (R_a values .35 and .66). Species examined and their hemoglobin numbers and R_a values were:
Figure 8: Composite diagram of all hemoglobin bands found in five families of bats. Bands are shown in the order of their relative mobilities from left to right from the origin (0). Average $R_a$ values are given in relation to the migration of human hemoglobin $A$ and are explained in the text. Figures in parentheses represent the number of species from the family investigated. The bottom line gives mobilities for human hemoglobins $C$ ($R_a$ value .61), $S$ ($R_a$ value .78), $F$ ($R_a$ value .91), and $A$. 
Group I: one hemoglobin ($R_a$ value .66)

Artebius hirsutus
Glossophaga soricina
Leptonycteris sanborni
Macrotus waterhousii
Pteronotus parnelli
Sturnira lilium

Group II: two hemoglobins ($R_a$ values .35 and .66)

Pteronotus davyi
Pteronotus psilotus

Natalidae

In one species examined, Natalus stramineus, two hemoglobins were found ($R_a$ values .35 and .58).

Emballonuridae

In one species examined, Balantiopteryx plicata, two hemoglobins were found ($R_a$ values .35 and .58).

**Hemoglobin Peptide Maps**

Hemoglobin peptide maps were made for the following vespertilionid species and their respective hemoglobin groupings.

Group I: (one hemoglobin, $R_a$ value .45)

Antrozous pallidus
Myotis fortidens
Myotis thysanodes
Myotis yumanensis

Group II: (one hemoglobin, $R_a$ value .66)
Nycticeus humeralis

Group III: (two hemoglobins, $R_a$ values .45 and .66)
Lasionycteris noctivagans
Plecotus townsendi

Group IV: (three hemoglobins, $R_a$ values .29, .45, and .66)
Pipistrellus hesperus

In order to make structural interpretations from comparative peptide maps of bat hemoglobins, for which the sequence is not known, it was first necessary to identify corresponding peptides of known sequence from a hemoglobin of another species. Human hemoglobin A tryptic peptides were separated and the resulting map is presented in Figure 9. Numbering of the various bat peptides was then based on the numbering of human hemoglobin A by Chernoff and Liu (1961). Presented in Figures 10, 11, 12, and 13 are peptide maps for the representatives of the four hemoglobin groups of vespertilionid bats investigated. Electrophoretically homogeneous hemoglobins all gave similar peptide mapping picture.

The electrophoretic mobilities of the individual hemoglobins found in Group I ($R_a$ value .45) and Group II
Figure 9: Human hemoglobin A typtic peptide map. The point of sample application was at the intersection of the ruled lines. Chromatography was performed first, for 22 hrs along the horizontal axis in the non-aqueous phase of a mixture of n-butanol (4 volumes), water (5 volumes), and glacial acetic acid (1 volume). High voltage electrophoresis was then carried out along the vertical axis with the cathode at the top. Electrophoresis continued for 1½ hrs at 2000V in a pH 1.8 solution of formic acid (1 volume), glacial acetic acid (4 volumes) and water (45 volumes).
Figure 10: Tryptic peptide map for vespertilionid bats belonging to hemoglobin Group I (one hemoglobin, Ra value .45). Homogeneous maps were found in four members of this group: Antrozous pallidus, Myotis fortidens, Myotis thysanodes, and Myotis yumanensis. Peptide mapping conditions were identical to those described in the legend to Figure 9.
Figure 11: Tryptic peptide map for the vespertilionid bat, Nycticeus humeralis, belonging to hemoglobin Group II (one hemoglobin, $R_a$ value .66). Peptide mapping conditions were identical to those described in the legend to Figure 9.
Figure 12: Tryptic peptide map for vesper-tilionid bats belonging to hemoglobin Group III (two hemoglobins, Pκ values .45 and .66). Homogeneous maps were found for two species belonging to this group: Lasionycteris noctivagans and Plecotus townsendi. Peptide mapping conditions were identical to those described in the legend to Figure 9.
Figure 13: Tryptic peptide map for the vespertilionid bat, Pipistrellus hesperus, belonging to hemoglobin, R₃ values .29, .45, and .66). Peptide mapping conditions were identical to those described in the legend to Figure 9.
(R_a value .66) are identical with the two hemoglobins for Group III. If the hemoglobins are identical in primary structure then one would expect the peptide map for Group III (Figure 12) to be a composite of Groups I and II (Figures 10 and 11). This proved to be true with the exception that the peptide designated as number 22 for Group I was not visible in the maps of Group II and Group III. Peptide number 22 may be present in these groups but was not demonstrated due to masking by other peptides.

The map for Group IV (Figure 13) contained the peptides found in Groups I, II, and III plus those present in this group's third hemoglobin, namely peptides 42-49. In comparing the Group IV map with the others it is evident that peptides which were designated the same in all maps (e.g. 15 and 16) are somewhat displaced in the map for Group IV. One of the major difficulties in peptide mapping is that poorer peptide separation occurs when larger numbers of peptides are present. Individual peptides may interfere with the separation of others and often overlapping peptides make interpretation difficult. Complete isolation and identification was often difficult despite the fact that specific color reactions were used to identify peptides in the large central mass which remain together during two-dimensional separation. Peptide number 29 was not found in the Group IV map. This may indicate that it was not present,
or that it was hidden by another peptide, or that it was incorrectly identified.

The peptides which reacted with the specific stains for amino acids are presented in Table I. The only variations noted in the color reactions occurred in peptides 9, 13, and 24 of Group II. Peptides 9 and 13 failed to give a positive arginine test and peptide 24 gave no reaction for tryptophan. One interesting observation was the absence of methionine positive peptides. The positive reaction of purified methionine controls demonstrated that the test was valid. Methionine residues, though not common in occurrence, have been reported as present in hemoglobins of other species of vertebrates. Kitchen, et al. (1968), investigating four sheep hemoglobins and two deer hemoglobins, found a range of 1-4 methionine positive peptides in the six species studied. Hemoglobins containing methionine residues are also reported in lamprey and carp (Braunitzer 1966), while human hemoglobin A contains a total of six methionine residues, two in the \( \alpha \)-chain and one in the \( \beta \)-chain. Few primary structures have been determined for the hemoglobins of naturally occurring animals and thus this absence of methionine containing peptides in bats cannot be interpreted as being unusual.
Table 1. Specific staining amino acid residues in tryptic peptides of vespertilionid hemoglobins. Presented are peptides which reacted with specific stains for amino acids and which were found in peptide maps from vespertilionid bats belonging to the four hemoglobin groupings (see text).
Specific Staining Amino Acid Residues in Tryptic Peptides of Vespertilionid Hemoglobins

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DISCUSSION

Some Possible Physiological Functions
of Multiple Hemoglobins in Bats

Knowledge of the occurrence of multiple hemoglobins is not new, but no experimental evidence that multiple hemoglobins offer physiological advantages over a single hemoglobin has been found. Suggestions have been made that multiple hemoglobins could differ physiologically and these differences would permit adaptation to a variety of environmental situations. Possible properties of multiple hemoglobins which would be advantageous to bats, include increased oxygen affinity and a reverse Bohr effect.

The tetrameric protein portion of hemoglobin has a definite heme-heme interaction which facilitates oxygen loading. All four heme residues in the hemoglobin molecule are capable of binding oxygen, but their respective binding constants are dependent on one another. As successive oxygen molecules are bound a conformational change is produced which allows the next residue to have a greater affinity for oxygen and, therefore, to load more readily. If a second hemoglobin were present in an organism and if this hemoglobin had a greater heme-heme interaction, the additional hemoglobin oxygen affinity would be an advantage.
in adapting to environmental conditions of low oxygen partial pressures. Such conditions occur at high elevations and in stagnant cave atmospheres. Mammals are known to adapt to high elevations by having increases in erythrocyte numbers and hemoglobin content, but altitudinal differences in hemoglobin types have not been thoroughly investigated. Ahl (1968) electrophoretically compared hemoglobins from three altitudinal populations of *Peromyscus maniculatus nebrascensis*, the deer mouse. In this comparison she found two electrophoretically distinct hemoglobins. Hemoglobin type D was found in deer mice at all three altitudes (3000 ft, 7000 ft, and 11000 ft). Hemoglobin type S was found in deer mice at the two higher altitudes. When comparing quantities of hemoglobins D and S in those populations where both were present, it was found that type S hemoglobin was more common than type D in the mice trapped from higher altitudes. Thus, it is possible that type S hemoglobin gives the higher altitudinal population some advantage in conditions of lower oxygen partial pressures.

Of the seven vespertilionid species found to possess multiple hemoglobins four (*Lasiurus borealis*, *Lasiurus cinereus*, *Lasiurus ega*, and *Lasionycteris noctivagans*) are migratory and have wide altitudinal ranges. If their second hemoglobin has a higher oxygen affinity than one would expect the concentration of this hemoglobin to vary with
the altitudinal range for the species or, possibly, to be associated with migratory behavior. Only *Lasiurus borealis* and *Lasiurus cinereus* were found to have equal quantities of both hemoglobin types and both migrate distances which are great in relation to those for the other two species. Both *L. borealis* and *L. cinereus* range in Canada and the United States in summer but ordinarily winter farther south (Hall and Kelson 1959, p. 188).

This hypothesis partially ignores the non-migratory species found with multiple hemoglobins: *Pipistrellus hesperus*, *Pipistrellus subflavus*, and *Plecotus townsendi*. It also does not explain the fact that many *Myotis* range to high altitudes yet have only one hemoglobin type.

Another possible adaptive advantage of multiple hemoglobins in bats would be the presence of a hemoglobin with a reverse Bohr effect. This would allow a condition in which increased carbon dioxide levels would directly facilitate loading of the heme residues with oxygen (rather than indirectly causing the dissociation of oxyhemoglobin as is true for hemoglobins which have a normal Bohr effect). Thus far no reverse Bohr effects have been shown for vertebrate hemoglobins, but there are examples of this occurring in some invertebrate respiratory pigments. The snail *Fusitriton* and horseshoe crab *Limulus* both have hemocyanin
as their respiratory pigment and both of these have reverse Bohr effects (Manwell 1964).

Many bats live under conditions in which oxygen levels are lower and carbon dioxide levels are higher than those of atmospheric air. The most widely known example is the common guano bat, Tadarida brasiliensis, which often inhabits caves in large numbers. With Tadarida populations sometimes reaching into the millions, the occupied cave may have abnormal levels of oxygen, carbon dioxide, and ammonia. One such cave was investigated by Mitchell (1964) in Sonora, Mexico. This cave, a volcanic blowhole, contained up to three million Tadarida brasiliensis in addition to other species (Cockrum and Bradshaw 1963). During the periods of heaviest occupancy the carbon dioxide level increased tenfold (.03% to .3%) and at the same time oxygen levels fell to approximately one-half the normal value (13% as compared to the normal 21%).

In the initial phase of this study, hemoglobins from members of four bat families (Emballonuridae, Molossidae, Natalidae, and Phyllostomatidae) were separated electrophoretically and compared (Figure 8). Of the twelve species examined all are cave dwellers, but of those with multiple hemoglobins only Tadarida brasiliensis live under adverse conditions in which a hemoglobin with a reverse Bohr effect would be an advantage. Among these species only Tadarida
brasiliensis showed an equal distribution of hemoglobin types. However, after quantitation of hemoglobin types for the seven vespertilionid species found to possess multiple hemoglobins (Figure 7) this hypothesis of a reverse Bohr effect hemoglobin was partially abandoned. Three of the seven vespertilionid species with multiple hemoglobins, the lasiurans, are tree dwellers and none of the seven species live under conditions in which a reverse Bohr effect hemoglobin would seem to be advantageous.

**Chiropteran Hemoglobins and Phylogeny**

Variations found among the hemoglobins which were examined were so slight at the specific and generic levels that little phylogenetic inference can be made. For the 23 species of vespertilionid bats studied only three electrophoretically distinct hemoglobins were found, and these occurred in four combinations (Figure 5). Further, peptide mapping studies (Figures 10-13) showed that electrophoretically homogeneous hemoglobins probably have the same primary structure. Thus, one must agree in general with Zuckerkandl and Pauling (1962) that proteins, as a rule, have evolved so slowly that there may be few differences in primary structure within closely related species. This can also be shown by comparing hemoglobins of horses, pigs, cattle, and rabbits to that of man. Zuckerkandl (1965), in comparing the number of differences of these four
mammalian hemoglobins with human hemoglobin found a mean value of 22 amino acid substitutions between the animals and man. The mean of 22 differences between the hemoglobin of human and any one animal implies an average total of eleven mutations per chain or about one change per seven million years of evolution. This mutation rate was based on an estimated 80 million years of time since these animals evolved from a common ancestor.

Bats may be as old phylogenetically as any members of the other modern orders of placentals. Simpson (1935, 1937) found what he judged to be phyllostomatid fossils from the Paleocene. On this basis one would expect the number of variations in primary sequence of bat hemoglobins to be comparable to that found for horses, pigs, cattle, and rabbits, in relation to human hemoglobin. With so little variation shown among orders, one would expect fewer differences if comparing families, and still fewer differences between members of the same family.

Five electrophoretically distinct hemoglobins were found in members of five families of bats (Figure 8). In all but the Vespertilionidae, the species sample is so small that one cannot definitely conclude that the results will be the same for all members of a given family. Enough consistency is present, however, to allow some consideration of relationships. On the basis of electrophoretic mobilities,
the family Emballonuridae (hemoglobin Ra values .35 and .58) and Natalidae (hemoglobin Ra values .35 and .58) can be placed into one group. Of the remaining families, Molossidae (hemoglobin Ra values .45 and .66) and Vespertilionidae (hemoglobin Ra values .29, .45, and .66) appear more closely related. The family Phyllostomatidae appears intermediate, with one hemoglobin (Ra value .35) found in the Emballonuridae-Natalidae grouping and another hemoglobin (Ra value .66) present in the Molossidae-Vespertilionidae grouping.

These associations found among these five bat families are in agreement with those suggested by Miller (1907) in his classical work on chiropteran classification. Miller based family divisions on anatomical specialization of different bats in relation to the megachiropterans which he considered to be the most primitive of all bats. Although he thought the structure of the teeth of megachiropterans presented high degree of specialization, he felt that the development of the wings and the form of the skull represented an earlier evolutionary stage. On the basis of these characters, the Megachiroptera more nearly resemble non-chiropteran mammals than do the Microchiroptera. In a group of volant animals the chief taxonomic importance must be assigned to the development of the wings.
Based on anatomical specialization associated with flight, Miller placed the five families considered in this study in the following evolutionary sequence (primitive < specialized): Emballonuridae < Natalidae < Phyllostomatidae < Molossidae < Vespertilionidae. He called the Emballonuridae the most primitive of all New World microchiropterans. Natalidae, based on skull similarities appears phylogenetically near the Phyllostomatidae. Molossidae and Vespertilionidae were considered among the most specialized of all bats. A very high evolutionary position was given to the Vespertilionidae because of the structure of the wing. In this character the family stands further removed from megachiropterans and from nonvolant mammals than any other group except the Molossidae and Mystocopidae. In the extreme reduction of the ulna it even surpasses both of these.

On the bases of numbers and electrophoretic properties, hemoglobins appear to complement the phylogenetic associations suggested by Miller. His phylogeny was based entirely on anatomical changes associated with functional changes. For hemoglobins no such phylogeny can be made. Until physiological differences are found for hemoglobins that have varying physico-chemical properties it cannot be determined if one hemoglobin is more primitive or advanced than another. No phylogenetic tree, then, can be made from the hemoglobins shown by this study. There does appear to be a taxonomic correlation between anatomical specializa-
tion associated with flight in bats and electrophoretic mobilities and numbers of hemoglobins.
SUMMARY AND CONCLUSIONS

This study was conducted to investigate some of the physico-chemical properties of bat hemoglobins and the possibility of their use in biochemical taxonomy. Emphasis was placed on members of the family Vespertilionidae with hemoglobins from 23 species electrophoretically isolated and compared. Hemoglobins from members of four other bat families were also investigated: Emballonuridae (1 species), Natalidae (1 species), and Phyllostomatidae (8 species). Parameters investigated and results obtained were:

1) Analysis of hemoglobin numbers and electrophoretic mobilities revealed no population or geographic variation. In the cases where more than one species was examined in a given genus, variations in hemoglobin numbers were found in three of the five genera.

2) Variations in hemoglobin numbers and electrophoretic mobilities in vespertilionids examined allowed the 23 species to be divided into four groups: Group I, one hemoglobin (R_a value .45), 15 species; Group II, one hemoglobin (R_a value .66), one species; Group III, two hemoglobins (R_a values .45 and .66), six species; and Group
IV, three hemoglobins (Rα values .29, .45, and .66), one species.

3) Peptide mapping studies of representative hemoglobins from the four vespertilionid hemoglobin groups showed that electrophoretically homogeneous hemoglobins are probably the same in primary structure.

4) Color tests for specific amino acids present in bat hemoglobin peptides gave the following:
   a) the quantities of arginine, histidine, tyrosine, and tryptophan positive bat hemoglobin peptides appears similar to that of other vertebrate hemoglobins; and b) no methionine containing peptides were found in any of the hemoglobins studied by the peptide mapping technique.

5) Quantitation of hemoglobins for the seven vespertilionids with multiple hemoglobins gave varying results. In four of the six species found with two hemoglobins, the slower electrophoretically migrating hemoglobin was the most common. The other two species had hemoglobins of equal concentrations. For *Pipistrellus hesperus*, the only species with three hemoglobins, the fastest electrophoretically migrating hemoglobin was the major portion of the total complement.
6) Possible physiological advantages of multiple hemoglobins in bats may be associated with conditions of low oxygen partial pressure. Of the seven vespertilionids found with multiple hemoglobins, four are migratory and have wide altitudinal ranges.

7) In comparing all hemoglobins found among the five families of bats investigated in this study certain relationships are suggested. On the bases of similarities of hemoglobin numbers and electrophoretic properties the following families are considered more closely related: Emballonuridae-Natalidae, Molossidae-Vespertilionidae. The Phyllostomatidae occupy an intermediate position between the two groupings.

8) Results from this study suggest that hemoglobins may be considered to be a phylogenetic conservative character in bats. Their use in understanding chiropteran phylogeny appears to lie at the family level.
APPENDIX I

SPECIMENS EXAMINED

Below are listed the collection sites of specimens examined in this study along with the depositories of skins and institutional catalogue numbers. Abbreviations used are as follows: UA (University of Arizona); UAX (University of Arizona - catalogue number not yet assigned); NAU (Northern Arizona University); A (are deposited at CSIRO, Division of Wildlife Research, Canberra City, Australia, with no catalogue numbers available).

Emballonuridae

**Balantiopteryx plicata**

NAYARIT: Cerro Contaduria, 1 mi E San Blas (2 males, 2 females) (UA16985, UA17243-UA17245).

Molossidae

**Tadarida brasiliensis**

ARIZONA: Mohave Co. 1 mi S Kingman (2 males, 2 females).

Pinal Co. Railroad Bridge S. of San Carlos Lake (2 males, 2 females).

Natalidae

**Natalus stramineus**

SONORA: Mina Armolillo, 4.5 mi NNW Alamos, 2 mi S Piedras Verdes (3 males, 3 females).
Phyllostomatidae

Artibeus hirsutus
SONORA: Hacienda Las Delicias, Alamos (1 male) (UA16871).

Glossophaga soricina
SONORA: La Aduana Mine, 5 mi W Alamos (1 male, 1 female); Mina Armolillo, 4.5 mi NNW Alamos, 2 mi S Piedras Verdes (1 male, 1 female).

Leptonycteris sanborni
SONORA: Mina Armolillo, 4.5 mi NNW Alamos, 2 mi S Piedras Verdes (2 males, 2 females).

Macrotus waterhousii
ARIZONA: Pima Co. Fortuna Mine, Silver Bell Mts. (3 males, 2 females).

Pteronotus davyi
SONORA: La Aduana Mine, 5 mi W Alamos (1 male, 1 female) (UA16869-16870).

Pteronotus parnellii
SONORA: La Aduana Mine, 5 mi W Alamos (1 male, 1 female) (UA17239-UA17240).

Pteronotus psilotus
SONORA: La Aduana Mine, 5 mi W Alamos (1 male, 2 females) (UA16743, UA16872, UA16917).

Sturnira lilium
SONORA: Rio Alamos, 8 mi S Alamos (1 female) (UA17116).
Vespertilionidae

**Antrozous pallidus**


**Eptesicus fuscus**

ARIZONA: Cochise Co. Herb Martyr Dam, Chiricahua Mts. (2 females); Southwest Research Station, Chiricahua Mts. (2 males). Coconino Co. Turkey Tanks, 3-3/4 mi N old Hwy 66 off Leupp Rd (2 males). Pima Co. Pueblo High School, Tucson (1 male, 4 females); Railroad Bridge 5 mi S Continental (4 females); Rose Canyon Lake, Santa Catalina Mts. (1 male).

MISSOURI: McDonald Co. Henderson Cave, 1 mi W Jane (1 male, 1 female) (UA16990, UA17019).

**Lasionycteris noctivagans**

ARIZONA: Cochise Co. Herb Martyr Dam, Chiricahua Mts. (2 males) (UA16865-UA16866); Southwest Research Station, Chiricahua Mts. (2 males, 2 females) (A).
Lasiurus borealis
SONORA: Rio Alamos, 8 mi by road S Alamos (1 male) (UA17267).

Lasiurus cinereus
ARIZONA: Cochise Co. Herb Martyr Dam, Chiricahua Mts. (2 males, 3 females) (UA16863, UA16864, 3A).
Coconino Co. Turkey Tanks, 3-3/4 mi N old Hwy 66 off Leupp Rd (1 male) (UA17274). Pima Co. Upper Sabino Canyon, Coronado National Forest (2 males) (UA16877, UA16878).

Lasiurus ega
ARIZONA: Cochise Co. Herb Martyr Dam, Chiricahua Mts. (1 female) (UA17011).
SONORA: Rio Alamos, 8 mi by road S Alamos (1 male) (UA16862).

Myotis californicus
ARIZONA: Pima Co. Canyon Del Oro High School (2 females) (UAX); Mine at entrance to Madera Canyon (1 male, 4 females) (UA17197-UA17201). Pinal Co. Morning Glory Mine, Hi Jinks Rd., 4 mi E Oracle (1 male, 1 female) (UA16988, UA17074); Tungsten Mine, 3 mi S Oracle, Coronado National Forest (1 female) (UA16987).
**Myotis fortidens**

SONORA: Hacienda Las Delicias, Alamos (4 males, 1 female) (UA17073, UA17230, UA17231, UA17266, 1UAX).

**Myotis grisescens**

KENTUCKY: Edmonson Co. James Cave near Park City (3 males, 3 females) (UA16999, UA17022, UA17023, UA17111, UA17192, 1UA1).

MISSOURI: McDonald Co. Henderson Cave, 1 mi W Jane (1 male) (UA17255).

**Myotis lucifugus**


PENNSYLVANIA: Bucks Co. Durham Mine, Durham (3 males, 3 females (UA17094, UA17095, UA17109, UA17119 UA17121).

**Myotis sodalis**

KENTUCKY: Edmonson Co. James Cave near Park City (2 males, 2 females) (UA17024-UA17026, UA17096).

**Myotis subulatus**


COLORADO: Larimer Co. Tunnel 6 mi NW Livermore (1 male, 3 females) (UA17098-UA17100, UA17123).
**Myotis thysanodes**

ARIZONA: Cochine Co. Pyatt Cave ca. 1 mi S West Gate Ft. Huachuca (1 male) (UA); Coconino Co. Boulin Tank, R3E, T23N, Sect 11 (1 female) (UA16263). Pima Co. Mine at entrance to Madera Canyon (4 males) (UA7193-UA17196); Mine ca. 1/2 mi E Bench Mark 3332 in W Foothills Baboquivari Mts. (2 males) (UA). Yavapai Co. 8 mi E Camp Verde (1 male, 1 female) (NAU284, NAU285).

**Myotis velifer**

ARIZONA: Cochine Co. General Store at Cochine (5 males). Graham Co. Hwy Bridge 6.5 mi NW Pima (4 females). Pinal Co. Railroad Bridge S San Carlos Lake (1 female); Picacho Mine (1 male, 1 female).

**Myotis volans**

ARIZONA: Coconino Co. Boulin Tank R3E, T23N, Sect 11 (3 females) (UA16264-UA16266); Turkey Tanks 3-3/4 mi N old Hwy 66 off Leupp Rd (1 male, 1 female) (UA16992, UA17265).

**Myotis yumanensis**

ARIZONA: Pinal Co. Railroad Bridge S San Carlos Lake (3 males, 6 females) (UA16892-UA16896, UA17256-UA17258, UAX).
SONORA: Rio Alamos, 8 mi by road S Alamos (1 female) (UA16919).

_Nycticeus humeralis_

MISSOURI: Ozark Co. Hawksley Pond near Zononi (3 females, 1 male) (UAX).

_Pipistrillus hesperus_


SONORA: Hacienda Las Delicias, Alamos (3 females) (UA17227-UA17229); Rio Alamos, 8 mi by road S Alamos (1 male) (UA17202).

_Pipistrillus subflavus_

MISSOURI: McDonald Co. Henderson Cave, 1 mi W Jane (1 male) (UA16952).

PENNSYLVANIA: Berks Co. South Temple Cave (3 males, 3 females) (UA16976-UA16980, UA17007).

_Pizonyx vivesi_

SONORA: Partida Island, San Carlos Bay, Gulf of California (1 female) (UA17203).
Plecotus phyllotis

ARIZONA: Mohave Co. mine tunnel 2 mi W Union Pass, Black Mts. (2 males, 3 females) (UA16342-UA16345, UA16920).

Plecotus townsendi


COLORADO: Larimer Co. Tunnel 6 mi NW Livermore (1 female) (UA17098).

Rhogeesa parvula

SONORA: Rio Alamos, 8 mi by road S Alamos (4 males, 1 female) (UA16956, UA16981-UA16983, UA16991).
LITERATURE CITED


