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SYSTEMATIC STUDIES OF GROUND SQUIRRELS (Genus Spermophilus) BY COMPARATIVE SERUM PROTEIN ANALYSIS

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ROY DEWAYNE IKENBERRY

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SYSTEMATIC STUDIES OF GROUND SQUIRRELS (Genus Spermophilus)
BY COMPARATIVE SERUM PROTEIN ANALYSIS

APPROVED BY

Clyde E. Hopla

George C. Cozad

Howard Haines

J. K. Greer

DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

Systematic zoology has been remarkably enhanced by recent advances and improved techniques in cytogenetics, behavior, ecology, physiology, and biochemistry. Through judicious use of the methods available to him, the modern systematist can revaluate phylogenetic relationships that were formerly determined through morphological characteristics. Advances in genetics have led to the acceptance of the one gene-one polypeptide theory. Critical analyses of serum proteins have demonstrated that differences in the mobility and composition of the protein fractions may be the result of homozygous and heterozygous expression of several alleles (Nadler, 1968). Systematic analyses at the level of the gene will undoubtedly provide further insight into the phylogenies of biological species. Investigations at the level of the gene often require elaborate and expensive methods, therefore, emphasis has been placed on the primary product of gene action, the proteins. Numerous reliable and inexpensive methods of separation and characterization of proteins are available. Electrophoresis,

immunoelectrophoresis, and related immunological methods can be modified to give both quantitative and qualitative separation and characterization of serum proteins.

Electrophoretic studies have been applied to such diverse proteins as hemolymph of the developmental stages of lepidopterans (Loughton and West, 1965), hemolymph patterns as species characters in holothurians (Manwell and Baker, 1963), and avian egg-white proteins as taxonomic characters (Sibley, 1960). Extensive studies using electrophoretic methods on the serum proteins of numerous species of reptiles and amphibians have demonstrated differences at several taxonomic levels (Dessauer and Fox, 1956, 1962), and (Fox, Dessauer, and Maumus, 1961). Recently, electrophoretic methods have been applied to studies involving serum proteins of different populations of the white-footed deer mouse, Peromyscus maniculatus (Ahl, 1968) and to serum protein heterogeneity in inbred strains of laboratory rats (Rattus sp.) (Dolezalova and Brada, 1968). These studies reveal that electrophoretic methods are highly sensitive techniques in distinguishing subtle differences within species or strains of mammalian species. Although a number of investigations have utilized serological techniques in mammalian systematics (Levine and Moody, 1939), (Boyden, 1942), (Leone and Wiens, 1956), (Gerber and Birney, 1968), few investigations have utilized

electrophoretic and immunoelectrophoretic procedures. Nadler and Hughes (1966) and Nadler (1968) using single and two-dimensional starch gel electrophoresis were able to distinguish a number of taxonomic characteristics of serum proteins of some species of the genus Spermophilus. Nadler and Hughes (1966b) found nine major differences in the serum protein fractions of several species of ground squirrels of the subgenus Spermophilus. These investigators used two-dimensional electrophoresis to distinguish primarily transferrin, albumin, and gamma globulin fractions.

Histochemical characterizations and specific stains (PAS) can be used to characterize specific fractions, as well as, to distinguish taxonomic differences in the serum protein fractions. Esterase activity in dog sera was used by Leone and Anthony (1966) to identify and separate 40 breeds of dogs through a combined technique of immunoelectrophoresis and histochemical characterization according to Uriel (1963).

Immunoelectrophoretic techniques have not been used to a great extent in mammalian systematics. Forman, Baker, and Gerber (1968) used immunoelectrophoretic analysis, in conjunction with karyotypes and sperm morphology to suggest that the vampire bats (Family Desmodontidae) are a subfamily of the leaf-nosed bats (Phyllostomatidae). The present investigation utilized immunoelectrophoresis as a corrobora-

tive method.

Interest in the relationships within the genus Spermophilus has increased recently as a result of numerous cytogenetic investigations by Nadler (1962, 1966a, 1966b), Nadler and Hughes (1966a), Nadler and Sutton (1962) and electrophoretic serum protein analyses by Nadler (1968) and Nadler and Hughes (1966b). Gerber and Birney (1968) recently published results of precipitation analyses within the genus Spermophilus. The results of the formerly cited investigations compared favorably with earlier investigations by Howell (1938), Bryant (1945), Durrant and Hansen (1954), Moore (1959), and Black (1963) who sought to determine, through cranial morphology, baculum morphology, pelage, distribution patterns, and paleontological information, the phylogenetic interrelationships of this genus, and to revise the current taxonomy of the genus.

The objectives of this investigation were to determine characteristics of serum proteins having taxonomic or phylogenetic relationships and to compare the phylogenetic implications with those obtained in previous investigations on this genus: 1.) by separation and characterization of the serum proteins of several subgenera of ground squirrels of the genus Spermophilus (= Citellus) through the use of agar gel electrophoresis; and 2.) to identify the serum protein fractions through immunoelectrophoretic analysis and

histochemical methods.

This study is an attempt to further elucidate the interrelationships of five of the eight subgenera of Spermophilus (Ictidomys, Otospermophilus, Callospermophilus, Xerospermophilus, and Ammospermophilus). Ammospermophilus is given generic ranking according to Hall and Kelson (1959) but, in this study was considered as a subgenus to facilitate data handling and comparisons within Spermophilus.

CHAPTER II

METHODS AND MATERIALS

Animals and Their Care

Serum samples were taken from nine species of ground squirrels, representing five subgeneric groups of the genus Spermophilus (= Citellus). The ground squirrels collected by the author included:

Subgenus Ictidomys

Spermophilus mexicanus parvidens (Mearns). Texas: Kermit Country Club, Kermit, Winkler County, 7 females and 4 males; Big Spring Cemetery, Big Spring, Howard County, 1 female and 2 males; Alpine Country Club, Alpine, Brewster County, 2 males. Total, 16.

Spermophilus tridecemlineatus arenicola Howell. Portales Country Club, Portales, Roosevelt County, New Mexico, 15 females. Total, 15.

Spermophilus tridecemlineatus texensis (Merriam). Norman, Cleveland County, Oklahoma, 7 females and 2 males. Total, 9.

Spermophilus spilosoma canescens (Merriam). Las Cruces, Dona Anna County, New Mexico, 5 females and 7 males. Total, 12.

Subgenus Ammospermophilus

Spermophilus leucurus escalante (Hansen). 7 mi, N. W. St. George, Washington County, Utah, 2 males. Total, 2.

The remainder of the ground squirrels were purchased from the Pet Corral, Tucson, Arizona and included:

Subgenus Ammospermophilus

Spermophilus harrisii harrisii (Audubon and Bachman). 8 females and 4 males. Total, 12.

Subgenus Xerospermophilus

Spermophilus tereticaudus tereticaudus (Baird). 6 females and 4 males. Total, 10.

Subgenus Otospermophilus

Spermophilus variegatus grammurus (Say). 5 males.

Subgenus Callospermophilus

Spermophilus lateralis arizonensis (V. Bailey). 8 females and 2 males. Total, 10.

These specimens were identified through Hall and Kelson (1959).

The ground squirrels were individually caged and provided with water ad libitum and food (Purina Laboratory Chow). The laboratory environment was stabilized at about 25°C, about 70% relative humidity, and a diel cycle of 10 hours light-14 hours darkness. All animals were maintained at these conditions for a period of six weeks or more, prior to preparation of the serum samples. All animals were adults with the exception of offspring of three S. tridecemlineatus arenicola, which were removed from the nesting burrow following capture of the adult female. The general physical condition of the animals was observed closely, those with noticeable parasitic infections or infected wounds were recorded. Individual S. tereticaudus had moderate to heavy intestinal nematode infections, manifest by the large number of helminths found in fresh fecal pellets. The rock squirrels (S. variegatus) had large, festering wounds on the rump and shoulders, presumably caused by the excessive activity of these large squirrels in small cages. These exceptions to good physical health were considered when the serum samples were tested and analysed.

Serum Collection and Antibody Preparation

Blood was obtained by cardiac puncture with sterile disposable syringes. Repeated bleedings were necessary to

obtain plasma, hemoglobin, and serum samples of adequate volume. Initial bleedings were made with heparinized syringes, needles, and collecting tubes. The whole blood was centrifuged at 3,000 rpm (International Centrifuge) for 7 minutes, the plasma removed by pipette, and preserved with a merthiolate solution (1:1,000 dilution). The erythrocytes were washed three times with a 0.85% saline solution and lysed with an equal volume of distilled water. The hemoglobin and plasma samples were stored at -76°C, to be analysed later (not included in this investigation).

The second cardiac puncture was performed after a three week recuperation period. This puncture involved removal of 0.5 ml whole blood from which serum was separated by centrifugation at 3,000 rpm for 7 minutes. The serum was recentrifuged at 18,000 rpm for 15 minutes in a refrigerated centrifuge (Servall), preserved with merthiolate and stored at -76°C in a freezer. The final bleeding was made three weeks later. The squirrels were exsaguinated at this bleeding and the sera was refined and treated following the procedure used in the second bleeding. The sera of S. tridecemlineatus, lateralis, and tereticaudus were high in lipid content, probably due to the pre-hibernatory state of the squirrels.

Serum samples from the following animals were used in the production of whole serum antibodies in rabbits (litter

mates of Dutch strain):

Species	Animal Number	Sex	Antiserum Number
S. mexicanus	3	male	Ab 12
	8	female	Ab 2
S. t. arenicola	20	female	Ab 3
S. spilosoma	51	female	Ab 4
	52	male	Ab 5
S. harrisii	66	female	Ab 6
	60	male	Ab 7
S. tereticaudus	73	female	Ab 8
	72	male	Ab 9
S. variegatus	83	male	Ab 10
S. lateralis	90	female	Ab 11

A 2 ml volume of a 1% solution of squirrel serum, in 0.85% saline solution, was injected into the lateral ear vein of the rabbit every other day until a total of nine injections were made. The rabbits were ear bled on day 25 after the initial injection and a titer of serum antibodies was determined by the ring precipitation test of Ascoli (1902). The titers of all the rabbits were found to be 1:1600 or higher. The rabbits were exsanguinated on day 31 and the titers were determined again. The final titers were identical to those of the previous test. The rabbit sera were centrifuged at 18,000 rpm for 15 minutes in a refrige-

rated centrifuge, preserved with merthiolate, separated into one dram screw-cap vials, and stored at -76°C.

Pooled samples were obtained by removing an equal volume (0.1 ml) from each sample of squirrel serum and collected in a serum tube. This procedure allowed for comparisons to be made between pooled and individual sera.

Electrophoresis

The electrophoretic method used was that described by Wieme (1959). The salient features of this method are: 1) ultramicro-electrophoresis on an agar medium coating a glass microscope slide; 2) reduction of electro-osmotic flow by formation of an agar to agar bridge between the electrophoretic block and agar within the electrode chambers; 3) prevention of evaporation of water from the medium by sealing with petroleum ether which allows for maintaining a cooling action on the agar plate through its volatility; 4) narrow application slits permit a greater degree of resolution; and 5) diffusion of the separating proteins is considerable, separation must be rapid, therefore, a high voltage is applied to the agar plate. The method may also be characterized by the features of the agar medium, as it is inexpensive, homogenous, firm yet flexible, possesses good transparency, can be dried quickly into a clear stable film, and is easily

integrated into immunoelectrophoretic and immunodiffusion analyses.

Serum proteins were electrophoresed in a 0.9% agar (Difco Special Agar) medium using a sodium barbital-barbiturate buffer (pH 8.6; ionic strength 0.05) (Fisher), in a Wieme chamber (Vitatron). A current of 25 milliamperes per slide was applied (Gelman Power Supply) and maintained throughout a 25 minute separation period. The temperature of the chamber was maintained at 7-10°C. Individual and pooled serum samples were electrophoresed; with repeated separations made until each sample showed good resolution, with no aberrancies due to sample application or slit distortion.

The electrophoretically separated serum protein fractions were fixed in an acetic acid-alcohol solution, the agar film dried onto the microscope slide overnight in a 37°C incubator, and stained with Amidoblack 10-B stain. Photodensitometric recordings were made of each sample by a Photovolt scanning-recording densitometer (Densicord #542) with an integrator (Integraph #49). The slides were scanned at the most sensitive response level (D-3) through a red filter (610 mu).

Each sample was analysed for: 1) the number of fractions, 2) migratory distance of each fraction from the point of application, 3) the density of each fraction

(relative to the blank slide), and 4) the percentage contribution of each fraction.

Ground squirrel serum protein fractions were classified by arbitrarily assigning them to one of the classes of human serum protein fractions according to their mobilities (i.e., fractions of squirrel serum migrating to the human alpha region were called alpha 1A, 1B, or alpha 2; those separating to the beta region, B_1, B_2, or B_3). Thus, the serum protein fractions of each species could be identified according to mobility, the absence or presence of a particular fraction, or by variations in the mobility of the fractions.

Single classification, one way analysis of variance (ANOVA) tests were performed on each fraction of the individually treated serum samples. The results of these tests were then subjected to Student-Newman-Kuhls and Duncan's Multiple Range tests for nonsignificant differences. These tests permitted classification of the serum protein fractions as taxonomic characteristics.

The mean mobilities and relative densities of the fractions of the individual samples were compared with the fraction mobilities and relative densities of the pooled samples.

The pooled serum samples were separated in a two-dimensional electrophoretic system which incorporated the

use of square agar plates 8 centimeters on the slide. The current, temperature, and duration of the runs were kept constant in both dimensions. These are not the criteria for true two-dimensional electrophoresis, however, this method was used to corroborate the presence of double albumin fractions in three of the subgeneric groups studied, as well as, the apparent multiple fractions in the alpha and beta regions. The resulting patterns were traced by hand, as the scanning stage of the photodensitometer could not accomodate the square slide.

Immunoelectrophoresis

Immunoelectrophoretic analyses were performed on both individual and pooled samples. The method devised by Scheidegger (1955) was used with modifications of the electrophoretic separation occurring in the Wieme chamber, and staining of the precipitin arcs. Individual serum samples were separated electrophoretically, followed by introduction of 0.2 ml of antiserum into the trough cut parallel to the line of migration. The precipitin arcs developed for 16-24 hours, at which time, the unreacted serum was washed from the slide with two 10-hour rinses in 0.85% saline solution followed by a 5-hour rinse in distilled water. The slides were dried overnight and the resulting arcs were stained with Amidoblack 10-B stain rather than

Ponceau Red as used by Scheidegger (1955). The slides were dried and stored subsequent to analysis.

The procedure for determing the phylogenetic relationships by immunoelectrophoretic analyses was:

1.) Individual serum samples were tested against each of the eleven antisera, thus constituting a series of cross reactivity tests.

2.) The resulting precipitin arcs of each test were compared with the arcs obtained when the serum used for antibody production was reacted with its homologous antiserum. For example, S. mexicanus #3 reacted with antiserum Ab 12 was a reference for all reactions from the use of antiserum Ab 12. The relative density of the arcs, the number of arcs, and the serum fraction eliciting the arc were compared.

3.) Precipitin arcs were identified by superimposing the immunoelectrophoretic product upon the electrophoretically separated and stained slide, thus the arc could be identified as the product of a reaction occurring between a distinct fraction and the antiserum used in the test.

4.) The number of individuals showing a particular arc was recorded as a proportion of the total number of individuals of each species. The

number of antisera eliciting a particular arc was expressed as a proportion of the total number of antisera used. Multiplying the two ratios resulted in an estimate of the probability of a particular antiserum eliciting a response to a particular fraction. The formula:

$$\frac{\text{Number of individuals with precipitin arc}}{\text{Total number of individuals in the sample}} \times \frac{\text{Number of antisera eliciting arc}}{\text{Total number of antisera}}$$

was named the antiserum correlation index (ASCI) by the author and the range of values obtained for any specific fraction was between 0 and 1.

5.) Phylogenetic relationships were demonstrated by adding the ASCI values of all the protein fractions of each species. These sums were ranked from highest (indicating the greatest degree of antigen-antibody reactivity) to lowest (indicating the least antigen-antibody reactivity).

The ASCI formula was modified to show relationships between pooled sera by using the ratio of the number of species exhibiting a particular arc to the total number of species used and multiplying this ratio by the right hand side of the original formula.

Histochemical Analyses

Pooled sera were reacted against species specific antisera and the resulting precipitin arcs were tested for the presence of beta napthyl esterase and cholinesterase activities according to the procedures developed by Uriel (1963). Specific serum-antiserum precipitin arcs were stained with Periodic Acid-Schiff reagent (PAS) (Uriel and Grabar, 1961) to identify glycoprotein complexes and with Oil Red O to identify lipoproteins.

Quantitative Precipitation

A quantitative precipitation test was performed as an adjunct to the electrophoretic analyses. A constant amount of S. spilosoma antiserum Ab 5, (0.2 ml) was added to each of 10 test tubes of a doubling dilution series of pooled serum (antigen) for each of the eight species used in the electrophoresis analyses. Dilutions were made in 0.5 ml of 0.85% saline solution, pH 7.0. Following addition of antiserum, the tubes were shaken and refrigerated at 4°C for 48 hours, during which time, the tubes were intermitantly shaken. The precipitates were centrifuged at 3,000 rpm for 15 minutes and were washed in three changes of 0.85% saline. The precipitates were allowed to dry overnight, then were digested and Nesslerized according to Lanni, Dillon, and Beard (1950). The final solutions were scanned in a

spectrophotometer (Spectronic 20, Bausch and Lomb) at a wavelength of 440 mu. The amount of protein nitrogen of each tube was calculated by comparing the optical density of a reference standard (0.10 mg N) to that of the unknown. The protein nitrogen values of all tubes were expressed as a percentage of the equivalence dilution tube number (the dilution at which the quantity of antigen is proportional to the quantity of antibody) of the control species, S. spilosoma.

CHAPTER III

RESULTS

Measures were taken to assure that the protein fractions were genetically controlled and of a potential taxonomic value. To eliminate physiological or environmental effects on the serum proteins, the ground squirrels were maintained in a nearly constant environment for 6-15 weeks. No differences in the serum protein fractions were observed in respect to age or sex of the animal. No pregnant animals were used, although some of the Spermophilus tridecemlineatus arenicola females were lactating when collected, lactation was not observed when serum was taken from these females. The serum proteins of infected animals did not deviate from those of healthy squirrels and were included in the analyses.

Electrophoresis of Individual Sera

According to the nomenclature and mobilities of human serum proteins, the eight separate fractions that exhibited qualitative variation were: albumin, alpha 1 A, alpha 1 B, alpha 2, beta 1, beta 2, beta 3, and gamma.

The mean mobilities and relative densities of the grouped individual serum protein fractions (Figure 1.) reveal the following information:

1.) The albumin fraction is the most rapid anodally migrating serum protein. The albumin mobilities and densities are similar in the nine species studied. S. lateralis, S. harrisii, and S. leucurus exhibited a double albumin fraction; the remaining species showed a single fraction. S. mexicanus and S. tereticaudus exhibited slower albumin mobilities.

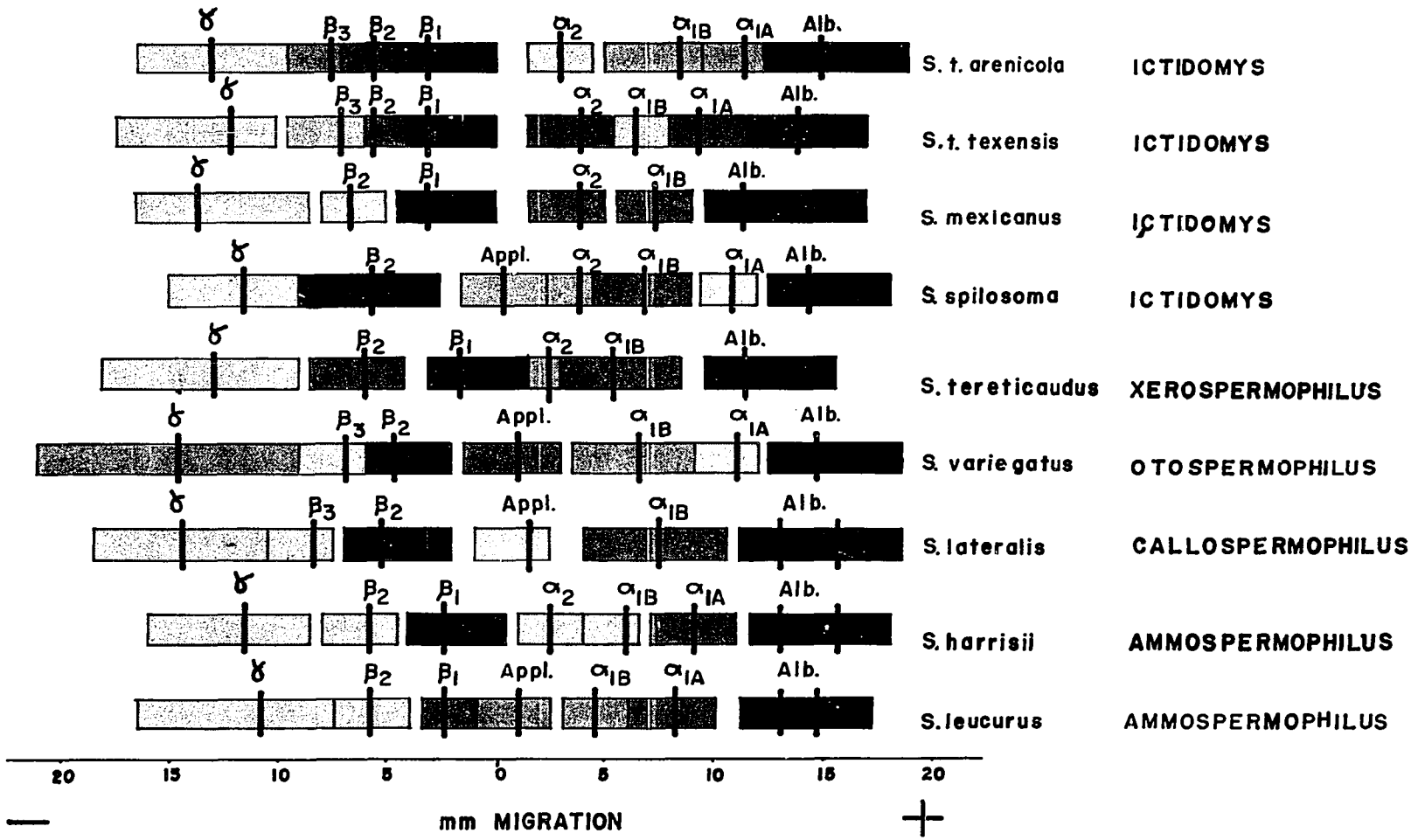
2.) The alpha 1 A fraction was not exhibited by S. mexicanus, S. tereticaudus, and S. lateralis. There is a difference in the mobilities of the alpha 1 A fraction between the two subspecies of S. tridecemlineatus. S. t. arenicola alpha 1 A is more rapid than that of S. t. texensis. Little difference between the mobilities of the alpha 1 A fractions of S. harrisii and leucurus is observed.

3.) The alpha 1 B fraction is present in all of the sera studied. S. t. arenicola and texensis exhibited individual variation of mobilities. The degree of variation in the mobility of this fraction does not permit distinctions to be made between any of the species studied.

4.) The alpha 2 region is represented in all species

Figure 1. Composite electrophoretic patterns of nine Spermophilus species. The solid vertical bar is the mean mobility of the individual fraction. Shading is representative of the relative densities of each fraction(black, 100%; four grades of shading: 80%, 60%, 40%, and 20%).

GROUPED INDIVIDUAL SERA



except S. variegatus, lateralis, and leucurus. The fraction labeled "Application" (Figure 1.) in these forms is probably the alpha 2 fraction, which fails to migrate any appreciable distance from the point of application (0). The mobilities of the alpha 2 fractions of the four species of the subgenus Ictidomys (S. t. texensis, S. t. arenicola, S. mexicanus, and S. spilosoma) are quite similar. S. tereticaudus and S. harrisii mobilities are identical.

5.) The beta 1 fraction is exhibited by the tridecemlineatus species group (S. tridecemlineatus subspecies and S. mexicanus) but not in S. spilosoma. S. tereticaudus and both S. harrisii and S. leucurus exhibit the beta fraction, whereas, S. lateralis and S. variegatus do not. Therefore, the Ictidomys excepting S. spilosoma), Xerospermophilus, and Ammospermophilus subgenera share this fraction in common. The beta 1 fraction mobilities of the two Ammospermophilus species is identical.

6.) The beta 2 fraction is present in all species studied and is the least variable serum protein fraction in this study. This fraction is referred to as a generic trait, lacking comparisons with other Sciurids.

7.) The beta 3 fraction is present in only three sub-

generic groups (Ictidomys, Otospermophilus, and Callospermophilus). Within the Ictidomys only the two S. tridecemlineatus subspecies exhibit this fraction. 8.) The gamma fraction of ground squirrel sera is present in all species and represents the most cathodally migrating fraction. It is highly variable, and no relationships are indicated.

The results of a one way single classification analysis of variance of the mobilities showed nonsignificant differences in the application and beta 3 fractions. The mean fraction mobilities of the other fractions were significantly ($P = .05$) to highly significantly ($P = .01$) different according to this analysis (Table 1).

The Student-Newman-Kuhls and Duncan's tests for nonsignificant differences between means yielded identical results, although the Duncan's test is more sensitive (Steel and Torrie, 1960). These tests provided little taxonomic information when the species represented were handled individually. However, when subspecies (S. t. arenicola and texensis), species groups (tridecemlineatus and spilosoma) species (S. leucurus and harrisii), and subgeneric taxa were treated separately, phylogenetic relationships were indicated. The beta 2 fractions exhibit the least variability. Only S. variegatus (Otospermophilus) differs from the other subgenera in this trait. S. mexicanus differs

TABLE 1

Analysis of variance of mobilities of each serum protein fraction.

Fraction	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Calculated F value	Tabular F value
Albumin	Among species	8	519.85	64.98	19.98	3.04
	Within species	71	233.54	3.29		
	Total	79	753.39			
Alpha 1 A	Among species	5	238.21	47.64	12.12**	4.46
	Within species	42	165.27	3.93		
	Total	47	403.48			
Alpha 1 B	Among species	8	335.79	41.97	14.28**	3.04
	Within species	66	194.13	2.94		
	Total	74	529.92			
Alpha 2	Among species	5	109.17	21.83	11.43**	4.46
	Within species	49	93.74	1.91		
	Total	54	202.91			
Application Point	Among species	3	22.45	7.48	6.86 n.s.	8.65
	Within species	22	24.01	1.09		
	Total	25	46.46			

Beta 1	Among species	5	187.56	37.51	42.62**	4.43
	Within species	51	45.28	.88		
	Total	56	232.84			
Beta 2	Among species	8	61.42	7.68	9.60**	3.04
	Within species	71	56.78	.80		
	Total	79	118.20			
Beta 3	Among species	3	22.31	7.44	4.22 n.s.	8.65
	Within species	22	38.81	1.76		
	Total	25	61.12			
Gamma	Among species	8	306.76	38.34	12.13**	3.04
	Within species	54	170.89	3.16		
	Total	62	477.65			

Tabulated F values are taken at the 95% confidence level.

** highly significant difference ($P \leq .05$)

n.s. nonsignificant difference ($P > .05$)

from the other representatives of the tridecemlineatus species group by this fraction. These tests would suggest the beta 2 fraction to be a generic trait.

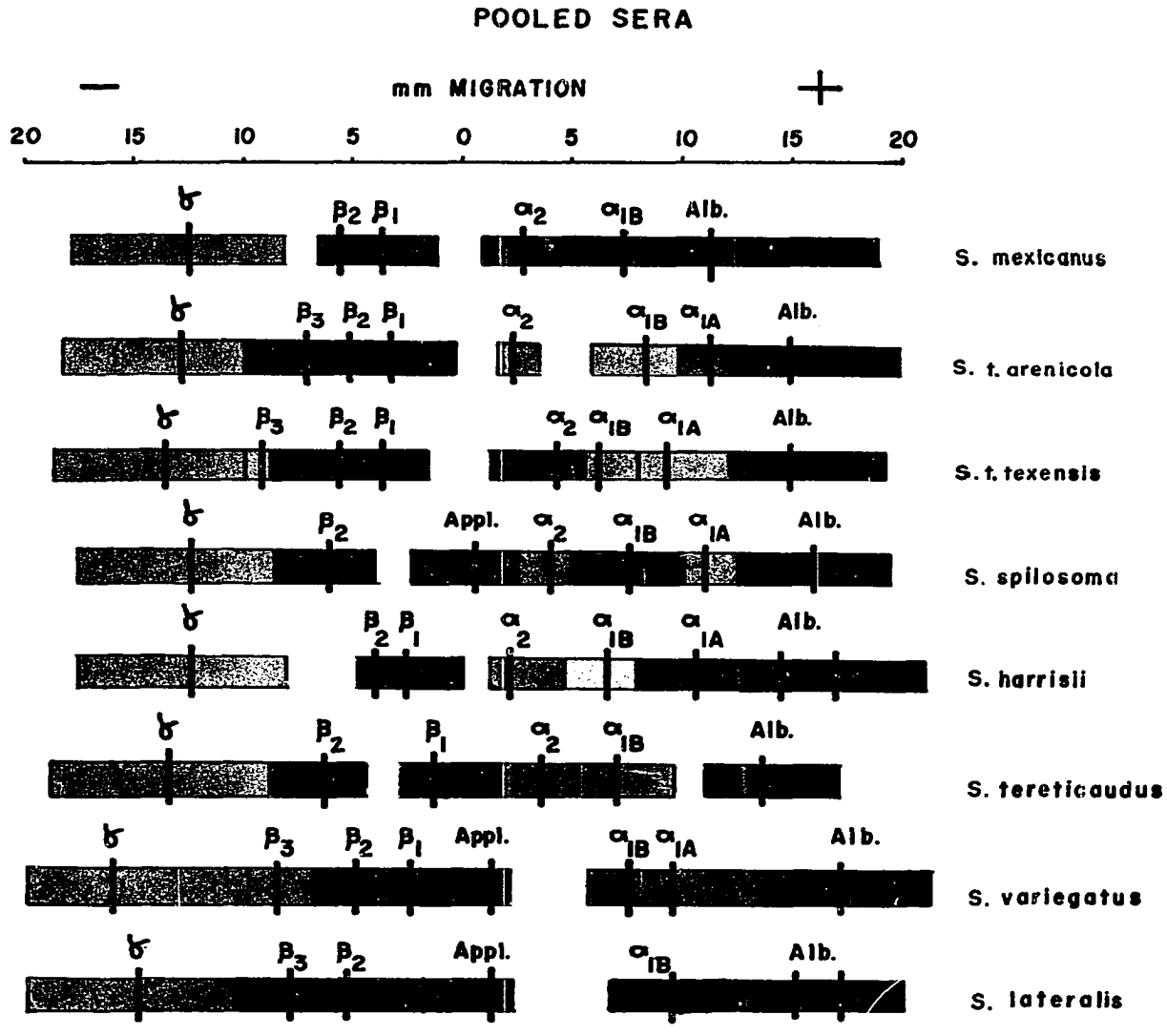
Ictidomys, Otospermophilus, and Callospermophilus exhibit nonsignificant differences in the mean mobility of the alpha 1 B fraction. Ammospermophilus and Xerospermophilus alpha 1 B fraction mobilities are nonsignificantly different from each other, but are significatnly different from the other three subgenera. The alpha 1 B and alpha 2 fraction mobilities are not significantly different within the Ictidomys subgenus. These fractions are considered to be subgeneric traits of this group when compared to the other subgenera. The beta 1 fraction mobility of S. mexicanus, S. t. arenicola, and S. t. texensis are not significantly different. This fraction may be regarded as a species group trait for the tridecemlineatus species group as S. spilosoma does not exhibit this fraction. The beta 3 fraction is represented in only the S. tridecemlineatus subspecies within the subgenus Ictidomys and is regarded as a species specific trait. The alpha 1 A fractions of S. t. arenicola and texensis are significantly different and may be a subspecific trait. Designation of this fraction as a trait for distinguishing between the two subspecies is rather tenuous, as both subspecies show some variation in this fraction.

The Ammospermophilus subgenus, represented by two species, had certain serum protein fraction mobilities having taxonomic relevance. Two albumin fractions, a major and minor fraction, are exhibited by this subgenus. The alpha 1 B fraction mobility is significantly different from that of the other subgenera, with the exception of Xerospermophilus. The mobilities of the alpha 1 B fraction and the presence of two albumin fractions may be considered collectively to be subgeneric traits, to distinguish Ammospermophilus from the other subgenera. The alpha 2 mobilities are significantly different between S. leucurus and harrisii and may be considered to be species specific characteristics. There are nonsignificant differences between the mobilities of beta 1, beta 2, and gamma fractions within this subgenus.

Pooled Sera Electrophoresis

Pooled serum electrophoresis of eight species of ground squirrels (S. leucurus was not analysed) yielded protein patterns that were essentially identical to those obtained from individual serum protein separations (Figure 2.), the only difference being the presence of the beta 1 fraction in S. variegatus in the pooled pattern. Mobility differences of fractions are considered to be due to variations in the conditions under which the pooled serum proteins were separated.

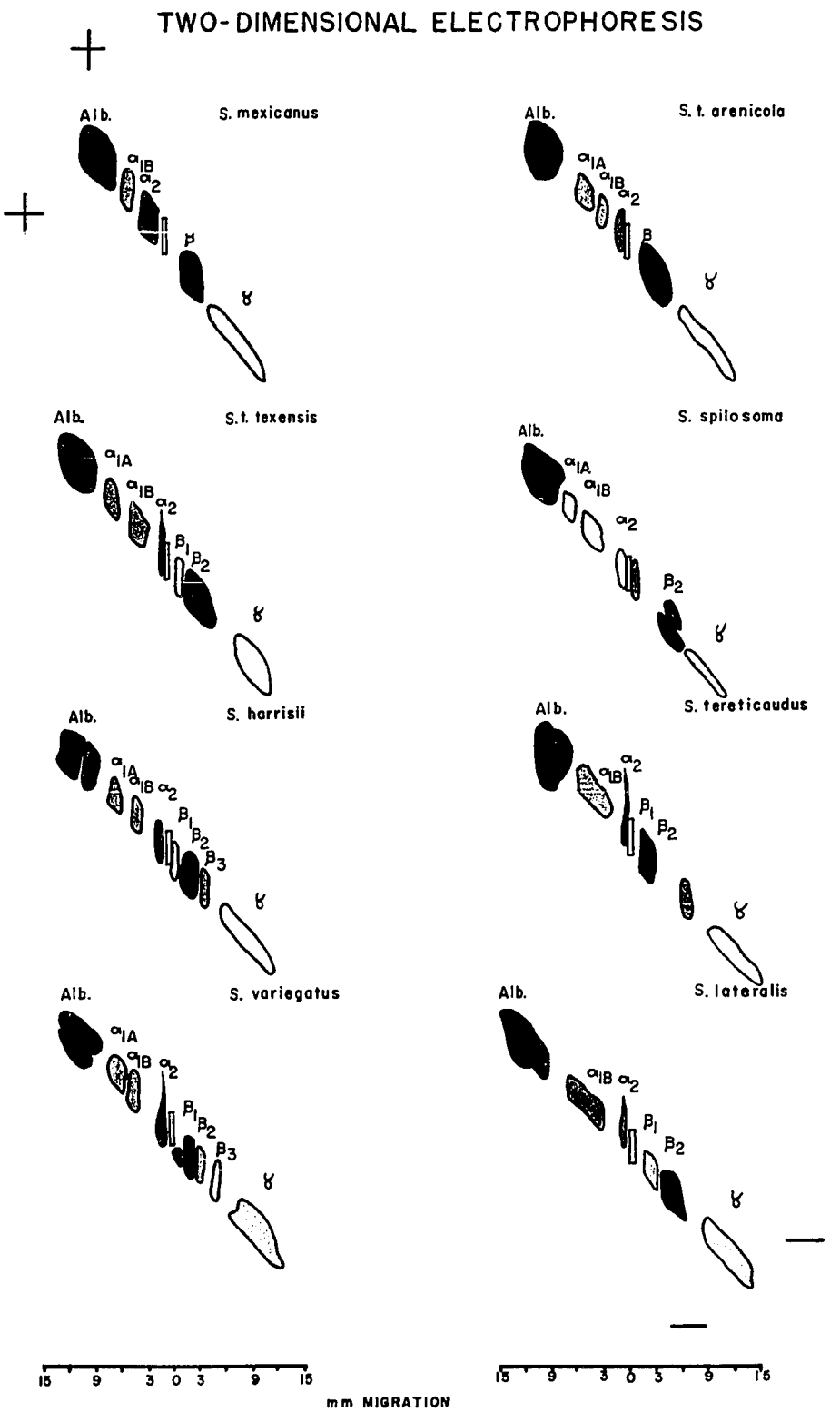
Figure 2. Electrophoretic patterns of serum proteins of the pooled sera of eight Spermophilus species. The solid vertical bar is the actual mobility of each fraction. Shading is representative of the relative densities of each fraction (black, 100%; four grades of shading, 80%, 60%, 40%, and 20%).


POOLED SERA
mm MIGRATION
20 15 10 5 0 5 10 15 20
γ β2 β1 α2 α1B Alb.
S. mexicanus
γ β3 β2 β1 α2 α1B α1A Alb.
S. t. arenicola
γ β3 β2 β1 α2 α1B α1A Alb.
S. t. texensis
γ β2 Appl. α2 α1B α1A Alb.
S. spilosoma
γ β2 β1 α2 α1B α1A Alb.
S. harrisii
γ β2 β1 α2 α1B Alb.
S. tereticaudus
γ β3 β2 β1 Appl. α1B α1A Alb.
S. variegatus
γ β3 β2 Appl. α1B Alb.
S. lateralis

The serum protein patterns shown by two-dimensional electrophoresis (Figure 3.) are similar to those obtained by one-dimensional separations. The alpha 2 (or application fraction due to lack of sensitivity in this method) fraction exhibits a tailing in S. t. arenicola, S. t. texensis, S. tereticaudus, S. variegatus, and S. lateralis. The beta fractions observed in one-dimensional separation of S. mexicanus and S. tridecemlineatus arenicola do not appear following two-dimensional electrophoresis. The double albumin fractions of S. harrisii and S. lateralis are separate and distinct. The beta 2 fraction of S. spilosoma and the beta 1 fraction of S. variegatus are shown to be two separate fractions, however, this is probably an aberration caused by improper application of serum into the application slit. This type of separation is always exhibited in electrophoretic preparations in which the agar application slit is distorted during applications (Wieme, 1965).

The apparent absence of any significant differences in the mobilities of individual and pooled species fractions led to testing the differences in mobilities by the "t" test procedure of Sokal and Rohlf (1969) for comparing a single observation with the mean of a sample (Appendix I). The alpha 1 B fraction of the pooled sera of S. tereticaudus was the only fraction that showed a significant difference

Figure 3. Two-dimensional electrophoretic patterns of pooled sera of eight species of Spermophilus. The open rectangle is the point of application. Shading represents the actual densities of each fraction.


TWO-DIMENSIONAL ELECTROPHORESIS
Alb.
S. mexicanus
α_{1B}
α_2
β
γ
Alb.
S. t. arenicola
α_{1A}
α_{1B}
α_2
β
γ
Alb.
S.t. texensis
α_{1A}
α_{1B}
α_2
β_1
β_2
γ
Alb.
S. spilosoma
α_{1A}
α_{1B}
α_2
β_2
γ
Alb.
S. harrisii
α_{1A}
α_{1B}
α_2
β_1
β_2
β_3
γ
Alb.
S. tereticaudus
α_{1B}
α_2
β_1
β_2
γ
Alb.
S. variegatus
α_{1A}
α_{1B}
α_2
β_1
β_2
β_3
γ
Alb.
S. lateralis
α_{1B}
α_2
β_1
β_2
γ
15 9 3 0 3 9 15
15 9 3 0 3 9 15
mm MIGRATION

($P = 0.05$).

The fraction mobilities, the presence or absence of a fraction, and double fractions are suggestive of relationships among the subgenera included in this study. The subgenus Otospermophilus is similar to Callospermophilus in the mobilities and densities of the gamma, beta 3, beta 2, application (alpha 2) and alpha 1 B fractions (Figures 1. and 2.). The presence of the alpha 1 A fraction in Otospermophilus representative species is not exhibited by the Callospermophilus species. The Callospermophilus species has a double albumin fraction that is not exhibited by the Otospermophilus species but is present in both Ammospermophilus species studied (Figures 1. and 2.). The Ammospermophilus serum protein fractions: gamma, beta 2, beta 1, application (alpha 2), and alpha 1 B are similar in mobility to those of the Xerospermophilus species (Figures 1. and 2.). The representatives of the Ictidomys subgenus, taken collectively, appear to be more similar to Ammospermophilus and Xerospermophilus in mobility and density of the gamma and beta 2 fractions. Close similarity is exhibited by the mobilities of alpha 1 B fractions between Ictidomys, Otospermophilus, and Callospermophilus (Figure 1.). The albumin fraction mobilities of S. t. arenicola, S. t. texensis, S. spilosoma (Ictidomys) and S. variegatus (Otospermophilus) are similar, whereas, the albumin fractions

of S. mexicanus (Ictidomys) and S. tereticaudus (Xerospermophilus) are dissimilar (Figures 1. and 2.).

The subgenus Ictidomys is well represented by both species groups and two subspecies of S. tridecemlineatus. The representative species of this subgenus show similarity of mobilities in the gamma, beta 2, alpha 2, and alpha 1 B fractions. The tridecemlineatus species group differs from the spilosoma group by the presence and identity of the beta 1 fraction (Figures 1. and 2.). The tridecemlineatus species differ from others of the subgenus by the presence of a beta 3 fraction. The tridecemlineatus and mexicanus species differ in the beta 3, alpha 1 A, and the albumin fractions.

Individual Serum Immunoelectrophoresis

The results of immunoelectrophoretic characterization of the serum protein fractions of individual sera were treated by using the Antiserum Correlation Index (ASCI) (Chapter 2) (Table 2). The probability that alpha 1 B fraction would elicit a precipitin arc against any of the eleven antisera is 0.954 or 95% of the time. This fraction is considered to be the most reactive fraction in the antigen-antibody reactions observed by this method. The second most reactive fraction is the beta 2 (o.529) and the third is alpha 2 (0.413). The addition of the ASCI values, of all

TABLE 2

Antiserum Correlation Index Values of Each Serum Protein Fraction That Demonstrated a Precipitin Arc.

Species	Serum Protein Fractions							
	Beta 3	Beta 2	Beta 1	Application	Alpha 2	Alpha 1 B	Alpha 1 A	Albumin
S. mexicanus	.182	.581	.091	.104	.364	.983	.273	.273
S. t. arenicola	.091	.527	.000	.012	.339	.885	.273	.326
S. t. texensis	.000	.538	.081	.091	.429	.940	.252	.242
S. spilosoma	.000	.542	.174	.114	.454	1.000	.265	.182
S. harrisii	.182	.364	.083	.129	.436	.949	.265	.076
S. tereticaudus	.091	.511	.091	.124	.455	1.000	.265	.076
S. variegatus	.091	.535	.091	.234	.455	.969	.236	.091
S. lateralis	.091	.636	.091	.273	.370	.906	.182	.091
Mean value for each fraction	.091	.529	.088	.135	.413	.954	.251	.170

serum proteins eliciting response to antisera, for each species indicates the degree of cross-reactivity (Table 3). The larger the ASCI sum, the greater the cross-reactivity of the species with the eleven antisera used; the smaller the sum, the lesser the cross-reactivity. Ranking the subgenera, according to summed ASCI values, results in:

Otospermophilus	2.611	
Ictidomys	2.584	(mean value of all species)
Callospermophilus	2.549	
Xerospermophilus	2.522	
Ammospermophilus	2.302	

Pooled Serum Immunoelectrophoresis

The precipitin arc patterns produced by pooled sera samples were similar to those produced by individual sera. The most prominent feature of the pooled sera-antisera reactions is that each antiserum is a separate and individual test as demonstrated in Figures 4, 7, and 8, whereas antisera produced against the serum proteins of different individuals of the same species of ground squirrel produce radically different precipitin arc patterns.

The precipitin arcs exhibited by reacting Ab 12 (anti-mexicanus antiserum) with the serum proteins of each of the Spermophilus species (Figure 4.) shows:

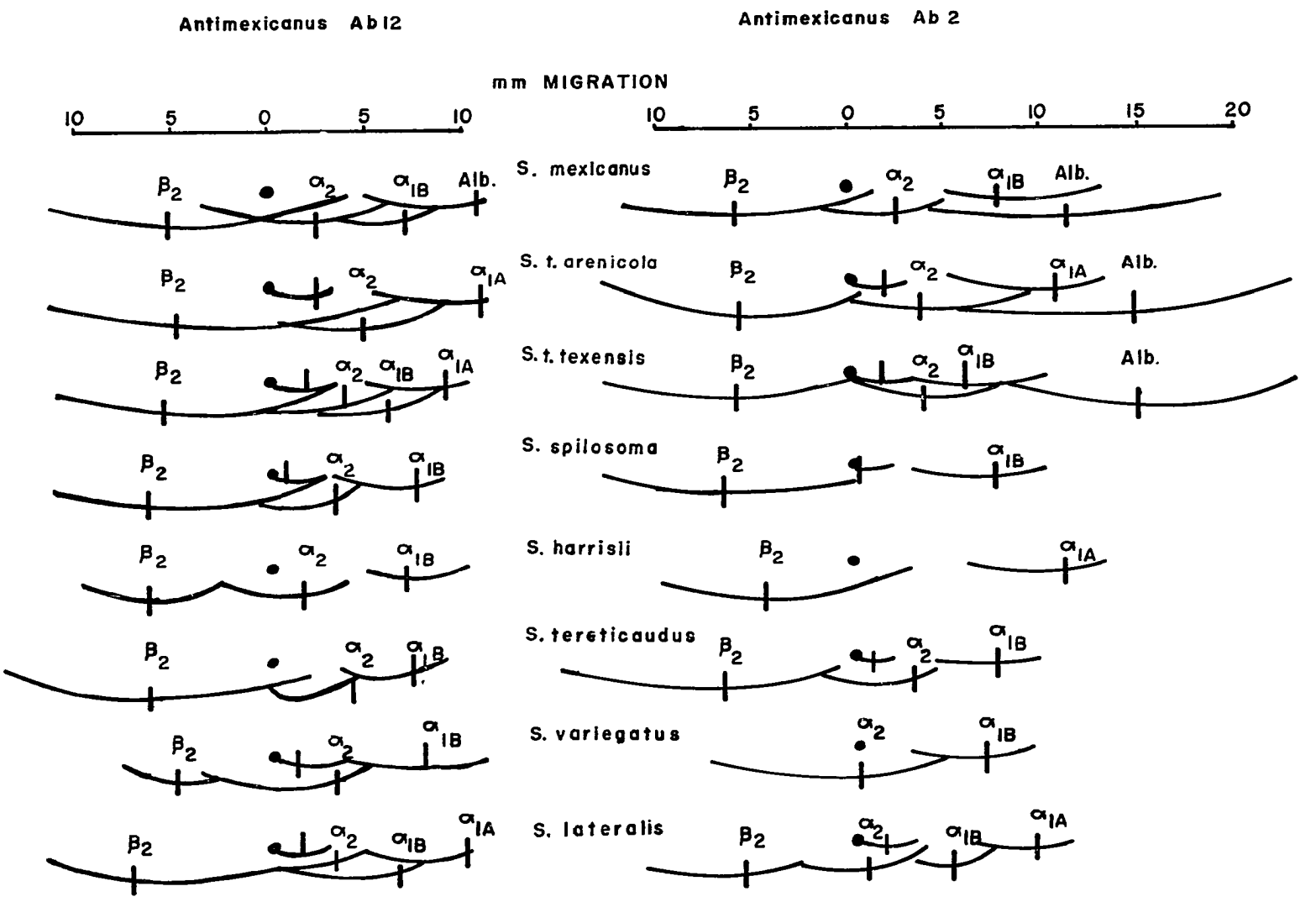
1.) Beta 2 arc in all eight species.

TABLE 3

Antiserum Correlation Index values added within each species

Species	ASCI values of each species	ASCI values for each subgenus	Subgenus
S. mexicanus	2.669		
S. t. arenicola	2.362	2.584	Ictidomys
S. t. texensis	2.573		
S. spilosoma	2.731		
S. harrisii	2.302	2.302	Ammospermophilus
S. tereticaudus	2.522	2.522	Xerospermophilus
S. variegatus	2.611	2.611	Otospermophilus
S. lateralis	2.549	2.549	Callospermophilus

Figure 4. Immunoelectrophoretic precipitin arc patterns exhibiting cross-reactivities between eight species sera and antimexicanus antisera. The solid vertical bars indicate electrophoretic mobility. The black dot is the point of application of serum.


Antimexicanus Ab12
Antimexicanus Ab 2
mm MIGRATION
10 5 0 5 10
10 5 0 5 10 15 20
S. mexicanus
S. t. arenicola
S. t. texensis
S. spilosoma
S. harrisii
S. tereticaudus
S. variegatus
S. lateralis
β2
α2
α1B
α1A
Alb.

2.) Application point arc (unlabeled) in all but S. harrisii, S. tereticaudus, and S. mexicanus.

3.) Alpha 2 arc in all species.

4.) Alpha 1 B arc in all species but S. t. arenicola.

5.) Alpha 1 A arc in only the two S. tridecemlineatus subspecies and S. lateralis.

6.) Albumin arc in S. mexicanus only.

Serum proteins reacted with Ab 2 (Figure 4.) show:

1.) Beta 2 arc in all but S. variegatus.

2.) Application point arc (unlabeled) in both S. tridecemlineatus subspecies, S. tereticaudus, and S. lateralis.

3.) Alpha 2 arc in all but S. harrisii and S. spilosoma.

4.) Alpha 1 B arc in all but S. t. arenicola.

5.) Alpha 1 A arc in S. t. arenicola, S. harrisii, and S. lateralis.

6.) Albumin arc in S. mexicanus, S. t. arenicola and S. t. texensis.

The precipitin arc patterns produced by Ab 3 (anti-arenicola) (Figure 5.) show:

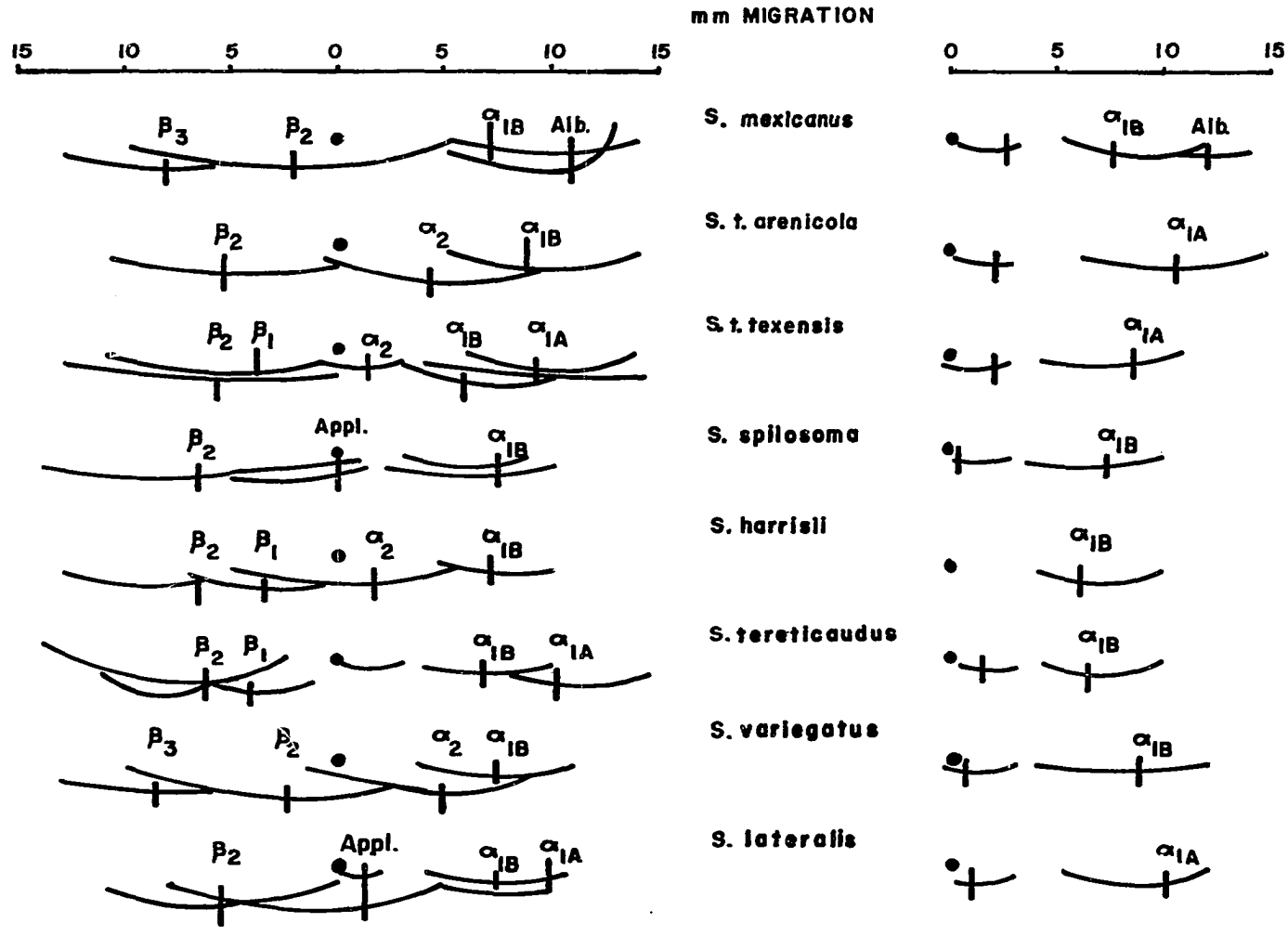
1.) Beta 3 arc in S. mexicanus and S. variegatus.

2.) Beta 2 arc in all eight species.

3.) Beta 1 arc in only S. t. texensis.

4.) Alpha 2 arc in S. t. arenicola and texensis, and

Figure 5. Precipitin arc patterns exhibiting cross-reaction between eight species sera and antiarenicola (left) and antispilosoma (right) antisera. Vertical bars indicate fraction mobilities. The solid dot is the point of application of serum.


Antiarenicola Ab 3
Antispilosoma Ab 4
mm MIGRATION
15 10 5 0 5 10 15
0 5 10 15
S. mexicanus
S. t. arenicola
S. t. texensis
S. spilosoma
S. harrisii
S. tereticaudus
S. variegatus
S. lateralis
β3
β2
β1
α2
α1B
α1A
Alb.
Appl.

S. harrisii. Application arcs of S. spilosoma and S. lateralis are considered to be alpha 2 arcs, as they are not contiguous with the application point.

5.) Alpha 1 B arc in all species.

6.) Alpha 1 A arc in S. t. texensis, S. tereticaudus, and S. lateralis.

7.) Albumin arc in S. mexicanus only.

The arc patterns of antispilosoma antiserum (Ab 4) are illustrated in Figure 5 and indicate few, if any, relationships except that the application arc is present in all but S. harrisii. This antiserum was considered to be faulty and was not evaluated any further.

The antispilosoma Ab 5 antiserum produced a number of precipitin arcs against all species of ground squirrels, with the exception of S. harrisii (Figure 6.):

1.) S. harrisii is the only species that does not show a beta 2 arc.

2.) S. mexicanus, S. t. arenicola, and S. t. texensis show a beta 1 arc.

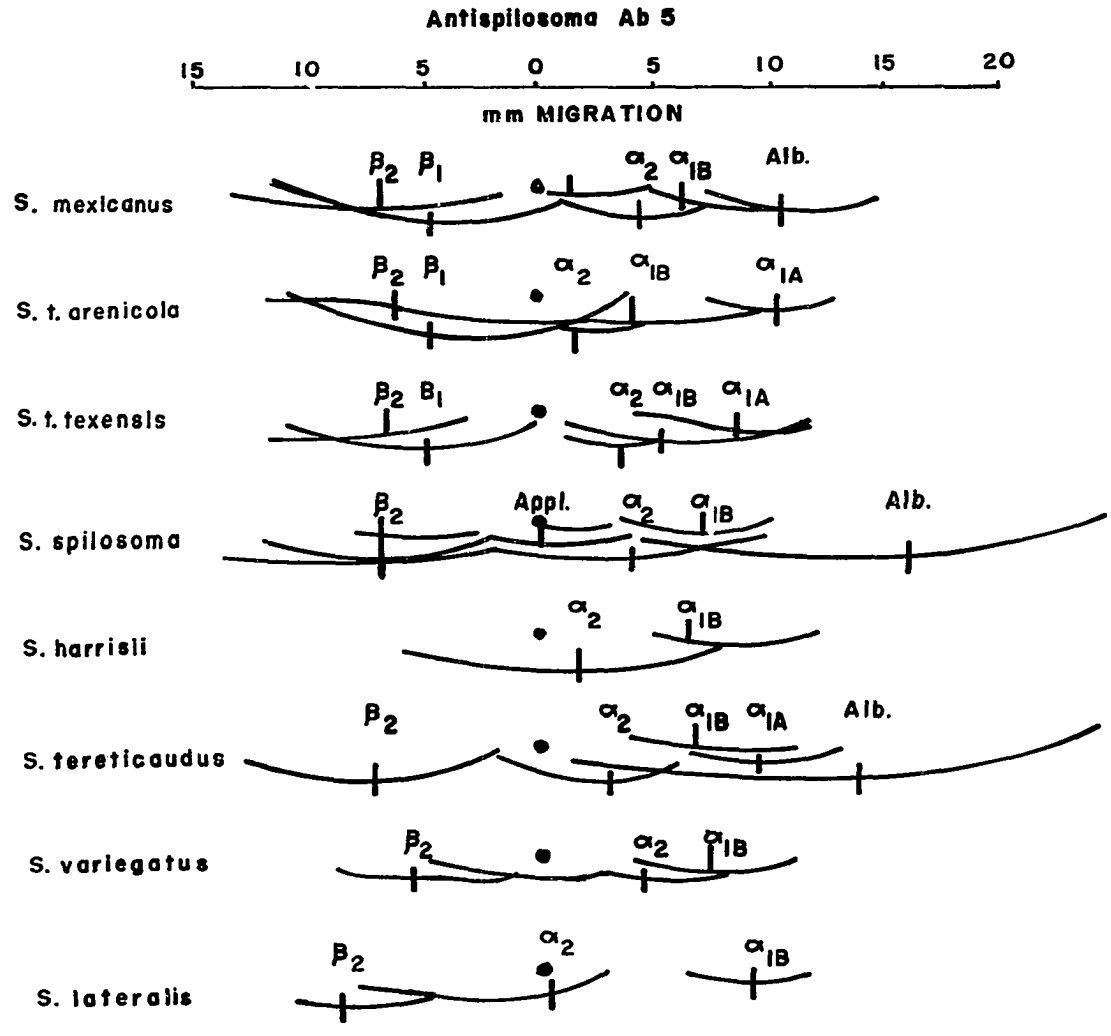
3.) Application point arc in S. mexicanus and S. spilosoma.

4.) Alpha 2 arc in all eight species studied.

5.) Alpha 1 B arc in all eight species.

6.) Alpha 1 A arc in only S. t. arenicola and

Figure 6. Precipitin arc patterns exhibiting cross-reaction between eight species sera and antispilosoma antisera. Vertical bars represent electrophoretic mobilities of each fraction. Solid dot is point of application of serum.


Antispilosoma Ab 5
15
10
5
0
5
10
15
20
mm MIGRATION
S. mexicanus
S. t. arenicola
S. t. texensis
S. spilosoma
S. harrisii
S. tereticaudus
S. variegatus
S. lateralis
Alb.
Appl.

texensis and S. tereticaudus.

7.) Albumin arc in S. mexicanus, S. spilosoma, and S. tereticaudus.

The precipitin arcs produced by antiharrisii antisera (Figure 7.) show little similarity in arc production. Few relationships can be inferred from the results and the opinion of this investigator is that the lack of relationships may be of taxonomic value. The subgenus Ammospermophilus according to these results is only distantly related to the other subgenera studied and therefore, the antisera reactivity is negligible.

The reduced reactivity observed in the antiharrisii antisera is also exhibited by the antitereticaudus antisera (Figure 8.). The only arcs produced when this specific antiserum is reacted with the specific antigenic sera (antitereticuadus vs. S. tereticaudus serum) are the alpha 1 arcs. The alpha 1 B arc is demonstrated in all species by both antisera (Ab 8 and Ab 9), except for the S. mexicanus versus Ab 8 reaction.

The precipitin arc resolution by antivariegatus antiserum (Figure 9.) shows:

1. Beta 2 arc in all but S. variegatus, which exhibits a beta 1 position arc.
2. Alpha 2 arc in all species except S. mexicanus and S. spilosoma.

Figure 7. Precipitin arc patterns exhibiting cross-reaction between eight species sera and antiharrisii antisera. Vertical bars represent electrophoretic mobilities of each fraction. The solid dot is the point of application.

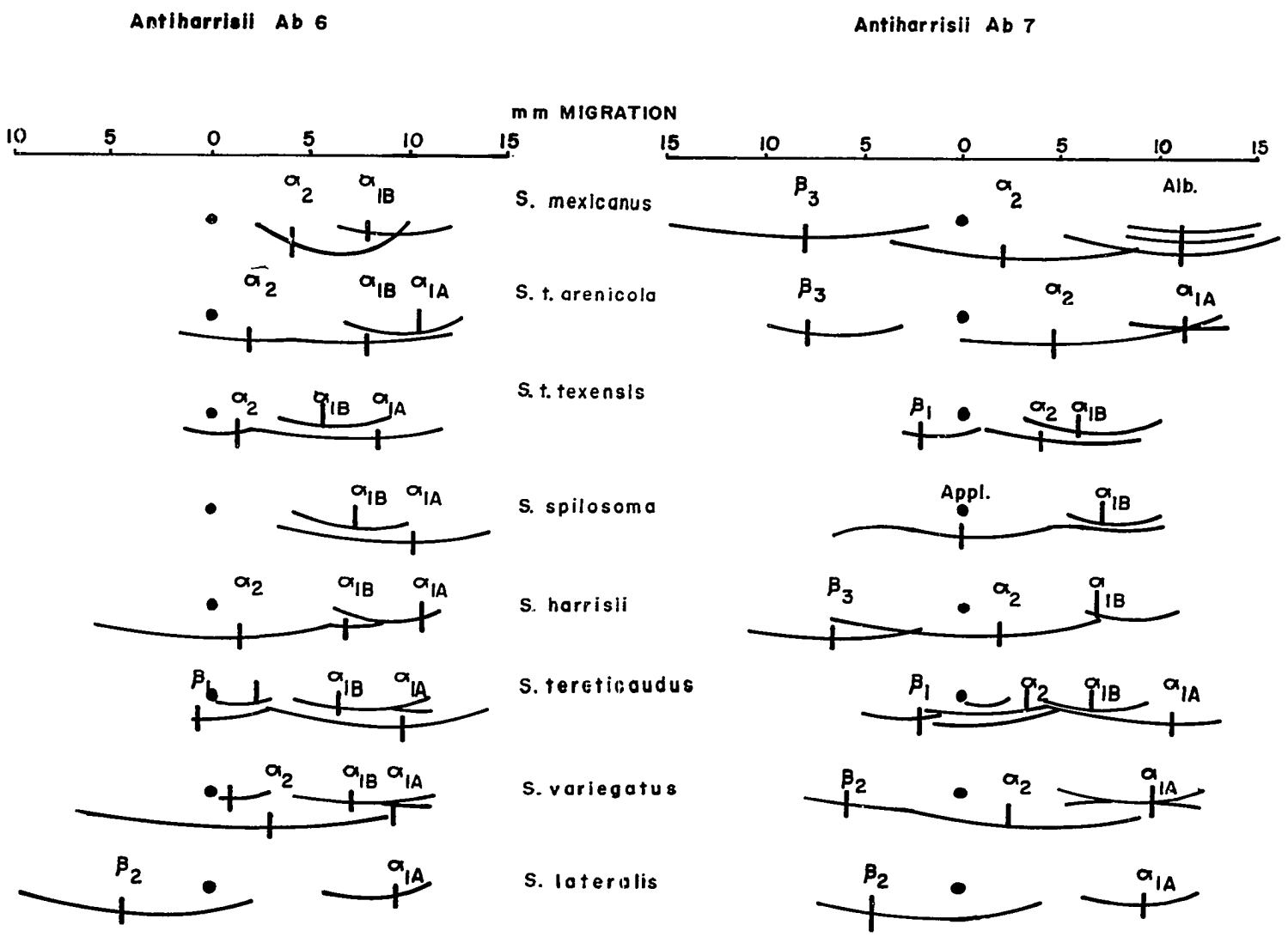

Antiharrisii Ab 6
Antiharrisii Ab 7
mm MIGRATION
10 5 0 5 10 15
15 10 5 0 5 10 15
S. mexicanus
S. t. arenicola
S. t. texensis
S. spilosoma
S. harrisii
S. tereticaudus
S. variegatus
S. lateralis
Alb.
Appl.

Figure 8. Precipitin arc patterns exhibiting cross-reaction between eight species sera and antitereticaudus antisera. Vertical bars represent electrophoretic mobilities of each fraction. The solid dot is the point of application.

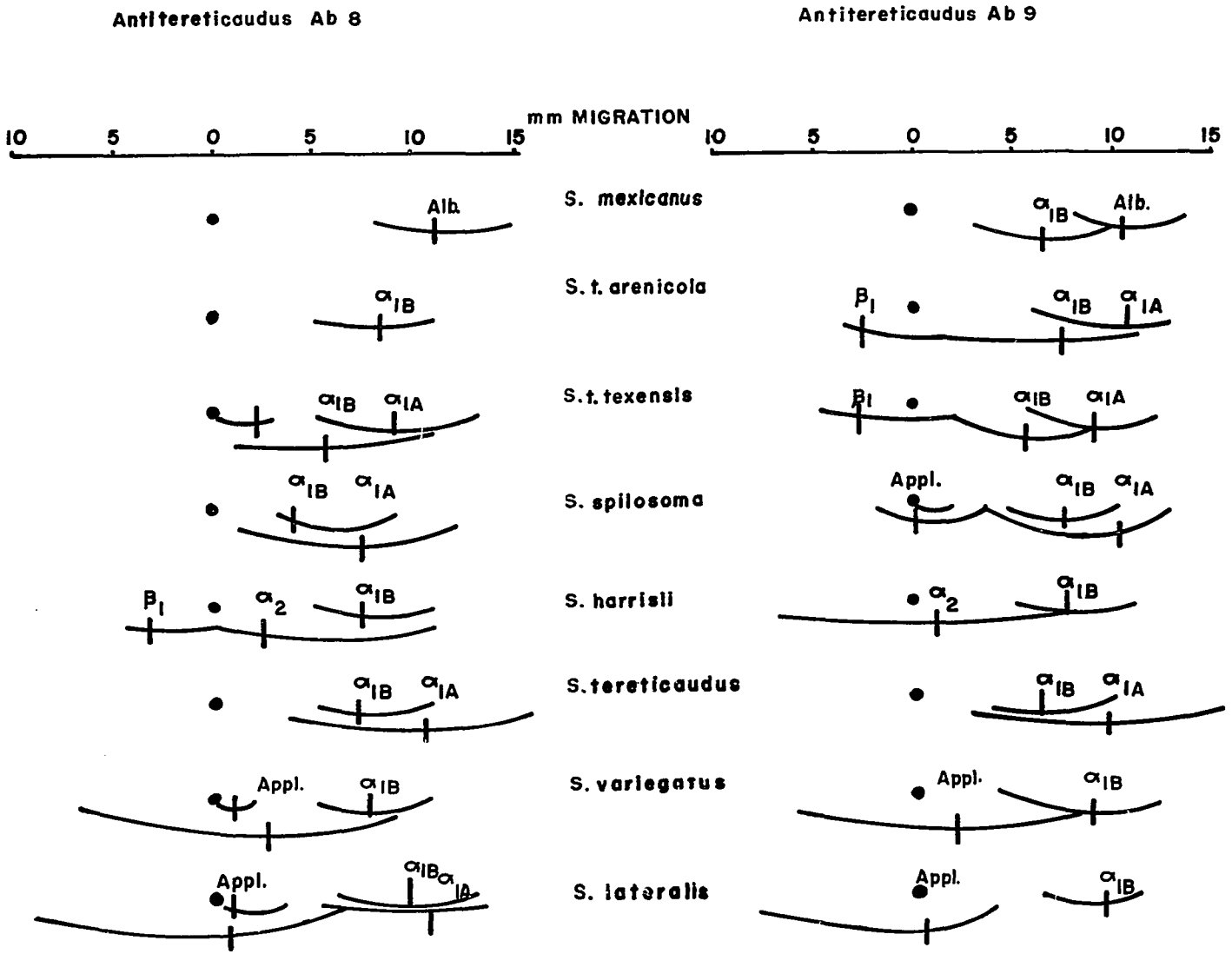
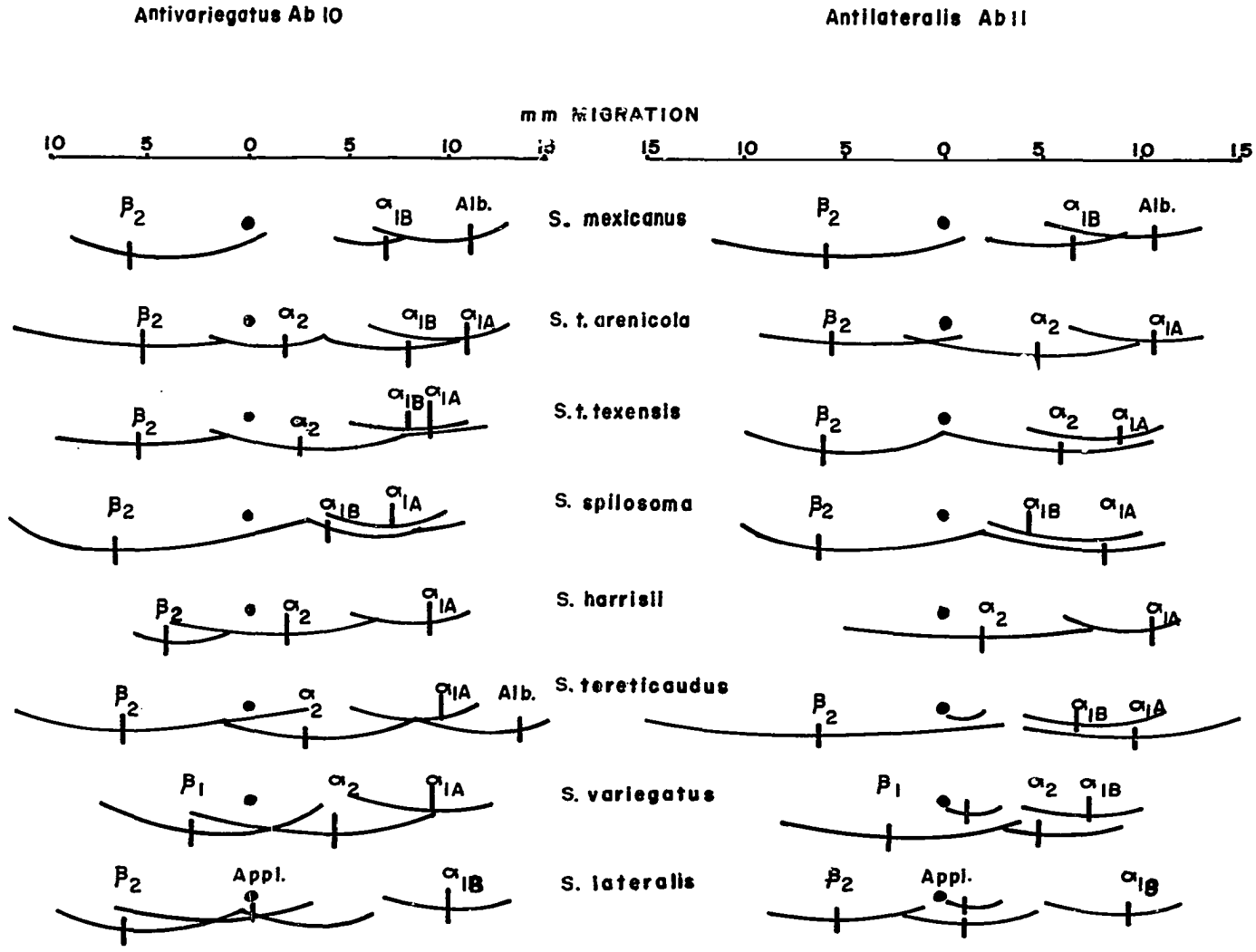

Antitereticaudus Ab 8
Antitereticaudus Ab 9
mm MIGRATION
10 5 0 5 10 15
10 5 0 5 10 15
S. mexicanus
S. t. arenicola
S. t. texensis
S. spilosoma
S. harrisii
S. tereticaudus
S. variegatus
S. lateralis
Alb.
α_{1B}
α_{1A}
β_1
α_2
Appl.

Figure 9. Precipitin arc patterns exhibiting cross-reaction between eight species sera and antivariegatus (left) and antilateralis (right) antisera. Vertical bars indicate fraction mobility. The solid dot is the point of application.


Antivariegatus Ab 10
Antilateralis Ab 11
mm MIGRATION
10 5 0 5 10 15
15 10 5 0 5 10 15
S. mexicanus
S. t. arenicola
S. t. texensis
S. spilosoma
S. harrisii
S. tereticaudus
S. variegatus
S. lateralis
Alb.
Appl.

3.) Alpha 1 B arc in all members of the Ictidomys subgenus and S. lateralis, but not in the other species.

4.) Alpha 1 A arc in all but S. mexicanus.

5.) Albumin arc in only S. mexicanus and S. tereticaudus.

The response of the antilateralis antisera (Figure 9.) shows:

1.) Beta 2 arc in all species except S. harrisii and S. variegatus.

2.) Beta 1 arc in S. variegatus only.

3.) Alpha 2 arc in all but S. mexicanus, S. spilosoma, and S. tereticaudus.

4.) Alpha 1 B arc in all species but S. harrisii and the two S. tridecemlineatus subspecies.

5.) Alpha 1 A arc in all species but S. mexicanus, S. variegatus and S. lateralis.

6.) Albumin arc in S. mexicanus only.

The degree of reactivity observed with antilateralis antisera indicates that this subgeneric group is not closely related to the other subgenera utilized in this study. The similarity of the precipitin arc patterns elicited by anti-variegatus and antilateralis antisera indicate a closer relationship between Otospermophilus and Callospermophilus subgenera, than between either of these subgenera and the

others represented in this study.

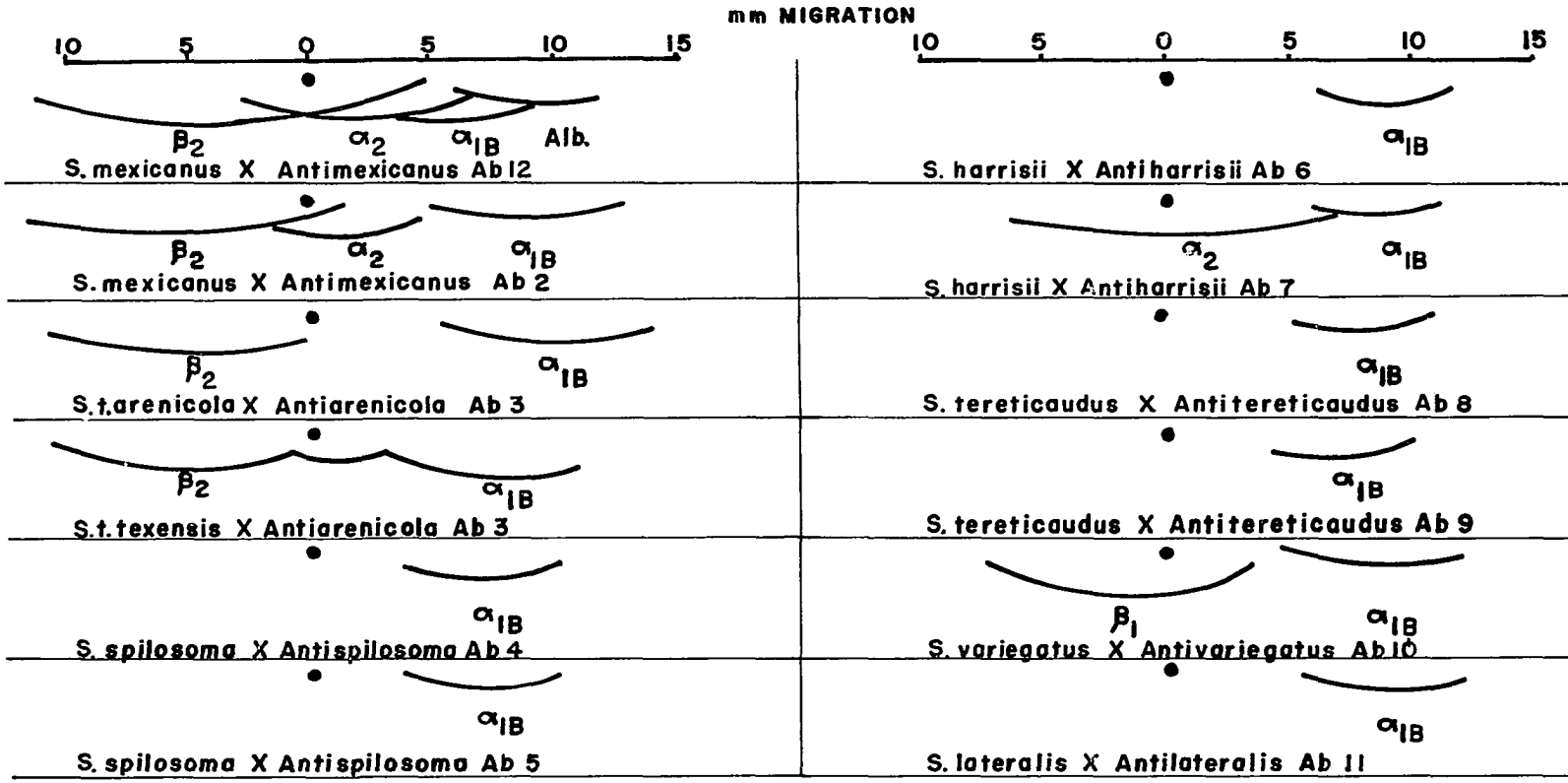
Histochemical Analyses

Detection of glycoproteins by the PAS test has a two-fold purpose; particular precipitin arcs could be distinguished as having a carbohydrate moiety, and phylogenetic implications could be inferred. Figure 10 illustrates the PAS test results. The alpha 1 B arc of the eight species tested, contains glycoprotein. The beta 2 arc of S. mexicanus, S. t. arenicola and texensis and the beta 1 fraction of S. variegatus show a positive reaction to the PAS reagents, as does the alpha 2 arc of S. mexicanus and S. harrisii. The results of the PAS test indicate only that carbohydrate reactivity is seen in these fractions.

Interpretation of these results include the fact that alpha 1 B is an ubiquitous serum fraction and may be a generic trait of the species selected for this study. The tridecemlineatus species group can be distinguished from the spilosoma species group by the presence of carbohydrate in the beta 2 fraction. S. mexicanus can be distinguished from S. tridecemlineatus and S. spilosoma by the alpha 2 fraction. Little can be stated as to the relative importance of the alpha 2 fraction of S. harrisii and the beta 1 fraction of S. variegatus, as similar comparisons would have to be made with other closely related species prior to any

Figure 10. PAS reactions indicative of carbohydrate moieties in serum protein fractions exhibited by specific immunoprecipitin reactions. The arcs exhibiting a positive reaction are labeled.

PERIODIC ACID-SCHIFF REACTION



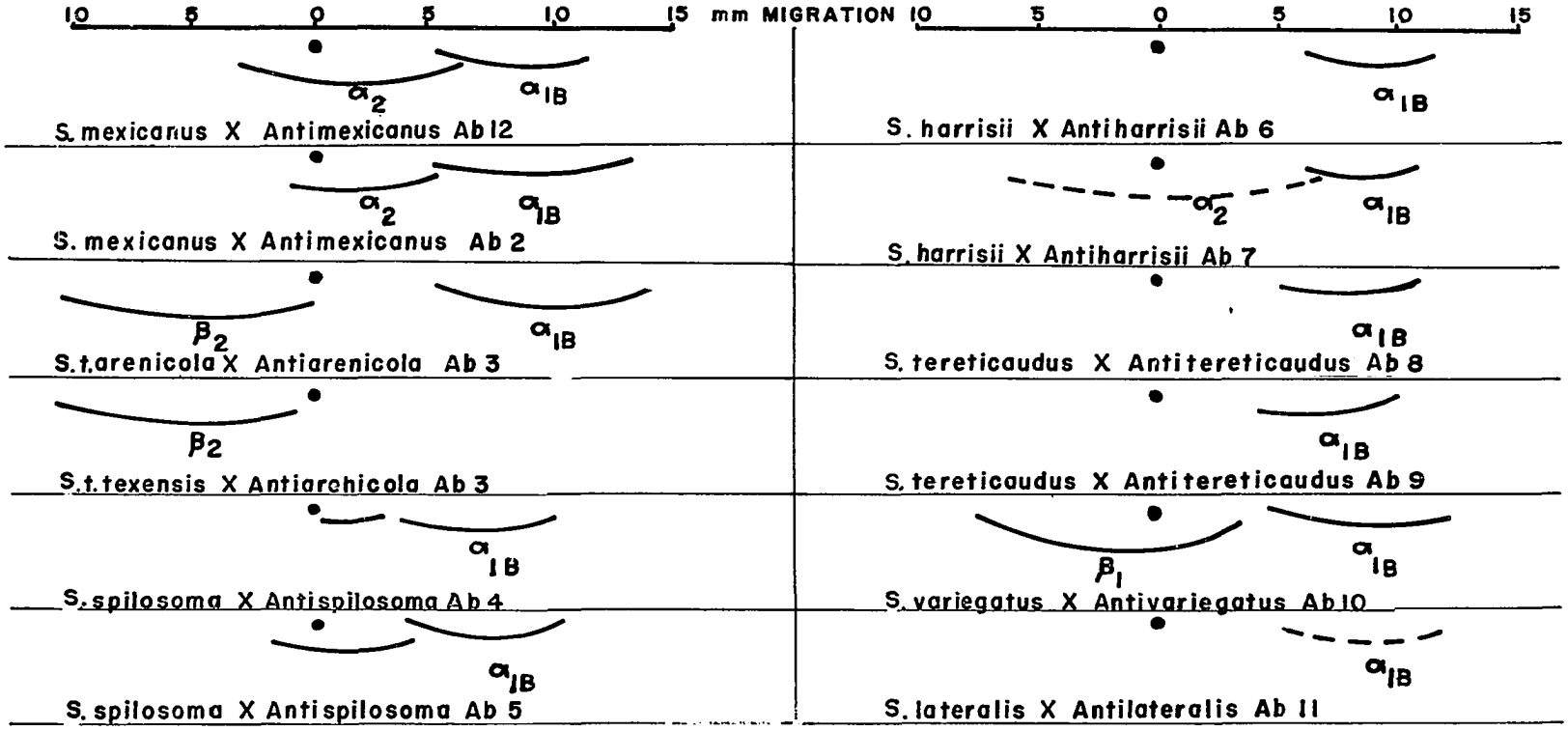
judgement on the taxonomic relevance of these fractions.

Detection of the presence of carboxylic esterase enzymes in the protein fractions by characterization with a beta-naphthyl ester substrate also reveals taxonomic characteristics (Figure 11.). Again, all species studied exhibit enzymatic activity in the alpha 1 B fraction, except for S. t. texensis. This subspecies was not available for study when antisera were produced, therefore, the antiserum used in this test is not species specific. This may account for the lack of enzyme activity in the alpha 1 B fraction. Both S. t. arenicola and texensis show an esterase activity in the beta 2 fraction. The alpha 2 fractions of S. mexicanus, S. spilosoma, and S. harrisii exhibit esterase activity. S. variegatus shows esterase activity in the beta 1 fraction. The relationships inferred by the PAS test are not as evident in this histochemical test. The separation of the species in the Ictidomys subgenus by this test is conceivable, however, without other representatives of the remaining subgenera no concrete taxonomic characteristics can be determined.

Histochemical characterization using Oil Red O stain for lipoproteins and cholinesterase activity produced negative results. These test procedures were not repeated.

Figure 11. Beta-naphthyl esterase activities demonstrating enzymatic components of the serum protein fractions through specific immunoprecipitin reactions. Arcs exhibiting a reaction are labeled.

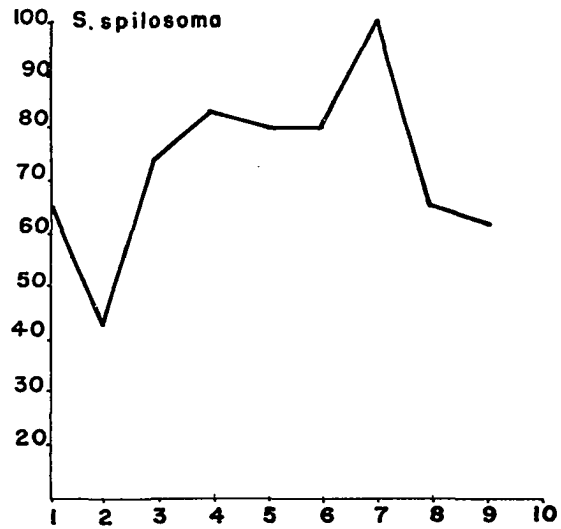
β-NAPHTHYL ESTERASE ACTIVITY

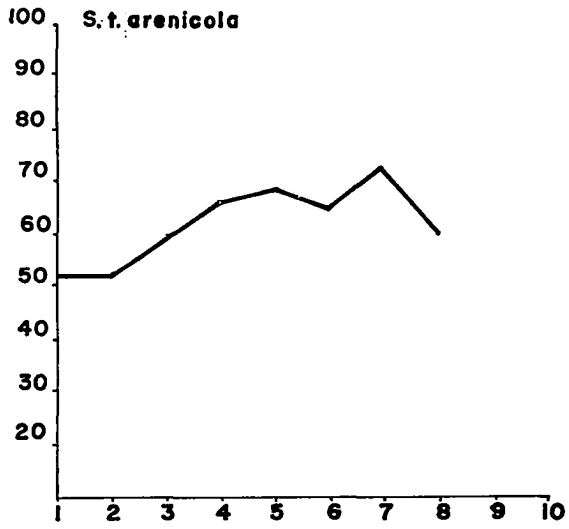


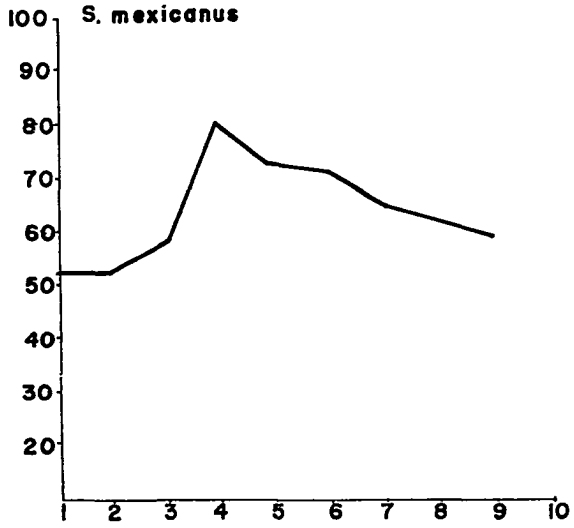
Quantitative Precipitation

The quantitative precipitation test results are plotted in Figures 12 and 13. Only antispilosoma Ab 5 antiserum was used as a test reagent, for it gave the highest titers in the precipitin ring test, the greatest immunoelectrophoretic reactivity, and was available in an adequate volume. The amount of protein nitrogen in each dilution tube of each species is expressed as a percentage of the amount present in the equivalence tube of the antispilosoma-spilosoma precipitin reaction. The equivalence point is the point in the dilution series where the proportions of antigen (serum proteins) are equal to the proportions of antibody (antiserum Ab 5), and is represented as dilution tube number 7 in S. spilosoma (Figures 12. and 13.). Dilution tube numbers lower than that of equivalence represent an excess of the antigen; tube numbers higher represent antibody excess. The quantitative precipitation test results are interpreted by using the percent protein nitrogen and dilution tube number of the equivalence point of each species and comparing these values. The S. spilosoma, S. t. arenicola and S. t. texensis equivalence points are seen to be at tube number 7, whereas, S. mexicanus equivalence falls at tube number 4 (Figure 12.). The protein nitrogen at equivalence of S. mexicanus and the

Figure 12. Precipitation curves indicating immunological relationships of the species of the subgenus Ictidomys. Ordinate values are expressed as percentages of the observed values relative to the reference precipitation test (S. spilosoma). The abscissa values are the dilution tube numbers of a doubling dilution series.


S. spilosoma
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10


S. t. arenicola
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10


S. mexicanus
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10

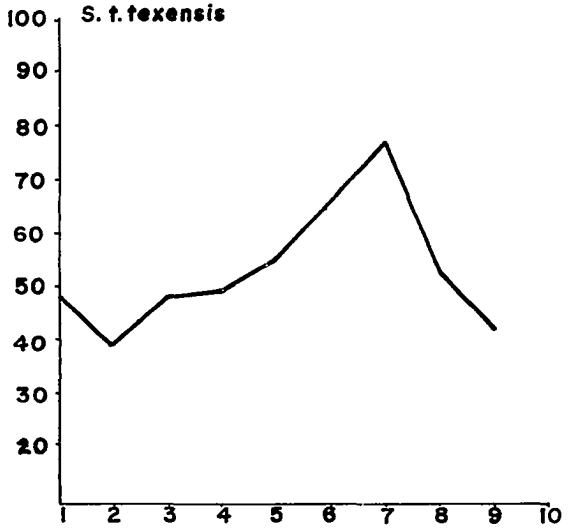
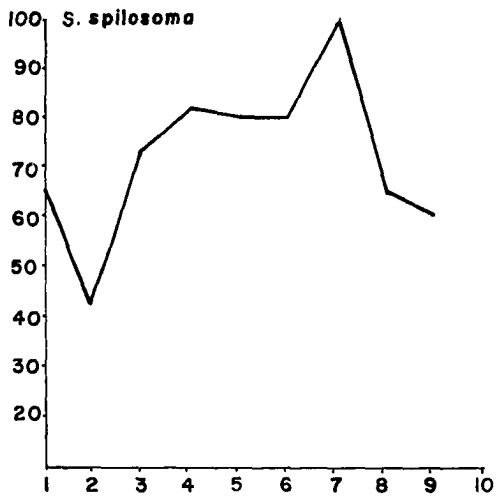
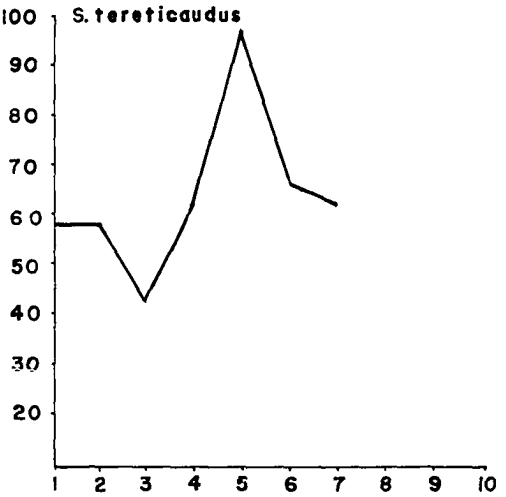
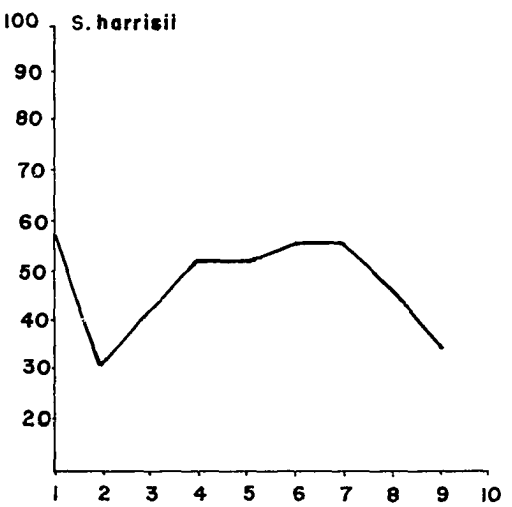
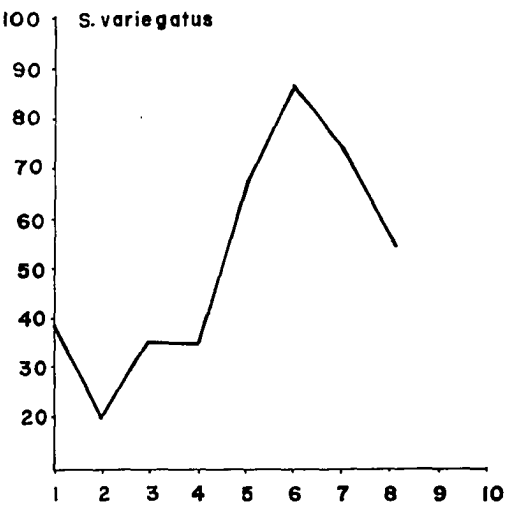

S. t. texensis
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10

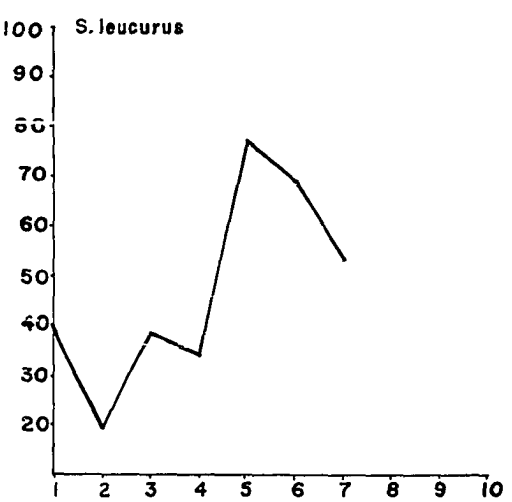
Figure 13. Precipitation curves indicating immunological relationships of six species representing five subgenera of ground squirrels. Ordinate values are expressed as percentages of the observed values relative to the reference precipitation test (S. spilosoma). The abscissa values are dilution tube numbers of a doubling dilution series.

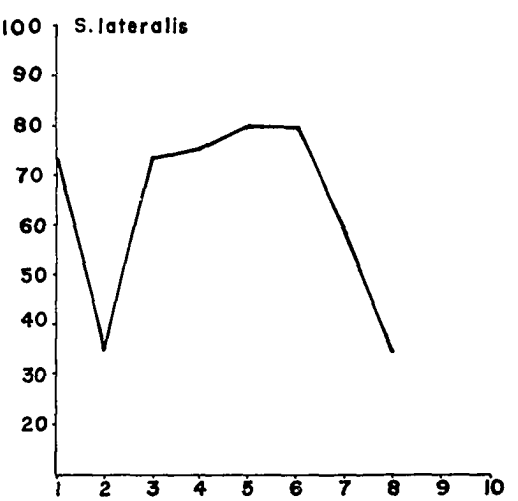

S. spilosoma
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10


S. tereticaudus
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10


S. harrisii
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10


S. variegatus
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10


S. leucurus
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10


S. lateralis
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10

two subspecies of S. tridecemlineatus is approximately 80% that of S. spilosoma.

When S. spilosoma specific precipitation is compared to the species representing the other four subgenera, some relationships are indicated (Figure 13.). The points of equivalence of the representatives of the subgenus Ammospermophilus fall between tubes 5 and 6 at 75% and 55% for S. leucurus and S. harrisii respectively. S. tereticaudus (Xerospermophilus) exhibits a 97% equivalence at tube 5. S. variegatus and S. lateralis (Otospermophilus and Callospermophilus respectively) show an equivalence at tube 6 with a protein nitrogen value ranging between 80 and 90 percent that of S. spilosoma. None of the other subgenera show an equivalence point at tube 7, which indicates differences in the total serum protein antigenicity that is two to four times less in these subgenera than that represented within the Ictidomys subgenus. The subgenera Ammospermophilus and Xerospermophilus appear to be more closely related to each other than to any of the other subgenera represented. The subgenera Otospermophilus and Callospermophilus also appear to be more closely related to each other by the results of this test. The taxonomic implications of this test are also demonstrated within the Ictidomys subgenus. The tridecemlineatus and spilosoma species groups can be separated by percentages of protein

nitrogen, and S. mexicanus can be separated from S. tridecemlineatus subspecies by equivalence tube number. The subspecies of tridecemlineatus cannot be individually characterized by this method.

CHAPTER IV

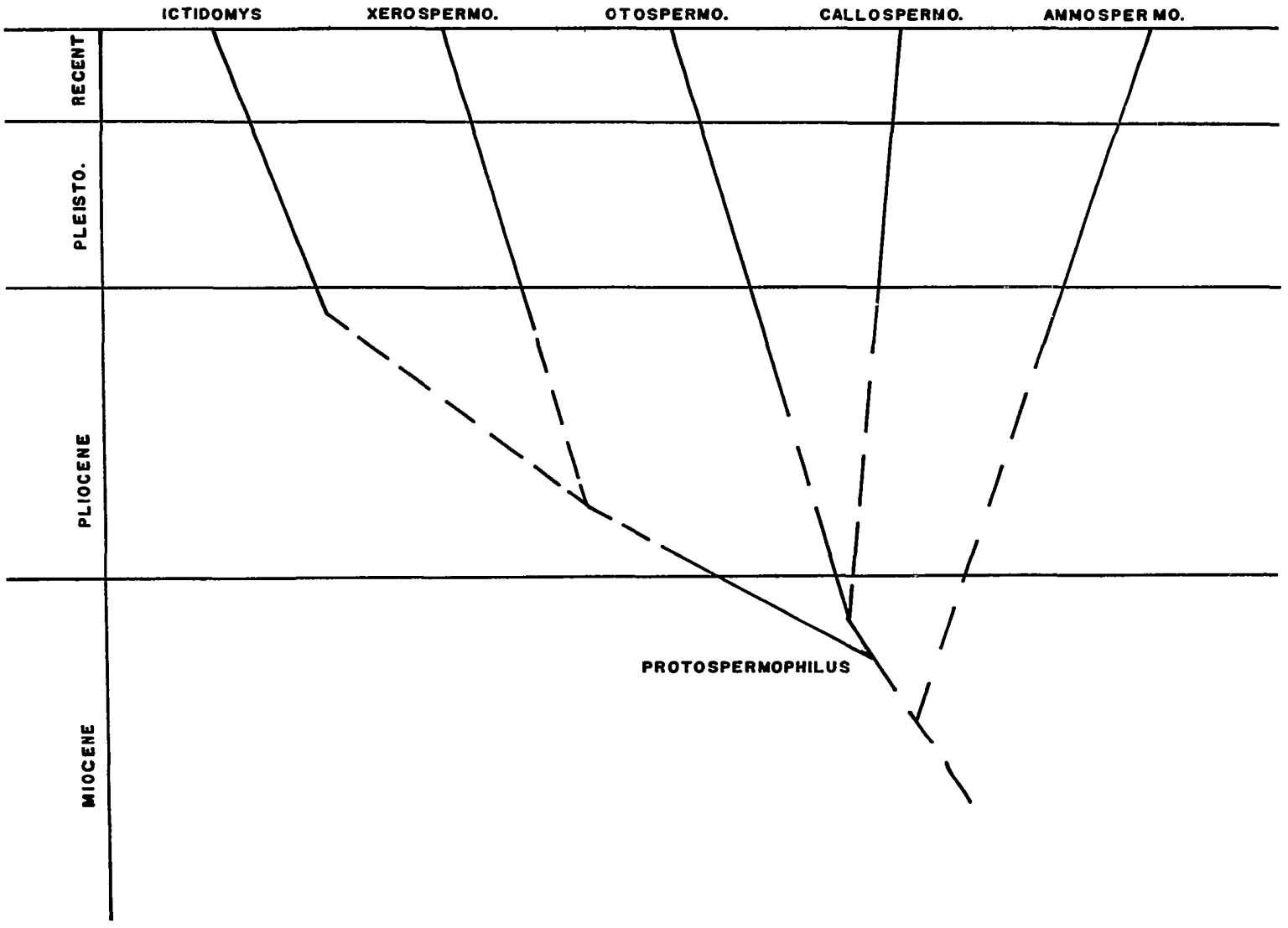
DISCUSSION

Howell (1938) revised the genus Spermophilus (= Citellus) to include eight subgenera: Citellus, Ictidomys, Poliocitellus, Otospermophilus, Notocitellus, Ammospermophilus, Xerospermophilus and Callospermophilus. Since this work, Ammospermophilus has been raised to generic rank and the accepted genus name is Spermophilus, the type subgenus is Spermophilus (Hall and Kelson, 1959). Howell (1938) utilized pelage, cranial and bacular morphology as the bases for this revision. Taxonomic relationships of these subgenera were not clearly defined until Bryant (1945) and Black (1963), using a number of morphological features obtained from fossil and Recent specimens, revealed a number of taxonomic relationships within this genus. Both investigators consider Otospermophilus to be the oldest and least specialized of the ground squirrel line. Bryant (1945) considers Otospermophilus and Callospermophilus to be more closely related than they are to the other subgenera of ground squirrels. Ictidomys and Spermophilus, according to Bryant are the most recently evolved and the most highly

specialized of the subgenera and are closely related. Bryant (1945) states that the species assigned to the Ictidomys subgenus are not as closely related to each other as the species of the other subgenera are. He suggests that S. tridecemlineatus and S. mexicanus are more similar to each other than either is to S. spilosoma, according to baculum and pelage characteristics. Bryant (1945) suggests that Xerospermophilus is related in an intermediate fashion between the more primitive and the more specialized groups, and although he places Ammospermophilus in generic rank, he intimates that these two groups share a number of features including dentition (Figure 14.). Black (1963), in a treatise on the Tertiary Sciuridae, regards Otospermophilus to be the ancestral stock of the Xerospermophilus and Callospermophilus subgenera. Black (1963) concurs with the view that Ictidomys is more closely related to Spermophilus than to the other subgenera and he infers that Ammospermophilus (generic rank) arose in early Pliocene times from a Spermophilus ancestral stock, however, he offers little fossil evidence.

Recent taxonomic investigations by Nadler (1966b) using mitotic chromosome morphology and numbers, demonstrate that Otospermophilus with a low diploid number and no acrocentric chromosomes does not display a "primitive" karyotype, namely, a high diploid number and numerous acrocentric chromosomes. The status of Otospermophilus as

Figure 14. Bryant's scheme of the phylogenetic relationships of five subgenera of the genus Spermophilus. Those subgenera not included in the present study are excluded (from Bryant, 1945).

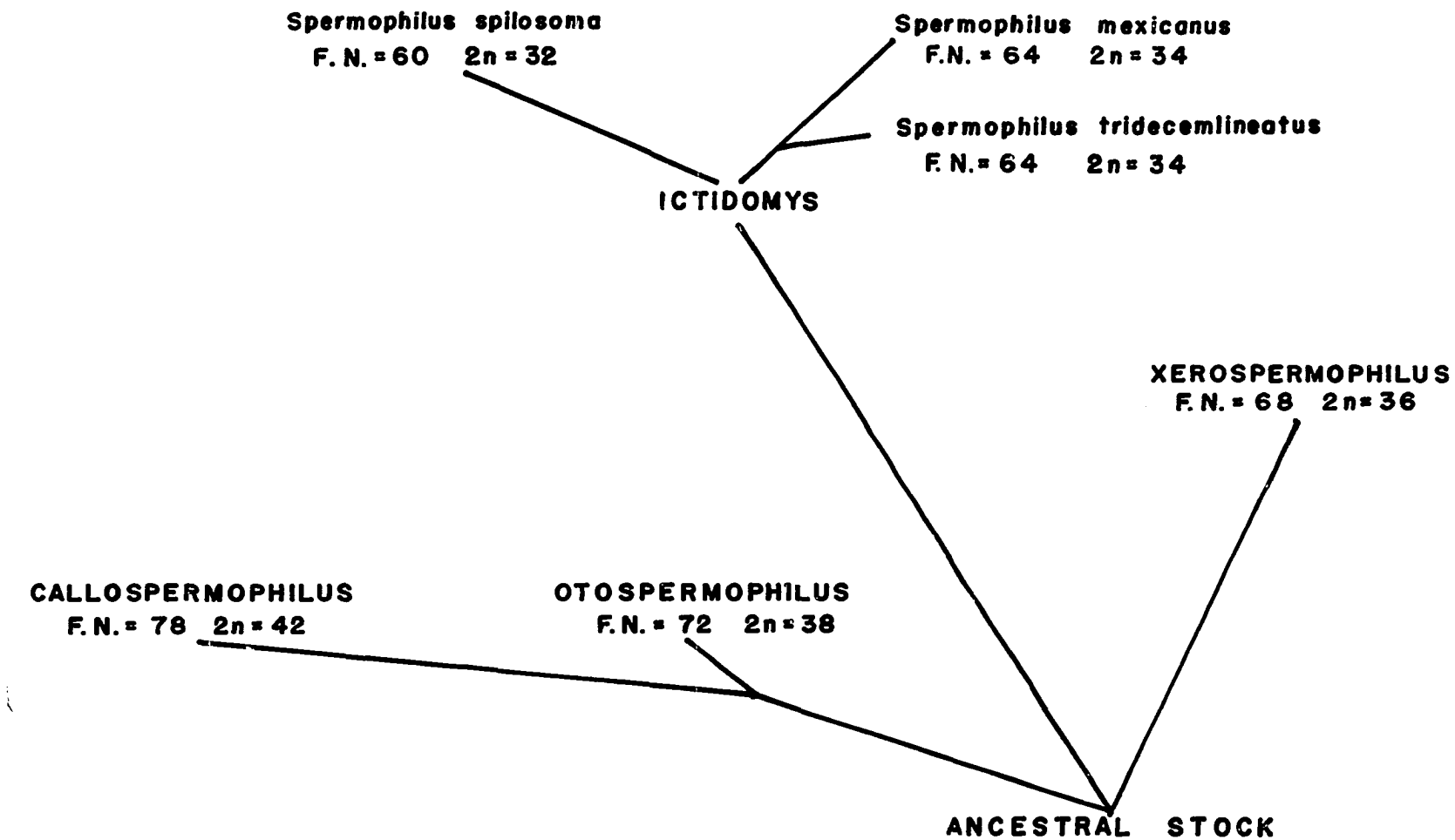

ICTIDOMYS
XEROSPERMO.
OTOSPERMO.
CALLOSPERMO.
AMMOSPERMO.
RECENT
PLEISTO.
PLIOCENE
MIOCENE
PROTOSPERMOPHILUS

the ancestral stock of the genus is questioned. However, the fundamental number (F.N.) of chromosomes indicates close relationships between Otospermophilus and Callospermophilus (Figure 15.). The F.N. values for Xerospermophilus indicate that this subgenus is not closely related to Callospermophilus as determined by Black (1963). Nadler (1966) supports the views of both Bryant (1945) and Black (1963) that Ictidomys is more closely related to Spermophilus than to the other subgenera on the basis of fundamental and diploid chromosomal numbers.

Nadler and Hughes (1966a) demonstrated close similarity between the three species of the Ictidomys subgenus through morphological similarities of submetacentric and metacentric chromosomes, as well as, similarity in the number of submetacentric chromosomes. These authors offer supporting evidence for the presence of two species groups within this genus. Nadler (1962) was able to differentiate between the karyotypes of S. tridecemlineatus and S. mexicanus while still showing close relationships between the two species (Figure 15.).

Gerber and Birney (1968) found Otospermophilus serum protein reactivities, in quantitative precipitation analyses, indicated that this subgenus apparently diverged earlier in the genetic lineage of the genus and has been evolving independently for a longer period of time than the other

Figure 15. A phylogenetic scheme compiled, by this author, from the results of karyotypic studies by Charles F. Nadler showing relationships between the five subgenera used in the present study. F.N. is the fundamental number of chromosomes; 2n is the diploid number.


Spermophilus spilosoma
F. N. = 60 2n = 32
Spermophilus mexicanus
F.N. = 64 2n = 34
Spermophilus tridecemlineatus
F. N. = 64 2n = 34
ICTIDOMYS
XEROSPERMOPHILUS
F. N. = 68 2n = 36
CALLOSPERMOPHILUS
F. N. = 78 2n = 42
OTOSPERMOPHILUS
F. N. = 72 2n = 38
ANCESTRAL STOCK

ground squirrel subgenera. These investigators support the views of previous researchers that Callospermophilus and Otospermophilus are more closely related than either is to Ictidomys or Spermophilus. Ictidomys and Spermophilus were shown to be related to each other through data from precipitin tests. However, Gerber and Birney agree with Bryant (1945) that the two subgenera must be the result of separation of two evolutionary lines in the Pliocene.

The biochemical separation and characterization of serum proteins utilizing several modifications of micro-electrophoresis reveal taxonomic characters, by which phylogenetic relationships of ground squirrels may be postulated. The valid use of serum proteins as taxonomic characters is theoretical, and is based on the assumption that the proteins are under genetic control. Thus, differences in genotype will be reflected by an alteration in the chemical structure and behavior of the serum proteins. The acceptance of these characters as reliable indicators of taxonomic relationships is dependent upon exclusion of any protein differences brought about by developmental stages or physiological states of the species being studied (Dessauer and Fox, 1964). In the present study, in an effort to minimize nongenetic variation, no pregnant animals were used, all specimens were classed as adults, and no differences in serum proteins were attributable to sexual

differences.

The implication throughout this study is that each method of protein characterization must be evaluated separately and any taxonomic inferences must be drawn from these tests individually. The serum protein fractions exhibiting major similarities and differences both in electrophoretic mobility and immunoelectrophoretic reactivity are alpha 1 B, alpha 2, and beta 2. However, a definite difference is observed between the two methods when those fractions or arcs that appear to be shared by all eight species are compared. The beta 2 fraction (Figures 1. and 2.) is shared by all species in this investigation when mobilities are used as taxonomic characters, whereas, the alpha 1 B arc is shared by all the species when reactivities are compared (Figures 4. through 9.)

Electrophoretic mobilities of the serum protein fractions exhibit differences that are of taxonomic importance. The beta 2 fraction is present in all tested species and is here considered to be a generic trait. Within the subgenus Ictidomys, the significance of fraction mobilities is most clearly demonstrated. The alpha 1 B and alpha 2 fractions are clearly subgeneric traits of this group when compared to the other subgenera. The tridecemlineatus species group, by the presence of the beta 1 fraction can be distinguished from the spilosoma group. The beta 3 fraction is a common

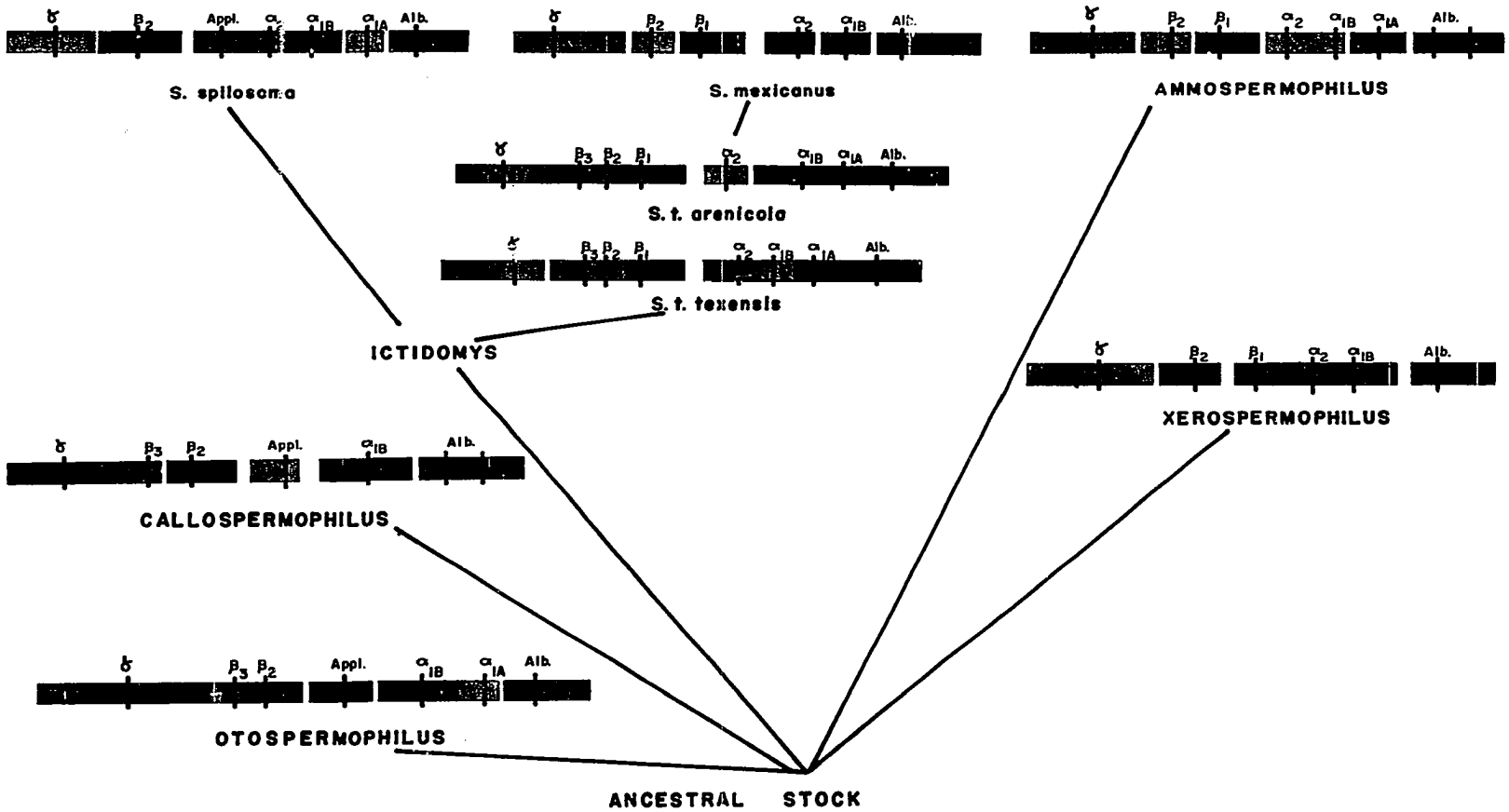
trait within the species tridecemlineatus and is not shared with the other members of the subgenus Ictidomys. The alpha 1 B and double albumin fraction mobilities are quire similar in both S. harrisii and leucurus and are probably subgeneric traits. The alpha 2 fraction is probably a taxonomic difference between the two species represented in this study.

The relationships of the five subgenera indicated by the electrophoretic mobilities of the fractions may be summarized collectively. Otospermophilus and Callospermophilus show close similarity in the gamma, beta 3, beta 2, application (alpha 2) and alpha 1 B fractions. Callospermophilus and Ammospermophilus are similar in the presence of a double albumin fraction. The double albumin fraction is present in the species of the subgenus Spermophilus according to Nadler (1968). Ammospermophilus and Xerospermophilus share fraction mobilities of the gamma, beta 2, beta 1, alpha 2, and alpha 1 B fractions; of which the gamma and beta 2 fractions are similar in mobility to those of the species in the subgenus Ictidomys. Ictidomys shares the mobility of the alpha 1 B fraction with both Otospermophilus and Callospermophilus. Thus, Otospermophilus and Callospermophilus are judged to be more closely related to each other than to the other three subgenera. Xerospermophilus and Ammospermophilus serum protein mobilities infer a close

relationship between these two subgenera. Ictidomys does not exhibit close similarity to any of the other subgenera in this study and it is assumed that this subgenus is the result of a distinct phylogeny. The electrophoretic mobilities of the serum protein fractions clearly distinguish the two species groups represented in the subgenus Ictidomys. The two subspecies of S. tridecemlineatus can be distinguished by this method.

The relationships of the five subgenera used in this study are diagrammed in Figure 16. The basis of this phylogenetic scheme is the presence of the beta 2 fraction in all the species studied. The ubiquitous representation of this fraction indicates that this protein is an ancestral character, and that it has been retained with only slight chemical modifications resulting in differences in net charge and molecular size. The relative densities of each fraction are indications of the quantity of protein present in each fraction, according to the properties of the stain used. When densities of the beta 2 fraction are compared, Callospermophilus and Otospermophilus are the only subgenera demonstrating this fraction in the greatest quantity (Figure 16.). The other subgenera demonstrate beta 1 to be present in a greater quantity than beta 2. S. spilosoma of the genus Ictidomys is an exception, as it exhibits only the beta 2 fraction in equal quantity with Callospermophilus

Figure 16. A proposed scheme of the relationships of the five subgenera of gound squirrels used in this study, based on the electrophoretic patterns and particularly on fraction mobilities and densities.


γ β2 Appl. α2 α1B α1A Alb.
S. spilosoma
γ β2 β1 α2 α1B Alb.
S. mexicanus
γ β2 β1 α2 α1B α1A Alb.
AMMOSPERMOPHILUS
γ β3 β2 β1 α2 α1B α1A Alb.
S. t. arenicola
γ β3 β2 β1 α2 α1B α1A Alb.
S. t. texensis
ICTIDOMYS
γ β2 β1 α2 α1B Alb.
XEROSPERMOPHILUS
γ β3 β2 Appl. α1B Alb.
CALLOSPERMOPHILUS
γ β3 β2 Appl. α1B α1A Alb.
OTOSPERMOPHILUS
ANCESTRAL STOCK

and Otospermophilus (Figure 16.). The shared beta 3 fraction between S. tridecemlineatus, Callospermophilus, and Otospermophilus indicates that the subgenus Ictidomys may have been derived from a fimilar, if not the same, ancestral stock. S. mexicanus and S. spilosoma have secondarily lost the beta 3 fraction and are considered to be more specialized than S. tridecemlineatus (Figure 16.). Ammospermophilus is considered to be more specialized than the other subgenera examined in this study by virtue of the double albumin fraction and the beta 2-beta 1 quantity difference (Figure 16.). Xerospermophilus is considered to be a moderately specialized subgenus by the beta 2-beta 1 quantity difference and the loss of the alpha 1 A fraction. Xerospermophilus, Ammospermophilus, and Ictidomys share several features (including gamma mobility, alpha 2 mobility, beta 1 mobility and quantity, and alpha 1 B mobility) and may have been derived from a similar ancestral species (Figure 16.).

Callospermophilus is considered to be moderately specialized through loss of the alpha 1 A fraction and the presence of a double albumin fraction, and was probably derived from the same ancestral species that gave rise to Otospermophilus (Figure 16.).

The subgenus Ictidomys poses a phylogenetic problem as each species demonstrates a divergent pattern when compared

to other species of this subgenus. However, S. tridecemlineatus appears to be the more generalized species, with S. mexicanus being derived from a similar ancestor species. S. spilosoma appears to be closer to the ancestral stock of this subgenus.

The immunoelectrophoretic procedure relates directly to the electrophoretic mobilities of the serum fractions for identification purposes, but is distinct in respect to the immunologic affinities of both the sera and antisera used (Crowle, 1961). The relationships elucidated by immunoelectrophoretic analysis are expressed by the ASCI values, which are indicative of the immunological cross-reactivities between the species studied, Otospermophilus exhibits the greatest degree of cross-reactivity of any of the subgenera tested. S. mexicanus and S. spilosoma also show high reactivities when the species are evaluated individually. This evidence is in agreement with the results of Gerber and Birney (1968) that Otospermophilus is close to the ancestral stock of the genus Spermophilus. Otospermophilus, Callospermophilus, and Ictidomys exhibit similar cross-reactivities and could be considered to be closely related, however, the immunoelectrophoretic arc patterns of Otospermophilus and Callospermophilus show a greater similarity to each other than either does to Ictidomys. Ammospermophilus, according to both arc patterns

and cross-reactivity value, is not closely related to any of the other subgenera but shows a distant relationship to Xerospermophilus by virtue of the two criteria employed.

Immunoelectrophoretic arc patterns and cross-reactivities indicate the suggested differences between the tridecemlineatus and spilosoma species groups of Ictidomys. The two subspecies of S. tridecemlineatus exhibit taxonomic differences by both arc pattern and ASCI values.

Taxonomically significant implications of the PAS test for glycoproteins are demonstrated by the members of the subspecies Ictidomys. The tridecemlineatus species group exhibits a carbohydrate component in the beta 2 fraction which is not present in this fraction in S. spilosoma serum. S. mexicanus does not exhibit a carbohydrate moiety in the alpha 2 fraction, whereas, S. tridecemlineatus and S. spilosoma alpha 2 fractions show a positive PAS reaction. The alpha 1 B fraction of all eight species contains a carbohydrate component and is significant as a generic trait. There are no other characteristic glycoprotein components of serum protein fractions that can be said to be of taxonomic importance. The PAS test results in this investigation do not provide adequate information to permit characterization or separation of the five subgenera. Based on the results obtained from the Ictidomys species, assumptions can be made that if a greater number of species

within each subgenus were analysed, this test might serve as a useful method for taxonomic distinction in the lower taxa.

Taxonomic separation based on carboxylic esterase enzyme characterizations in this investigation do not provide conclusive information as to the relationships of the species or subgenera studied. The separation of the species of Ictidomys is tenable, however, the absence of a shared component in the tridecemlineatus species group raises the question of the reliability of this type of characterization as taxonomically feasible. Perhaps more analysis with a greater number of representative species could provide the taxonomic relationships not observed in this study.

Quantitative precipitation tests results are quite similar to those of the other methods used in this investigation. The relationships between the spilosoma and tridecemlineatus species groups are demonstrated when the amount of protein nitrogen within the precipitate is compared. Theoretically, the equivalence point of all the species of Ictidomys should have been at dilution tube 7, however, this was not the case in S. mexicanus, sputtering in the digestion tubes could have caused such a divergent value. The equivalence points and percentage protein nitrogen provided two parameters for determining taxonomic relationships within the subgenera represented. Ammospermophilus and

Xerospermophilus are quite similar to each other in respect to both parameters. There is no similarity between these groups and the other three subgenera according to this analysis. Callospermophilus and Otospermophilus are quite similar in both parameters used. As S. spilosoma (Ictidomys) was used as the test antiserum, Ictidomys can not be compared to the other four subgenera. This method of taxonomic separation is not useful below the species taxon, as demonstrated by lack of appreciable differences between S. t. arenicola and texensis.

CHAPTER V

SUMMARY AND CONCLUSIONS

The serum proteins of eight species representing five subgenera of the ground squirrel genus Spermophilus are evaluated as indicators of taxonomic relationships. The principle analysis involves separation and characterization of the serum proteins by agar-gel electrophoresis.

Electrophoretic patterns of the serum proteins are diagnostic at the subgeneric level in Otospermophilus, Callospermophilus, Ictidomys, Xerospermophilus, and Ammospermophilus. These patterns provide diagnostic features at the species group and species levels as demonstrated within the subgenus Ictidomys. The use of electrophoresis as a systematic method is also indicated at the subspecies level by two subspecies of Spermophilus tridecemlineatus. Pooled serum samples of each species and individual serum samples provide identical patterns.

The subgenera Otospermophilus and Callospermophilus exhibit similar protein patterns which indicate close relationships between these forms. Otospermophilus is considered to have the more generalized protein pattern of the

subgenera examined, while Callospermophilus shows some specialization in the albumin and alpha protein fractions.

Ammospermophilus serum protein electrophoretic patterns are similar to that of Xerospermophilus with specialization of the albumin and alpha fractions. These subgenera are distinctly different from Otospermophilus and Callospermophilus, particularly in the beta fraction mobilities and densities.

The subgenus Ictidomys is represented by the three species of ground squirrel assigned to this taxon. Spermophilus mexicanus and S. tridecemlineatus patterns are similar, however, distinct differences in the beta protein fraction indicate S. tridecemlineatus to be the more generalized of the two species. S. spilosoma protein patterns indicate that this species shares a common ancestral relationship with the tridecemlineatus species group, and in fact, may be closer to the ancestral stock of this subgenus. The subgenus Ictidomys shares certain characteristics with the other four subgenera and represents an intermediate form in the phylogenetic history of the genus Spermophilus.

Immunoelectrophoretic, histochemical, and quantitative precipitation analyses are used as adjunct methods to corroborate the relationships determined by the serum protein patterns. The results of this investigation compare

favorably with previously published systematic investigations concerning the genus Spermophilus.

Conclusions based on the results of this study are:

1.) Agar-gel electrophoresis of serum proteins, under carefully controlled conditions, can yield patterns which may be used as taxonomic characteristics.

2.) Serum protein fraction mobilities can be subjected to statistical treatment and are of taxonomic value.

3.) Immunoelectrophoretically derived precipitin arc patterns can be used to indicate phylogenetic relationships.

4.) The use of an Antiserum Correlation Index, developed by the author, can provide information concerning immunological cross-reactivity between homologous and heterologous antisera and serum proteins, probabilities of the degree of reactivity expressed by each serum protein, and a numerical index that can elucidate taxonomic relationships.

5.) Quantitative precipitation tests distinguish relationships between species and subgenera, but are not sensitive or reliable below the species taxon.

6.) Histochemical characterization of serum protein fractions obtained by electrophoretic or immunoelectrophoretic techniques may be of taxonomic value, provided a series of these tests are used.

7.) The subgenus Otospermophilus is demonstrated to

be closer to the ancestral stock of the genus Spermophilus than the other subgenera studied, from the information obtained in this investigation.

8.) The subgenera Otospermophilus and Callospermophilus show close relationship by sharing a number of characteristics of the serum protein fractions.

9.) Ammospermophilus exhibits closer serum protein similarities to Xerospermophilus than to the other subgenera studied. The generic rank accorded to Ammospermophilus is questioned on the basis of the relationships observed in this investigation.

10.) Xerospermophilus exhibits serum protein characteristics that are intermediate to Ictidomys, Callospermophilus, and Otospermophilus.

11.) Ictidomys exhibits few similarities to the other subgenera studied, but is considered to be more closely related to Otospermophilus.

12.) The subgenus Ictidomys can be separated into two species groups (tridecemlineatus including S. mexicanus and S. tridecemlineatus, and spilosoma containing S. spilosoma) according to the methods utilized.

13.) The relationships indicated by the methods employed do not include information of the interrelationships of the Poliocitellus, Notocitellus, or Spermophilus subgenera, as these groups were not studied.

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APPENDIX

APPENDIX I

Differences in mobilities of serum protein fractions between pooled and individual samples.

Fraction	Species	Individual Values	Pooled Values	"t" Calculated	"t" Tabular
Albumin	S. t. arenicola n= 13, s = 1.71	29.92	30.00	.045	2.179
	S. t. texensis n= 7, s - 2.15	27.57	30.00	1.056	2.447
	S. spilosoma n - 12, s = 1.50	28.67	32.00	.832	2.201
	S. mexicanus n = 14, s = 1.29	23.14	23.00	.061	2.160
	S. tereticaudus n = 8, s = 2.10	22.88	27.00	1.847	2.365
	S. variegatus n = 5, s = 2.17	29.20	34.00	2.020	2.776
	S. lateralis n = 7, s = 1.80	26.29	26.00	.152	2.447
	S. harrisii n = 12, s = 2.24	26.08	29.00	1.250	2.201
Alpha 1 A	S. t. arenicola n = 10, s = 1.95	23.40	23.00	.196	2.262
	S. t. texensis n = 7, s = 2.58	19.00	19.00	.000	2.447
	S. spilosoma n = 12, s = 1.86	22.00	22.00	.000	2.201
	S. variegatus n = 5, s = 2.61	21.60	19.00	.915	2.776
	S. harrisii n = 12, s = 1.53	18.17	21.00	1.780	2.201

APPENDIX I Con't.

Fraction	Species	Individual Values	Pooled Values	"t" Calculated	"t" Tabular
Alpha 1 B	S. t. arenicola n = 11, s = 2.05	16.73	17.00	.108	2.228
	S. t. texensis n = 5, s = 1.34	13.60	13.00	.205	2.776
	S. spilosoma n = 12, s = 1.34	14.17	15.00	.597	2.201
	S. mexicanus n = 13, s = 2.14	15.62	15.00	.279	2.179
	S. tereticaudus n = 8, s = 0.92	10.63	14.00	3.470**	2.365
	S. variegatus n = 5, s = 0.89	13.40	15.00	1.649	2.776
	S. lateralis n = 7, s = 2.29	15.29	19.00	1.510	2.447
	S. harrisii n = 12, s = 1.51	11.50	13.00	.955	2.201
Alpha 2	S. t. arenicola n = 12, s = 1.35	6.00	5.00	.714	2.201
	S. t. texensis n = 7, s = 1.50	8.29	9.00	.441	2.447
	S. spilosoma n = 8, s = 1.60	8.38	8.00	.225	2.365
	S. mexicanus n = 13, s = 1.11	7.69	6.00	1.469	2.179
	S. tereticaudus n = 8, s = 1.00	5.00	7.00	1.887	2.365
	S. harrisii n = 12, s = 1.53	4.83	5.00	.107	2.201

APPENDIX I Con't.

Fraction	Species	Individual Values	Pooled Values	"t" Calculated	"t" Tabular
Application	S. spilosoma n = 12, s = 1.24	.50	1.00	.387	2.201
	S. lateralis n = 7, s = 0.97	2.57	2.00	.548	2.447
	S. variegatus n = 5, s = 0.44	1.80	2.00	.417	2.776
Beta 1	S. t. arenicola n = 13, s = 1.04	5.92	6.00	.074	2.179
	S. t. texensis n = 7, s = 0.69	6.14	7.00	1.162	2.447
	S. mexicanus n = 14, s = 1.31	5.79	7.00	.896	2.160
	S. tereticaudus n = 9, s = 0.53	2.44	3.00	1.000	2.306
	S. harrisii n = 12, s = 0.67	4.92	4.00	1.322	2.201
Beta 2	S. t. arenicola n = 13, s = 0.85	10.69	9.00	1.911	2.179
	S. t. texensis n = 7, s = 0.82	10.00	11.00	1.140	2.447
	S. spilosoma n = 12, s = 0.99	10.91	12.00	1.048	2.201
	S. mexicanus n = 14, s = 0.85	12.57	14.00	1.632	2.160
	S. tereticaudus n = 8, s = 1.31	11.50	13.00	1.079	2.365
	S. variegatus n = 5, s = 0.54	9.60	10.00	.680	2.776

APPENDIX I Con't.

Fraction	Species	Individual Values	Pooled Values	"t" Calculated	"t" Tabular
Beta 2	S. lateralis n = 7, s = 0.53	10.57	11.00	.758	2.447
	S. harrisii	11.75	12.00	.276	2.201
Beta 3	S. t. arenicola n = 10, s = 1.55	15.20	14.00	.737	2.262
	S. t. texensis n = 7, s = 1.13	14.43	18.00	2.950**	2.447
	S. variegatus n = 3, s = 1.15	14.33	14.00	.250	4.303
	S. lateralis n = 6, s = 1.17	16.83	16.00	.659	2.571
Gamma	S. t. arenicola n = 6, s = 0.82	26.33	25.00	1.501	2.571
	S. t. texensis n = 6, s = 1.64	26.50	27.00	.282	2.571
	S. spilosoma n = 6, s = 1.38	22.50	25.00	1.678	2.571
	S. mexicanus n = 12, s = 1.73	24.08	24.00	.044	2.201
	S. tereticaudus n = 8, s = 2.05	25.75	27.00	.575	2.365
	S. variegatus n = 5, s = 3.65	29.40	32.00	.655	2.776
	S. lateralis n = 7, s = 1.62	28.57	30.00	.826	2.447
	S. harrisii n = 11, s = 1.13	23.45	25.00	1.319	2.228

Tabulated "t" values are taken at the 95% confidence limit.
** Indicates significant difference.