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# AN ELECTROPHORETIC COMPARISON OF HEMOGLOBINS IN BATS

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**Abstract**—1. Three electrophoretically distinct hemoglobins, in four different patterns, were found in twenty-three species of vespertilionid bats.

2. Only two additional hemoglobins were found in four other bat families (one species each of Emballonuridae, Molossidae and Natalidae, and eight species of Phyllostomatidae).

3. Peptide mapping of hemoglobins from representatives of the four vespertilionid groupings showed that electrophoretically homogeneous hemoglobins probably have the same primary structure.

4. It appears that hemoglobins may be useful in determining chiropteran phylogeny at the family level, but not below.

## INTRODUCTION

ZOOLOGICAL taxonomy was once limited to direct measurements of morphological characters as a basis for classification, but has been expanded in recent years to include many other criteria, such as serum proteins, enzyme patterns, karyology and serological differences. One protein investigated as a possible taxonomic tool has been hemoglobin. Many have compared hemoglobin numbers and electrophoretic properties with varying results. In some instances, hemoglobins appear useful in separating species (Foreman, 1960, 1964, 1966, 1968; Guttman, 1967, 1969; Ahl, 1968), but in others they appear phylogenetically more conservative (Dunlap *et al.*, 1956; Kitchen *et al.*, 1968; Rasmussen *et al.*, 1968).

Few have considered the electrophoretic properties of bat hemoglobins as a possible supplement to chiropteran classification. Thus far, hemoglobins for only eleven species have been investigated. Manwell & Kerst (1966) and Mitchell (1966) compared hemoglobins from species of the bat family Vespertilionidae, and Valdivieso *et al.* (1969) expanded this to include members of the Phyllostomatidae and the Molossidae. This study compares hemoglobins from a larger sample of species and families in an attempt to determine their value as a biochemical character.

## MATERIALS AND METHODS

The sources of all specimens used in this study are listed in the Appendix.

Blood samples were drawn by heart puncture from live bats with a 0·5 $cm^3$ tuberculin syringe wetted with heparin solution. Hemoglobins were prepared for electrophoretic studies in the manner described in Seligson (1961), with the hemoglobin suspended in a

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0·06 M barbital buffer, pH 8·6 (10·3 g/l. Na barbital, 1·4 g/l. barbital in $CO_2$-free $H_2O$). Storing at −29°C for up to 1 year produced no varying results in electrophoretic properties.

Hemoglobins were separated electrophoretically in an EC470 Vertical Gel Electrophoresis Cell (E-C Apparatus Corp., Philadelphia, Pennsylvania). Samples in a 7% cyanogum gel were run for 2 hr at 0°C and 400 V constant voltage in a Tris–$Na_2$EDTA*–Borate buffer of pH 8·4. Hyland human hemoglobin electrophoresis controls A, C, F and S (Hyland Lab., Los Angeles, California) were subjected to electrophoresis simultaneously to serve as reference standards.

Hemoglobin bands were developed in the gel with a peroxidase stain as described in the E-C Corp. *Technical Bulletin* 141. When more than one hemoglobin fraction was present, stained bands were cut from the gel, each was thoroughly macerated in 4·0 ml glacial acetic acid and the mixture was centrifuged to remove the gel, and the optical density of the eluted dye was read at 405 mμ. The total optical density of all bands from each sample was taken as 100 per cent of the hemoglobin present, and the density of each individual hemoglobin fraction was represented as a percentage of this total.

The initial preparation of hemoglobin samples for peptide mapping was the same as used for the vertical acrylamide gel electrophoresis. Hemoglobin stored in 0·06 M barbital buffer, pH 8·6, was 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 purified by anion exchange column chromatography with DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) according to the method described by Leyko & Gondko (1963). Purity was ascertained by discontinuous gel electrophoresis following the procedure given in E-C Corp. *Technical Bulletin* 140.

Following purification, the samples were dialyzed against distilled water to remove salts used to elute them from the column and then digested with trypsin by the method of Chernoff & Lui (1961). Following digestion, the mixture was lyophilized to remove the volatile $(NH_4)_2CO_3$ buffer and to concentrate the resulting peptides.

For peptide mapping, 4·5–5 mg of peptide concentrate (1 mg peptides/10 μl 0·02 M $NH_4HCO_3$) were applied to Whatman No. 3MM paper in 10-μl aliquots. These samples were subjected to 22 hr of descending chromatography in n-butanol–acetic acid–water (4 : 1 : 5) in a standard chromatography cabinet, then to 1½ hr of high-voltage electrophoresis (2000 V) in a Savant E-800-213 electrophoresis tank (Savant Instruments Inc., Hickman, New York) in a formic acid–acetic acid buffer (formic acid 21 ml; glacial acetic acid 80 ml; $H_2O$ 900 ml, pH 1·9). Specific staining reactions as described by Easley (1965) were used to characterize the peptides.

## RESULTS

### *Electropherograms of bat hemoglobins*

Figure 1 gives a composite of the four hemoglobin patterns found for the twenty-three vespertilionid species examined. In this study, no intraspecific variations in hemoglobin numbers or mobilities were observed except in one instance. A gravid *Eptesicus fuscus* from one sample demonstrated two hemoglobin fractions; all other *E. fuscus* investigated had only one. Mitchell (1966) and Brush (1968) found one hemoglobin for this species, but Manwell & Kerst (1966) and Valdivieso *et al.* (1969) found two.

The electrophoretic values given in Fig. 1 are based on mean $R_a$ values (relative to migration of human hemoglobin A). For all twenty-three vespertilionid species studied, there were only three hemoglobin bands, present in four combinations:

* Disodium ethylenediaminetetraacetic acid.

Group I ($R_a$ value 0·45), *Antrozous pallidus*, *Eptesicus fuscus*, *Myotis californicus*, *M. fortidens*, *M. grisescens*, *M. lucifugus*, *M. sodalis*, *M. subulatus*, *M. thysanodes*, *M. velifer*, *M. volans*, *M. yumanensis*, *Pizonyx vivesi*, *Plecotus phyllotis*, *Rhogeesa parvula*; Group II ($R_a$ value 0·66), *Nycticeus humeralis*; Group III ($R_a$ values 0·45 and 0·66), *Lasionycteris noctivagans*, *Lariurus borealis*, *L. cinereus*, *L. ega*, *Pipistrellus subflavus*, *Plecotus townsendi*; Group IV ($R_a$ values 0·29, 0·45 and 0·66), *Pipistrellus hesperus*.

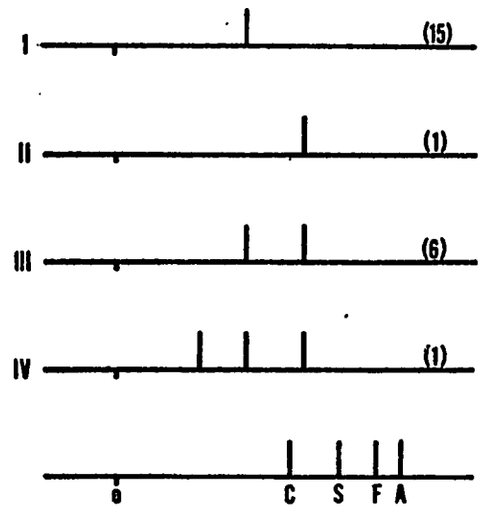


FIG. 1. Composite of hemoglobin bands of four patterns for twenty-three species of vespertilionid bats. Bands are shown in the order of their relative mobilities from the origin (O). Figures in parentheses represent the number of species showing this hemoglobin pattern. The bottom line gives mobilities for human hemoglobins C ($R_a$ value 0·61), S ($R_a$ value 0·78), F ($R_a$ value 0·91) and A.

The relative distribution of the multiple hemoglobin fractions in Groups III and IV were (slower migrating hemoglobin–faster migrating hemoglobin): *Lasiurus borealis*, *L. cinereus* (50–50 per cent); *L. ega* (67–33 per cent); *Lasionycteris noctivagans* (60–40 per cent); *Plecotus townsendi* (55–45 per cent); and *Pipistrellus subflavus* (58–42 per cent). *Pipistrellus hesperus*, the only species with three hemoglobins, had concentrations of 28–30–42 per cent.

Hemoglobins found in members of the other four families of bats examined were as follows. Molossidae: In the one species examined, *Tadarida brasiliensis*, two hemoglobins were found ($R_a$ values 0·45 and 0·66). Phyllostomatidae: In eight species examined, two hemoglobins were found—Group I (one hemoglobin, $R_a$ value 0·66), *Artebius hirsutus*, *Glossophaga soricina*, *Leptonycteris sanborni*, *Macrotus waterhousii*, *Pteronotus parnelli* and *Sturnira lilium*; Group II (two hemoglobins, $R_a$ values 0·35 and 0·66), *Pteronotus davyi* and *P. psilotis*. Natalidae: In one species examined, *Natalus stramineus*, two hemoglobins were found ($R_a$

values 0·35 and 0·58). Emballonuridae: In one species examined, *Balantiopteryx plicata*, two hemoglobins were found ($R_a$ values 0·35 and 0·58). Figure 2 shows a composite of the various hemoglobins by family.

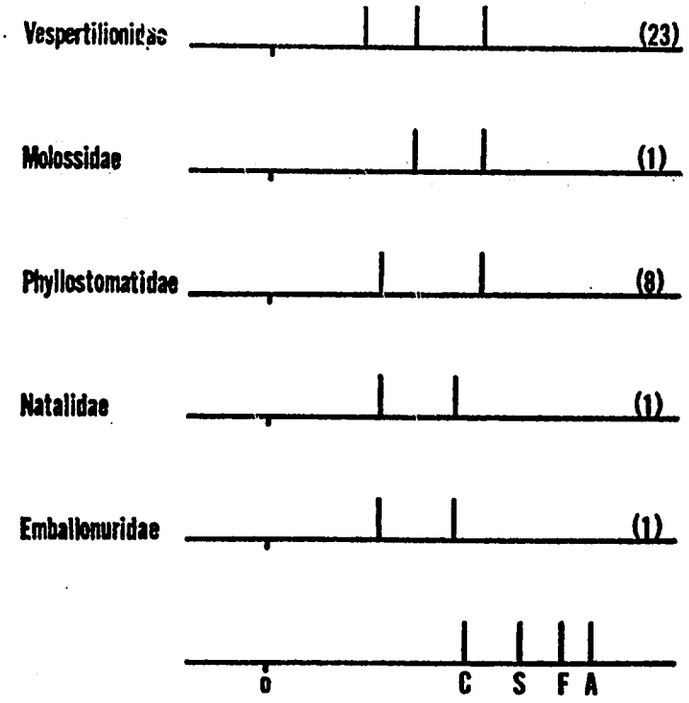


FIG. 2. Composite diagram of all hemoglobin bands found in five families of bats. Bands are shown in the order of their relative mobilities from the origin (O). Figures in parentheses represent the number of species from the family investigated. The bottom line gives mobilities for human hemoglobins C ($R_a$ value 0·61), S ($R_a$ value 0·78), F ($R_a$ value 0·91) and A.

*Hemoglobin peptide maps*

Hemoglobin peptide maps were made for eight vespertilionid species (Figs. 3–6). A map of human hemoglobin A tryptic peptides prepared at the same time is presented in Fig. 7 for comparison. Numbering of the various bat peptides is based on the numbering system for human hemoglobin A suggested by Chernoff & Liu (1961).

Electrophoretically homogeneous hemoglobins all gave similar peptide mapping pictures. The electrophoretic mobilities of the individual hemoglobins found in Group I ($R_a$ value 0·45) and Group II ($R_a$ value 0·66) were identical with the two hemoglobins for Group III. If the hemoglobins are identical in primary structure, one would expect the peptide map for Group III (Fig. 5) to be a composite of Groups I and II (Figs. 3 and 4). This proved to be true, except that the peptide

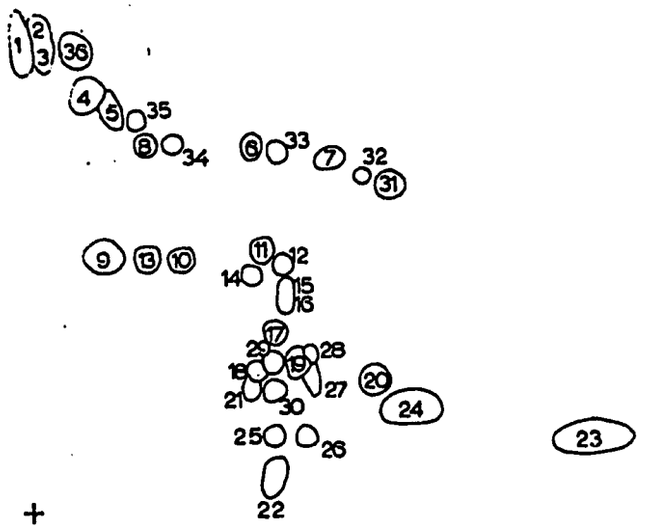


FIG. 3. Tryptic peptide map for four vespertilionid bat species belonging to hemoglobin Group I (one hemoglobin, $R_a$ value 0·45): *Antrozous pallidus*, *Myotis fortidens*, *M. thysanodes* and *M. yumanensis*. The point of sample application was at the intersection of the ruled lines. Chromatography was performed first along the horizontal axis and high-voltage electrophoresis was then carried out along the vertical axis.

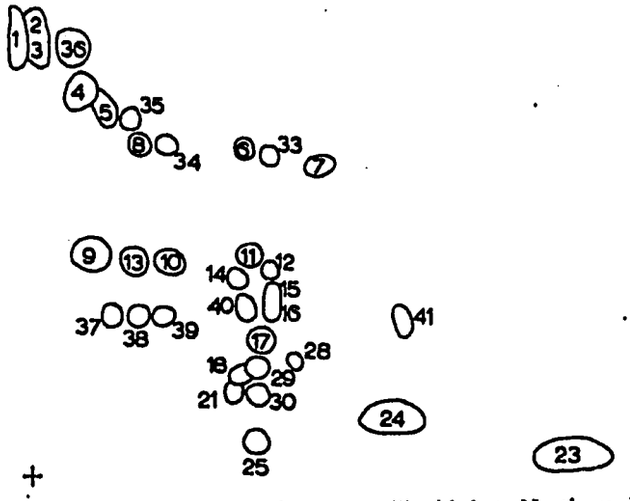


FIG. 4. Tryptic peptide map for the vespertilionid bat *Nycticeus humeralis*, belonging to hemoglobin Group II (one hemoglobin, $R_a$ value 0·66).

designated as number 22 in Group I was not visible in the maps of Groups II and III. Peptide 22 may be present in these groups but perhaps was masked by other peptides.

The map for Group IV (Fig. 6) included the peptides found in Groups I, II and III, plus peptides 42–49 from this group's third hemoglobin. In comparing the Group IV map with the others, it is evident that peptides designated the same

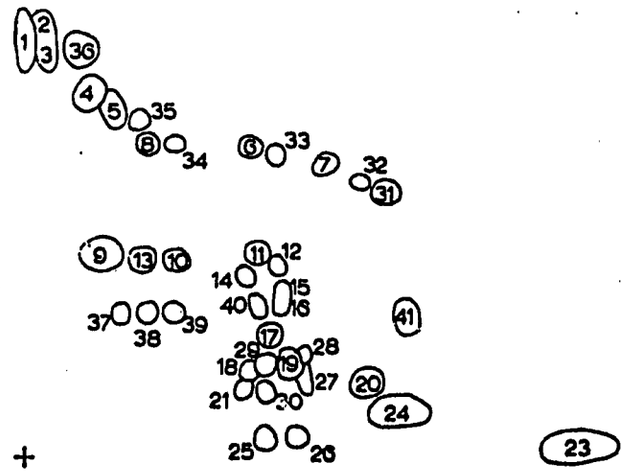


FIG. 5. Tryptic peptide map for two vespertilionid bat species belonging to hemoglobin Group III (two hemoglobins, $R_a$ values 0·45 and 0·66): *Lasionycteris noctivagans* and *Plecotus townsendi*.

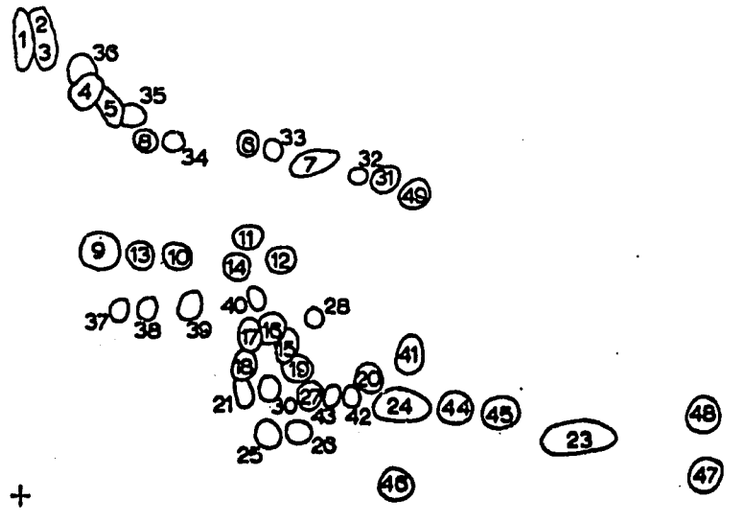


FIG. 6. Tryptic peptide map for the vespertilionid bat *Pipistrellus hesperus*, belonging to hemoglobin Group IV (three hemoglobins, $R_a$ values 0·29, 0·45 and 0·66).

in all maps (e.g. 15 and 16) are somewhat displaced in the map for Group IV. In addition, peptide 29 was not found in the Group IV map. This may indicate that it was not present, was hidden by another peptide or was incorrectly identified.

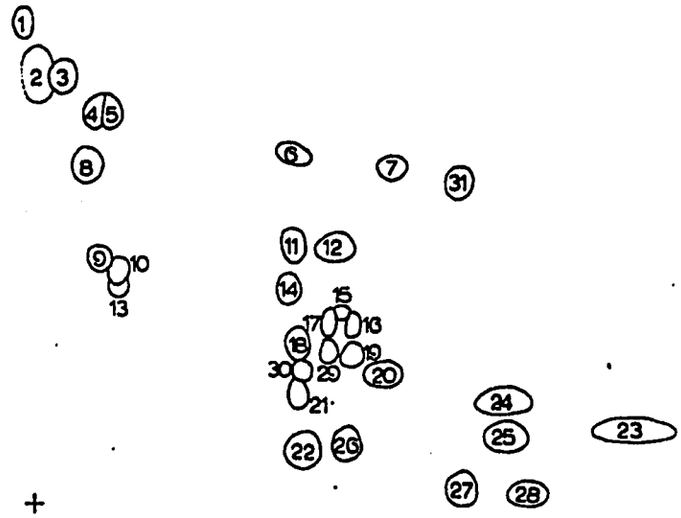


FIG. 7. Human hemoglobin A tryptic peptide map.

## DISCUSSION

Variations found among the hemoglobins examined were so slight at the specific and generic levels that little phylogenetic inference can be made. For the twenty-three species of vespertilionids studied, only three electrophoretically distinct hemoglobins were found, and these occurred in only four combinations (Fig. 1). Further, peptide mapping studies (Figs. 3–6) showed that electrophoretically homogeneous hemoglobins probably have the same primary structure. Thus, one must agree in general with Zuckerkandl & 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 was also shown by Zuckerkandl (1965), who found there was an average of twenty-two amino acid substitutions when he compared the hemoglobins of horses, pigs, cattle and rabbits with those of man. The mean of twenty-two differences between the hemoglobin of humans and that of any one of the four animals implies an average of eleven mutations per hemoglobin chain, or about one change per 7 million years (assuming 80 million years since these animals evolved from a common ancestor).

Bats may be as old phylogenetically as any 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 no greater hemoglobin variations in bats than horses, pigs, cattle and rabbits showed in relation to man. With so little variation shown among orders, one would expect few differences between families, still fewer between members of the same family.

Five electrophoretically distinct hemoglobins were found in members of five families of bats (Fig. 2). In all but the Vespertilionidae, the species sample was too small to conclude that the results will be the same for all members of a given family. Enough consistency is present, however, to allow some taxonomic speculation. On the basis of electrophoretic mobilities, the Emballonuridae and Natalidae (both with hemoglobin $R_a$ values of 0·35 and 0·58) can be placed in one group. Of the remaining families, Molossidae ($R_a$ values 0·45 and 0·66) and Vespertilionidae ($R_a$ values 0·29, 0·45 and 0·66) appear the most closely related. The family Phyllostomatidae appears intermediate, with one hemoglobin ($R_a$ value 0·35) found in the Emballonuridae–Natalidae grouping and another ($R_a$ value 0·66) found in the Molossidae–Vespertilionidae grouping.

These associations are in agreement with those suggested by Miller (1907) in his classical work on chiropteran classification. On the basis of 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. From skull similarities, he placed Natalidae near Phyllostomatidae. Molossidae and Vespertilionidae were considered among the most specialized of all bats.

On the basis of numbers and electrophoretic properties, hemoglobins appear to confirm the phylogenetic associations suggested by Miller. His phylogeny was based on anatomical changes associated with functional changes. No such phylogeny can be proposed from the hemoglobin data in this study. Until physiological differences are found associated with the different hemoglobins, it appears one cannot determine if one hemoglobin is more primitive or advanced than another.

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

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*Key Word Index*—Electrophoresis; hemoglobin; bats; peptide mapping; taxonomy; Vespertilionidae; Molossidae; Phyllostomatidae; Natalidae; Emballonuridae.

## APPENDIX

*Specimens examined*

Below are listed the collection sites of specimens examined in this study.

Emballonuridae

*Balantiopteryx plicata*

NAYARIT: Cerro Contaduria, 1 mile E. San Blas (2 ♂, 2 ♀).

Molossidae

*Tadarida brasiliensis*

ARIZONA: Mohave Co., 1 mile S. Kingman (2 ♂, 2 ♀). Pinal Co., railroad bridge S. of San Carlos Lake (2 ♂, 2 ♀).

Natalidae

*Natalus stramineus*

SONORA: Mina Armolillo, 4·5 miles N.N.W. Alamos, 2 miles S. Piedras Verdes (3 ♂, 3 ♀).

Phyllostomatidae

*Artibeus hirsutus*

SONORA: Hacienda Las Delicias, Alamos (1 ♂).

*Glossophaga soricina*

SONORA: La Aduana Mine, 5 miles W. Alamos (1 ♂, 1♀); Mina Armolillo, 4·5 miles N.N.W. Alamos, 2 miles S. Piedras Verdes (1 ♂, 1 ♀).

*Leptonycteris sanborni*

SONORA: Mina Armolillo, 4·5 miles N.N.W. Alamos, 2 miles S. Piedras Verdes (2 ♂, 2 ♀).

*Macrotus waterhousii*

ARIZONA: Pima Co. Fortuna Mine, Silver Bell Mts. (3 ♂, 2 ♀).

*Pteronotus davyi*

SONORA: La Aduana Mine, 5 miles W. Alamos (1 ♂, 1 ♀).

*P. parnelli*

SONORA: La Aduana Mine, 5 miles W. Alamos (1 ♂, 1 ♀).

*P. psilotis*

SONORA: La Aduana Mine, 5 miles W. Alamos (1 ♂, 2 ♀).

*Sturnira lilium*

SONORA: Rio Alamos, 8 miles S. Alamos (1 ♀).

Vespertilionidae

*Antrozous pallidus*

ARIZONA: Cochise Co., general store at Cochise (5 ♂, 3 ♀). Pinal Co., railroad bridge S. of San Carlos Lake (5 ♀).

*Eptesicus fuscus*

ARIZONA: Cochise Co., Herb Martyr Dam, Chiricahua Mts. (2 ♀); Southwest Research Station, Chiricahua Mts. (2 ♂). Coconino Co., Turkey Tanks, 3¾ miles N. old Highway 66 off Leupp Road (2 ♂). Pima Co., Pueblo High School, Tucson (1 ♂, 4 ♀); railroad bridge 5 miles S. Continental (4 ♀); Rose Canyon Lake, Santa Catalina Mts. (1 ♂).

MISSOURI: McDonald Co., Henderson Cave, 1 mile W. Jane (1 ♂, 1 ♀).

*Lasionycteris noctivagans*

ARIZONA: Cochise Co., Herb Martyr Dam, Chiricahua Mts. (2 ♂); Southwest Research Station, Chiricahua Mts. (2 ♂, 2 ♀).

*Lasiurus borealis*

SONORA: Rio Alamos, 8 miles by road S. Alamos (1 ♂).

*L. cinereus*

ARIZONA: Cochise Co., Herb Martyr Dam, Chiricahua Mts. (2 ♂, 3 ♀). Coconino Co., Turkey Tanks, 3¾ miles N. old Highway 66 off Leupp Rd (1 ♂). Pima Co., Upper Sabino Canyon, Coronado Nat. Forest (2 ♂).

*L. ega*

ARIZONA: Cochise Co., Herb Martyr Dam, Chiricahua Mts. (1 ♀).

SONORA: Rio Alamos, 8 miles by road S. Alamos (1 ♂).

*Myotis californicus*

ARIZONA: Pima Co., Canyon Del Oro High School (2 ♀); mine at entrance to Madera Canyon (1 ♂, 4 ♀). Pinal Co., Morning Glory Mine, Hi Jinks Road, 4 miles E. Oracle (1 ♂, 1 ♀); Tungsten Mine, 3 miles S. Oracle, Coronado Nat. Forest (1 ♀).

*M. fortidens*

SONORA: Hacienda Las Delicias, Alamos (4 ♂, 1 ♀).

*M. grisescens*

KENTUCKY: Edmonson Co., James Cave near Park City (3 ♂, 3 ♀).

MISSOURI: McDonald Co., Henderson Cave, 1 miles W. Jane (1 ♂).

*M. lucifugus*

ARIZONA: Coconino Co., Boulin Tank, *ca.* 10 miles N.E. Williams (1 ♂).

PENNSYLVANIA: Bucks Co., Durham Mine, Durham (3 ♂, 3 ♀).

*M. sodalis*

KENTUCKY: Edmonson Co., James Cave near Park City (2 ♂, 2 ♀).

*M. subulatus*

ARIZONA: Coconino Co., Boulin Tank, *ca.* 10 miles N.E. Williams (1 ♂).

COLORADO: Larimer Co., tunnel 6 miles N.W. Livermore (1 ♂, 3 ♀).

*M. thysanodes*

ARIZONA: Cochise Co., Pyatt Cave, *ca.* 1 mile S. West Gate Fort Huachuca (1 ♂). Coconino Co., Boulin Tank, *ca.* 10 miles N.E. Williams (1 ♀). Pima Co., mine at entrance to Madera Canyon (4 ♂); mine in W. foothills Baboquivari Mts. (2 ♂). Yavapai Co., 8 miles E. Camp Verde (1 ♂, 1 ♀).

*M. velifer*

ARIZONA: Cochise Co., general store at Cochise (5 ♂). Graham Co., Highway Bridge 6·5 miles N.W. Pima (4 ♀). Pinal Co., railroad bridge S. San Carlos Lake (1 ♀); Picacho Mine (1 ♂, 1 ♀).

*M. volans*

ARIZONA: Coconino Co., Boulin Tank, *ca.* 10 miles N.E. Williams (3 ♀); Turkey Tanks 3¾ miles N. old Highway 66 off Leupp Road (1 ♂, 1 ♀).

*M. yumanensis*

ARIZONA: Pinal Co., railroad bridge S. San Carlos Lake (3 ♂, 6 ♀).

SONORA: Rio Alamos, 8 miles by road S. Alamos (1 ♀).

*Nycticeus humeralis*

MISSOURI: Ozark Co., Hawksley Pond near Zononi (1 ♂, 3 ♀).

*Pipistrellus hesperus*

ARIZONA: Cochise Co., Southwest Research Station, Chiricahua Mts. (1 ♀). Mohave Co., pond *ca.* 1 mile S. Kingman Hilltop area (1 ♂, 5 ♀).

SONORA: Hacienda Las Delicias, Alamos (3 ♀); Rio Alamos, 8 miles by road S. Alamos (1 ♂).

*P. subflavus*

MISSOURI: McDonald Co., Henderson Cave, 1 mile W. Jane (1 ♂).

PENNSYLVANIA: Berks Co., South Temple Cave (3 ♂, 3 ♀).

*Pizonyx vivesi*

SONORA: Partida Island, San Carlos Bay, Gulf of California (1 ♀).

*Plecotus phyllotis*

ARIZONA: Mohave Co., mine tunnel 2 miles W. Union Pass, Black Mts. (2 ♂, 3 ♀).

*P. townsendi*

ARIZONA: Cochise Co., El Tigre Mine, Pinery Canyon, Chiricahua Mts. (2 ♀). Pima Co., rock slide N. side Sentinel Butte, Saguaro National Monument (3 ♂, 3 ♀). Pinal Co., Morning Glory Mine, Hi Jinks Rd., 4 miles E. Oracle (1 ♂).

COLORADO: Larimer Co., tunnel 6 miles N.W. Livermore (1 ♀).

*Rhogeesa parvula*

SONORA: Rio Alamos, 8 miles by road S. Alamos (4 ♂, 1 ♀).