HORIZONTAL STARCH GEL ELECTROPHORESIS AS A TECHNIQUE FOR EXAMINING GRAY SQUIRREL POPULATION GENETICS

by

Ralph A. Otto

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Approved:

Henry S. Mosby, Chairman

Peter P. Feret

Patrick F. Scanlon

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Blacksburg, Virginia 24061

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INTRODUCTION

Within similar environments, two populations of the same species should maintain similar reproduction rates and numbers. Occasionally, situations are found which contradict this. McDowell (1954) found that populations of wild turkeys (Meleagris gallopavo) in two habitats in Virginia possessed excellent population growth in one habitat, but poor growth in the other. He was unable to find significant environment differences between the two habitats. Mosby (personal communication) has observed a somewhat similar situation for gray squirrels (Sciurus carolinensis). He found that in two similar habitats, separated by only 200 yards, the population density of one population is consistently lower than the density of the other population. To date, he has been unable to find an explanation for this disparity.

Under similar conditions, two populations of the same species would be expected to have similar population dynamics. Yet, as indicated previously (McDowell, 1954, and Mosby, 1969), situations occur where some dissimilar aspects of population dynamics arise under apparently similar conditions. For the wildlife manager who is striving to maintain healthy populations of game animals, it is of practical interest to be able to identify underlying causes of populational dynamic differences.

Wilson and Bossert (1971) have indicated that two major causes of inter-population variance include genetics and environment. Hypothetically, then, population dynamics may be thought of as a function of genetics and environment.

In considering two populations where the environments of the two areas seem similar, but the population dynamics are dissimilar, it is logical to suspect that the genetic components of the two populations differ or that environmental measurements are not precise. Thus, in seeking the causes of populational differences,both the environmental and genetic characteristics should be examined.

Genetic studies usually necessitate long periods of investigation, especially if breeding experiments are performed to establish genotypic variation. If, however, it is sufficient to establish gross genetic differences, an examination of phenotypes may be satisfactory. Phillips (1928) cited a classic example of phenotypic investigation. Following a drastic reduction in numbers of northern bobwhite quail (Colinus virginianus virginianus) field biologists demonstrated that introduced Mexican quail (C. v. texanus) had interbred with the northern quail and thus had lowered the cold tolerance of the northern populations. Following the introduction of the Mexican type, varying plumage gradations of phenotype (from northern through various hybrids to typical Mexican) were observed, and further investigations in Pennsylvania indicated that the hybrid type was highly susceptible to severe cold (Latham et al. 1947).

Phenotypic differences are not always easily discerned. Many morphological characteristics are heavily influenced by environmental factors. Van Tets and Cowan (1966),in a study involving deer (Odocoileus) blood serum, noted that penned deer typically differed in form and size from wild deer. Their work suggested that electrophoresis of blood

sera might be a way to examine differences between individuals and populations.

In the past 25 years, a vast amount of information on proteins has been accumulated. Electrophoretic techniques developed in biochemical laboratories are now being employed as an aid in systematic classification (Dessauer, 1966a; 1966b; Manwell and Kerst, 1966; Nadler and Hughes, 1966). Although the amount of research using electrophoresis is massive, published work on the reproducibility of the results is lacking.

The objective of the present study was to investigate the use of horizontal starch gel electrophoresis as a technique for studying the population genetics of gray squirrels (Sciurus carolinensis). Several starch gel systems were tested and evaluated, a new method of staining starch gels was evaluated, and the reproducibility of squirrel blood serum electropherograms was examined. Squirrel populations from several discrete woodlots were compared on the basis of the most reproducible segment of the electropherograms. The woodlots from which squirrel populations were sampled are located on the grounds of Virginia Polytechnic Institute and State University.

LITERATURE REVIEW

Electrophoresis is a term derived from Greek roots which literally mean "carrying by electricity." The principle involved has been known since 1907, when Alexander Reuss passed electricity through a glass column containing water and clay and noted that colloidal clay particles migrated anodally (Gray, 1951). The first truly analytical use of electrophoresis did not occur until 1937. Tiselius (1937) discovered that the globulin component of mammalian blood is composed of three separate fractions, alpha globulin, beta globulin, and gamma globulin (Gray, 1951). With his results, Tiselius published the design of his apparatus, which operated through moving boundary electrophoresis. In this type of electrophoresis, protein samples are placed in the bottom of a U-tube, buffer is carefully placed in both arms of the tube, and then electrophoresis is performed by placing electrodes at the tips of each arm. Following electrophoresis, the different protein fractions show up as boundaries in the arms of the tube, and may be precisely located by optical scanning methods.

An improvement over moving boundary electrophoresis came in 1950 when Durrum employed paper strips as a support medium to separate blood serum into five fractions (Durrum, 1950). This type of separation resulted in bands or "zones" of relatively homogeneous protein fractions which could be fixed in place and stained. The method is termed zone electrophoresis.

Smithies (1955) improved the resolution of zone electrophoresis by using a starch gel medium. One of the primary advantages of this method

was the molecular sieving effect of the gel; that is, separations on the gel occurred not only due to charge differences but also because of differences in the molecular shape and size of the material being electrophoresed. Smithies (1955) developed both a horizontal starch gel apparatus, and a vertical starch gel apparatus (Smithies, 1959a). The major advantage of the vertical method was that samples could be inserted directly with no supporting substance, thus permitting greater resolution.

Several other media have been used for electrophoresis, including polyacrylamide gels, cellulose acetate, powdered glass and glass fiber. The development and use of these techniques have been reviewed by Smith (1968). It should be noted, however, that among these methods, only polyacrylamide gels allow resolution comparable to starch gels. Indeed, in many instances, polyacrylamide gels allow superior resolution, due primarily to excellent destaining properties.

Hubby and Lewontin (1966) outlined the rationale and justification for using electrophoretic techniques in studies of population genetics. A sample of protein, when placed on a support medium and subjected to an electric field, will migrate at a rate proportional to its net electric charge and size. Since differences in the electrostatic properties of a given protein at a specific pH are equatable with differences in the amino acid composition of the protein, then electrophoretic mobility differences are equatable with differences in DNA composition.

In the second paper of their series, Lewontin and Hubby (1966) tested this rationale on laboratory populations of Drosophila pseudoobscura. By electrophoresing a number of enzymatic and nonenzymatic proteins, and

then carrying out traditional breeding experiments, they showed that for D. pseudoobscura electrophoretic techniques correctly demonstrate homozygosity or heterozygosity for selected loci. Lewontin and Hubby further stated that this method was applicable to any organism of macroscopic dimensions.

Hubby and Throckmorton (1968) restated the assumptions underlying Hubby and Lewontin's (1966) earlier work. While Hubby and Throckmorton assumed single sites of enzymatic activity or of protein staining on a gel as representing the product of a single locus, they recognized that this is a simplification. Actually two, and rarely more than two, structural genes may contribute polypeptide subunits to a migrating band. However, Hubby and Throckmorton go on to point out that any electromobility difference which does occur clearly represents a change in a structural gene. Furthermore, identical electromobilities probably reflect identical base sequences in structural genes, and the probability that they do increases as the number of other common features increases.

Selander (1970a) using starch gel electrophoresis, examined aspects of the genetic structures of wild populations of the house mouse (Mus musculus) relative to the behavioral mechanism of territoriality. He found that natural populations of Mus are characterized by genetic subdivisions even in the absence of physical barriers to migration. On the surface, this is a remarkable finding, since it implies that genetic drift may operate in the presence of potential migration. Wright (1930) calculated that genetic drift could not operate on a population if immigration were of the order of one individual per every other generation. But De Fries and McClearn (1970) pointed out that if the effective

population size is considered, there are cases where drift might occur to offset migration. Specifically, their work on laboratory populations of house mice suggested that social dominance might reduce the effective population size to the extent that genetic drift would become important even with a migration rate as high as three percent. This may be significant in considering geographically close, but otherwise discrete, populations.

For example, Mosby (personal communication) found that migration occurred between two proximate woodlots of gray squirrels at a very small rate (less than one percent per annum). But Pack et al. (1967) demonstrated the existence of a social hierarchy among the squirrels of these two woodlots, and found evidence to indicate that most of the reproductive contributions came from relatively few high ranking individuals. It is possible that the effective population size for at least some wild animal populations is reduced through social interaction, and that genetic drift may be of importance in determining the genetic structures of populations.

Chapman and Morgan (1973) investigated the population genetics of the cottontail rabbit (Sylvilagus). For mid-Atlantic areas, working with serum proteins, they detected little protein polymorphism in the New England species (S. transitionalis) but found "ten polymorphic regions" on the electropherograms of the eastern cottontail (S. floridanus). Chapman and Morgan also found that subspecies usually have discrete serum protein patterns including some belonging to the parapatric subspecies of S. floridanus. Additionally, these workers used morphological characteristics to aid in their work.

Nadler (1968) employed two-dimensional electrophoresis to analyze serum proteins in ground squirrels (Spermophilus). To justify his work, Nadler, after reviewing the assumptions of Lewontin and Hubby (1966), stated that comparison of the total protein pattern can provide apparently reliable taxonomic data, especially when closely allied species are investigated. Additionally, Nadler cited work by Goodman (1963) demonstrating that starch-gel electrophoresis of primate proteins also gave reliable data.

Marsh et al. (1969), also investigating ground squirrels, analyzed transferrins for four species, five subspecies, and five laboratory-produced subspecific hybrids. Their study demonstrated the value of electrophoresis in confirming hybridization.

A number of authors have considered how to best analyze electrophoretic data. Lewontin and Hubby (1966) computed the proportion of loci at which the average individual in a population was heterozygous. They calculated this by taking the gene frequencies of all the alleles at a locus, computing the expected frequencies of heterozygotes from the Hardy-Weinberg equilibrium, and then averaging these over all the loci for each separate population being considered. The result, a measure of the mean heterozygosity per individual, could then be compared between populations.

Berry (1963) developed a "measure of distinctiveness" for comparing the genetics of populations. His method involves transforming the percentage incidence P of a given character into an angular value, θ:

$$\theta = \text{SIN}^{-1}(1 - 2P).$$

Given percent incidences of the same character in two populations of size N_1 and N_2, the measure of distinctiveness, D, is defined by:

$$D = (\theta_1 - \theta_2) - (1/N_1 + 1/N_2)$$

where $(1/N_1 + 1/N_2)$ represents additional variance due to random sampling fluctuations which would tend to increase the value of $(\theta_1 - \theta_2)$. Berry admitted that there are invalid assumptions involved in this procedure, but still thought that this calculation would provide a reasonable estimate of genetic divergence between populations.

Brown (1969), as cited by Singh and Jain (1971), developed a method to compare the degree of polymorphism between two sets of markers. The method involves calculating an "Index of Polymorphism" which is defined by:

$$Z = \frac{\Sigma p_i q_i}{n}$$

or in the case of multiallelic loci,

$$Z = \frac{1}{n} \sum_{i=1}^{n} m_i \sum_{j=1}^{m_i} P_{ij}(1 - P_{ij})$$

where n = number of loci, and m = number of alleles at i^{th} locus. Singh and Jain (1971) mention that the frequency data may be obtained by noting presence or absence of individual electrophoretic bands.

Several authors (e.g. Coates, 1967, Chapman and Morgan, 1973, Smithies, 1959b) have presented qualitative analyses of electrophoretic data. This approach is especially useful when considering gross genetic differences between populations as opposed to comparing the frequencies of a specific allele within two or more populations. Coates' method of presentation consisted of schematic electrophermograms of several

individuals in conjunction with a composite electropherogram of the pooled sera of all types of individuals. Smithies (1959b) presented photographs of electropherograms for comparative purposes.

Few studies of the reproducibility of electrophoretic results have been published. Van Tets and Cowan (1966), in a paper on the blood sera of deer (Odocoileus), stated that the variation within gels was numerically less than between gels, which in turn was less than that between individuals. In the same paper (Van Tets and Cowan, 1966:641), they described comparisons of electropherograms between two groups of deer, stating:

> "In each instance the captive animals differed from the wild animals in having two rather than one negatively moving fraction. The data do not permit more detailed comparisons."

These authors did not elaborate as to why these data did not permit more detailed comparisons, but they did investigate many sources of variation of the serum protein characteristics of deer. Among these influences were among-individual variation (all individuals from the same subspecies, Odocoileus virginianus texanus), and sex-related variation, both of which were exhibited as variation in protein mobilities. Age-related and season-related variation was present in protein concentration but did not influence protein mobilities. These workers also mentioned a study of female deer (Odocoileus virginianus), stating that while there were pronounced differences in the mobilities of many of the fractions, in every instance these proteins "were still identifiable with their probable equivalent in other individuals" (Van Tets and

Cowan, 1966:641). Finally, in discussing the application of their results to the systematic status of Odocoileus, Van Tets and Cowan (1966:641) concluded:

> "We have revealed many sources of influence on the serum protein characteristics of deer. In their sum they make it most difficult to obtain truly comparable samples that can be used to explore possible genetically based changes."

Smith et al. (1973) experienced difficulty in obtaining comparable electropherograms during a study on Peromyscus floridanus. To resolve this problem, they devised a method of calculating relative mobilities whereby a separate set of mobilities was calculated for each locus. For a given locus, the band occurring in highest frequency was designated 100 if it migrated anodally, or -100 if it migrated cathodally. Other bands at this locus were designated numerically as percentages of distances migrated relative to that of the 100 band. The authors (Smith et al., 1973:3) commented on the reliability of other methods of computing relative mobilities as follows:

> "We hasten to note that, because the absolute and relative mobilities of allozymes may be affected by even minor changes in composition and pH of the gel or electrode buffers, the particular brand and lot of starch employed, and many other aspects of electrophoretic technique, the numerical values assigned to alleles pertain only to the particular gel from which the measurements of the positions of

> the allozymes were taken. Hence, alleles cannot reliably be identified by measuring absolute or relative distances from the point of origin. For confident identification of alleles, there is no adequate substitute for direct side-by-side comparison of mobilities of allozymes electrophoresed on the same gel."

Krepp and Smith (1974) used the same method of computing relative mobilities during a study on Magicicada. These workers reported that approximately 50 percent of the proteins visible on the gels were consistently scorable, but did not define "consistently scorable". The authors examined several biochemical systems of Magicicada; within the general protein system, they detected two scorable proteins, and two other proteins which were not consistently scorable.

Thus, from the reported literature it appears that while electrophoresis is a valuable tool in genetic investigations, it is neither an exact nor a precise technique. For starch gel media in particular, Smith et al. (1973) suggest that side-by-side comparisons on the same gel are indispensable for reliable identification of alleles. A number of workers reported variation and/or inconsistency in portions of the results of electrophoretic studies.

METHODS AND MATERIALS

Several electrophoretic systems were examined in this study. The components of interest within these systems were the gel concentration, the buffer composition, and the staining procedure.

Gel Concentration

A series of gels were prepared at 10, 12.5, and 15 percent concentrations. Although Smithies (1955) had recommended a 8.8 percent concentration, later workers obtained more consistent results with higher concentrations (Marsh and Jolliff, 1968). Preliminary indications from the current study suggested that Marsh and Jolliff were correct, and thus the minimum concentration examined was 10 percent.

In evaluating gel concentration, two criteria were considered: ease of handling (avoidance of breakage), and band resolution. Since starch gels must be sliced in order to stain properly, it is important that they handle without tearing or breaking. Band resolution must be adequate so that separate protein fractions may be discerned from one another.

Gels were prepared in various sizes and were then used as media to electrophorese squirrel serum. Hydrolyzed potato starch from Connaught Medical Laboratories (Toronto, Canada) was used to prepare the gels. After several trials, micro gels (75mm x 25mm x 5mm) were selected as the most appropriate size due to their excellent handling properties. Amperage was held constant at 20ma with initial voltage equal to 250 V. During the gel concentration trials, the buffer system was lithium hydroxide as described below.

Thus, for the gel concentration trials, the following conditions applied:

1) Gel concentrations were 10, 12.5, and 15 percent (starch/buffer), using Connaught hydrolyzed potato starch;
2) The substance being electrophoresed was squirrel blood serum;
3) Gel size was 75mm x 25mm x 5mm;
4) A lithium hydroxide buffer system was used; and,
5) Amperage was constant at 20ma, with an initial voltage of 250 V.

Buffer Systems

Three buffer systems were evaluated: tris-hydrochloric acid (electrode buffer pH = 8.2; gel buffer pH = 8.5, Selander et al., 1971); lithium hydroxide (electrode buffer pH = 8.1; gel buffer pH = 8.4, Selander et al., 1971); and, tris-glycine (electrode buffer pH = 8.3; gel buffer pH = 8.7, Marsh and Jolliff, 1968). It was assumed that there was no interaction between buffer system and gel concentration; i.e., if the optimal gel concentration was chosen given a particular buffer system, then by using this optimal gel concentration while varying the buffer systems would lead to further improvement in the resolution.

The choice of the above systems was guided by the results of previous workers (Smithies, 1955; Smith, 1968; Marsh and Jolliff, 1968; Selander, 1971). The systems tested by no means included all possible systems available (see: Smith, 1968 and Selander et al., 1971).

Staining Procedures

Amido black (Naphthol blue-black, Buffalo black) is suitable as a general protein stain (Smith, 1968; Selander et al., 1971; Chapman and Morgan, 1973). Feret (personal communication) suggested that coomassie

brilliant blue might be equally suitable. Thus, both amido black and coomassie brilliant blue were examined as stains. In addition, ponceau S was tested in preliminary trials.

The method of examination was by visual comparison of electropherograms after staining in the given reagents. Initially, reconstituted standard normal human serum was used as the test protein to minimize variation from sources other than the stains themselves. Later trials employed squirrel blood sera, to assure the compatibility of stains between test sera. Electrophoresis was performed on micro gels, at 20ma, with the lithium hydroxide buffer system. It was assumed that there was no interaction between buffer systems, gel concentrations, and stains.

Following electrophoresis, two gels were stained with each of the above mentioned stains, using procedures recommended by the suppliers in the cases of ponceau S and coomassie brilliant blue, and after Selander et al. (1971) in the case of amido black. The ponceau S was supplied by Gelman Instrument Co., Ann Arbor, Mich.; coomassie brilliant blue was obtained from Colab Laboratories, Inc., Chicago Heights, Ill.; amido black was procured from Eastman Kodak Corp., Rochester, N. Y.).

The stained gels were then compared, noting resolution and intensity of staining. No attempt was made to determine quantitative properties of the stains by densitometry or otherwise.

Sample Collection

Collecting Squirrels

Animals in this study came from four woodlots on the grounds of VPI and SU in Blacksburg, Virginia (see Fig. 1) or from areas in or

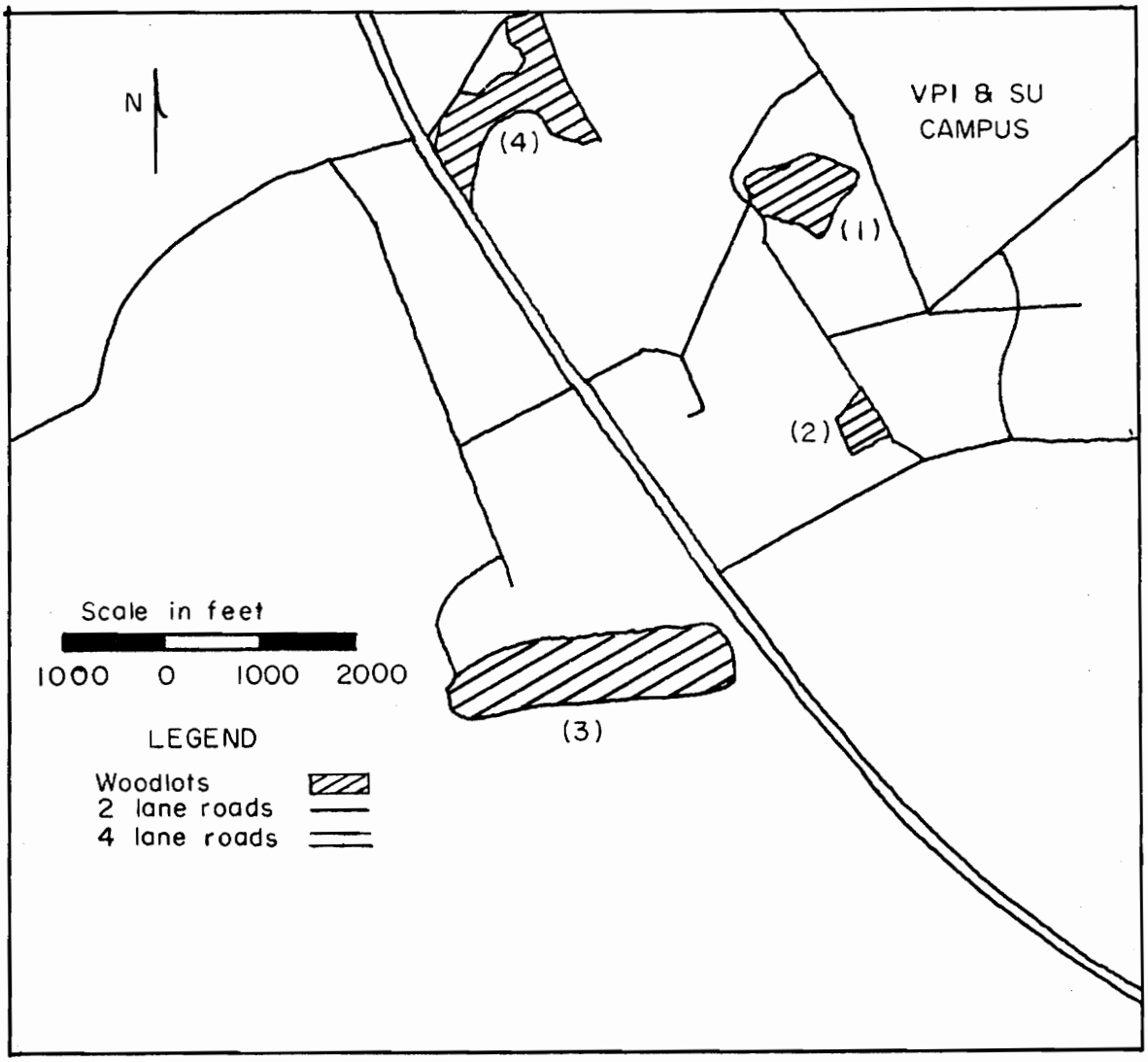

Fig. 1. The four primary woodlots of this study, on the VPI & SU College Farm: (1) President's Hill; (2) Turkey Pen; (3) Center; (4) Price's Fork.

close to Blacksburg. One woodlot, President's Hill, is perhaps best described as a semi-residential site with over-mature oak predominating. The understory was virtually devoid of shruberry, and was covered with well-mowed grass. The second woodlot, Turkey Pen Woods, was characterized by over-mature oak-hickory, with grasses and weeds which were heavily grazed by cattle. Center Woods, the third woodlot, contained a variety of tree species, including over-mature oak and hickory. The undergrowth was dense and was largely composed of seedlings and vines. The fourth woodlot, Price's Fork Woods, was similar to Turkey Pen Woods, but had an understory of brush and shrubs and a ground cover of high grass.

All squirrels were live-trapped in standard wooden box traps (Mosby, 1955), using dry corn kernels as bait. The first 36 animals to be trapped were transported to the laboratory and caged for three days before blood sampling. Initially, it was believed that the procedure would reduce stress and consequently reduce the number of animals lost through shock. During the course of the study, however, a shortage of laboratory space prohibited caging additional animals in the laboratory. Therefore, all animals beyond the 36th were bled in the field. Animals which were transported to the laboratory were fed water and Wayne Lab Blox ad libitum. Although it would have been preferable to maintain a single sampling procedure throughout the study, this was not feasible.

Squirrels were anesthetized using Methoxyflurane (Metofane; Pittman-Moore, Inc., Washington Crossing, N. J.). The captured animals were placed in a restraining cone, and the entire cone was inserted into a plastic bag (approximately 0.2 cubic feet in volume) containing

Methoxyflurane. Within four minutes, the animals were anesthetized. Most of the sampled squirrels were captured in April, 1974. Details regarding each specimen captured are shown in Appendix Table I.

Collection of Blood Samples

Blood samples (1-2 ml) were extracted by cardiac puncture using a 1 1/2 inch 20 gauge needle. The relatively large gauge needle permitted firm, straight insertion, and appeared to reduce hemolysis. The samples were left at ambient temperature in a test tube until clotting was observed; if the clot failed to retract, the inside of the test tube was gently reamed with a wooden probe. The samples were then refrigerated overnight (2 C) and serum was drawn off the following morning and frozen (-7 C). The samples of serum were kept frozen until immediately prior to electrophoresis. Freezing serum does not affect electrophoretic properties (Nadler, 1968).

Electrophoretic Procedure

Electrophoresis was performed on horizontal micro starch gel apparatus. The power supply was a canalco electrophoresis constant current source (Model 300B); the electrophoresis chamber was a Buchler Universal Electrophoresis Chamber; Dupont cellulose sponge cloths were used as wicks. The entire electrophoretic chamber was placed inside a refrigerator to prevent protein denaturation. Electrophoresis was performed at a constant 20ma with an initial voltage of 250 DC volts.

Serum samples were applied by pipette to wicks cut from Whatman No. 3 filter paper; wick dimensions were 5.0 x 10.0 mm. The wicks were then inserted into slots cut in the gels. Slots were cut with a 10 mm section of stainless steel razor blade to a depth of 5 mm.

Current was applied to the electrophoretic apparatus for a period of 2.5 hours. Immediately following electrophoresis, the gels were removed from the chamber and sliced for staining.

The gel slicer was a common cheese cutter with the original wire replaced by fine gauge wire. The gels were placed in a rectangular, bottomless tray with a number of blank microscope slides under the gel, the number of slides dependent on the thickness of slice required. Generally, the gels were sliced in half.

After slicing, the gels were placed in a fixing solution of 12.5 percent TCA (Chrambach, 1967). For one gel (i.e., two slices) 50 ml of fixing solution were used; each gel was placed in a separate container. Gels were fixed for 30 to 60 minutes. After fixation, 2.5 ml of 1 percent aqueous solution of coomassie brilliant blue were added directly to the fixing solution and then thoroughly (but gently) mixed. Gels were left in the stain for 60 minutes.

After staining, gels were placed in 10 percent TCA to destain and allow color intensification in the bands (Chrambach, 1967). It was found that gels were suitable for band recording 24 hours after being placed in the 10 percent TCA solution.

Recording Protein Fraction Bands

Protein fraction bands were recorded in the following manner: the stained gels were placed sliced-side up on a microscope slide and gently blotted with tissue paper to remove excess liquid. A clean labeled microscope slide was placed on top of the gel, with one end directly aligned with the sample insertion slot of the gel. Bands were then traced onto the top slide with a permanent ink felt-tip marking

pen. Finally, the slides were then traced onto a mimeographed form prepared for this purpose.

Reproducibility

A single sample of squirrel blood serum was divided into three subsamples, labeled and frozen. Shortly afterward, each subsample was electrophoresed across six micro gels within a single run. Thus, with three subsamples each being run on six micro gels, a total of 18 electropherograms were obtained of the same blood serum sample. The mean absolute mobilities of these electropherograms (by run) and their standard deviations were recorded.

Additional experiments were run to measure the reproducibility of the serum collection technique and the starch gel electrophoresis procedures used in this study. A sample of 4 squirrels was trapped (the small sample size reflects trapping difficulty). Three of the squirrels were bled four times, twice within a one minute period, and then twice again four days later. Each of the four blood samples per squirrel was divided into three separate samples of serum, labeled, and frozen. The other squirrel died following the first two bleedings, and thus provided only two samples (Table 1).

From these samples, 24 were randomly chosen to form four electrophoretic runs of six samples each (each sample run on a separate micro gel). The method of coding these samples is shown in Table 2. The samples not used in the reproducibility trial were employed in coelectrophoresis. Coelectropherograms showed so little resolution that they were not included as data in the present study.

In the reproducibility trial, the 24 samples were electrophoresed

Table 1. Bleeding dates of animals used in reproducibility trial.

Animal	Sex	Woodlot*	Capture date	Bleeding dates 1	2	3	4
A	M	PH	8/24/74	9/1	9/1	9/5	9/5
B	F	CW	8/29/74	9/1	9/1	**	**
D	M	TPW	8/31/74	9/1	9/1	9/5	9/5
E	F	CW	9/01/74	9/1	9/1	9/5	9/5

* PH = President's Hill, CW = Center Woods, TPW = Turkey Pen Woods.

** Animal C died 9/02/74.

Table 2. Method of coding squirrel samples used in reproducibility trial.

Squirrel Code	Bleeding number 1, 2, 3, or 4	Sample Code
A	1	A11
		A12*
		A13
	2	A21
		A22*
		A23*
	3	A31
		A32*
		A33
	4	A41
		A42
		A43*
D	1	D11
		D12*
		D13*
	2	D21*
		D22
		D23
	3	D31
		D32*
		D33
	4	D41
		D42*
		D43
C	1	C11*
		C12*
		C13*
	2	C21
		C22*
		C23
E	1	E11*
		E12
		E13*
	2	E21*
		E22*
		E23
	3	E31*
		E32
		E33*
	4	E41
		E42*
		E43*

*Sample used in reproducibility trial.

in the sequence shown in Table 3. For each of the resulting electropherograms, relative mobilities were calculated using three standards: (1) the distance from the point of insertion to the cathodal edge of the albumen band; (2) the same measurement to the anodal edge of the albumen band; and (3) the mean of these two measurements. For the purpose of comparing the three types of relative mobility with the absolute mobility, coefficients of variation (standard deviation/mean X 100 percent) were computed for each band position within each of the four classes of mobilities.

Another approach to analyzing the same data was made adapting the method of Smith et al. (1973). The mean absolute mobilities of the four animals were depicted schematically (Fig. 2). Series of bands on the four schematics were then assigned to band sets on the arbitrary basis of the qualitative similarity of their patterns. Albumen, the most anodal band, was not assigned to any group, but was depicted as the wide shaded band in Fig. 2. The band sets of Fig. 2 which were identified by the same code (i.e., 1, 2, or 3) were thought to be similar protein groups. Band sets 4c and 5c were unique to animal C. Groupings were arbitrarily made, and were designed to facilitate comparisons among samples.

Relative mobilities were calculated for each protein within each band set. Within each band set, the most anodal band of the band set was used as the standard, and mobilities were calculated relative to this standard. Thus, for each band set, the most anodal band possessed a relative mobility of 1.0, with the other bands displaying proportionately lower mobilities.

Table 3. Arrangement of samples as they were electrophoresed (6 samples per run) during reproducibility trial.

Run number	Samples					
1	A23	A43	E13	E21	E33	E43
2	C11	D23	C12	D43	E11	E31
3	A12	A22	A32	D12	D32	D42
4	C13	C22	D13	D21	E22	E42

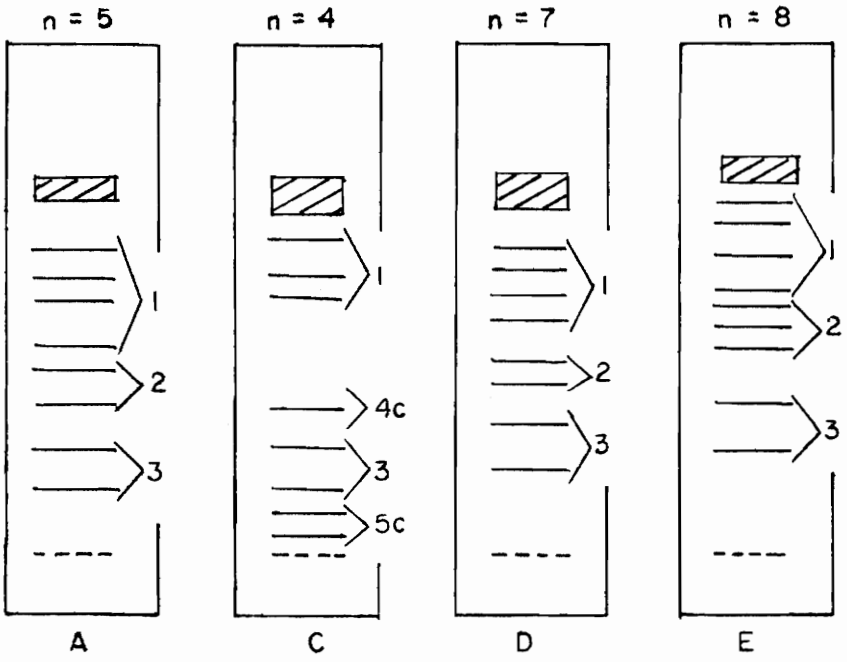

Fig. 2. Schematic diagram of mean absolute mobilities for the blood serum electropherograms of squirrels A, C, D, and E from the reproducibility trials. Figure shows one method of grouping bands.

Relative mobilities for the protein bands in electropherograms of the 46 squirrels investigated were also computed using the method adapted from Smith *et al*. (1973). Identifications of proteins were made on the basis of these relative mobilities, and chi-square contingency tables were then used to test the independence of protein frequencies *vs*. the woodlots in which the frequencies were observed.

RESULTS

Selection of Procedures

After a number of trials, a gel concentration of 12.5 percent appeared to be the most suitable of those tested. At this concentration, electromobility was adequate and the gel was sufficiently strong to withstand the rigors of slicing, handling, and staining. At 10 percent concentration, the gels tended to tear or break, particularly when slices were being separated. At 15 percent concentration, the gels handled well, but reduced the permitted migration length, and hence the band resolution.

The buffer system selected for the squirrel blood serum protein analysis of this study was the tris-glycine system (Marsh and Jolliff, 1968). This system yielded excellent results in terms of band resolution. It was found that in 2.5 hours, at 20ma (initial voltage of 250 V.), the tris-glycine system permitted migration to proceed almost to the anodal wick, thus yielding maximum resolution. The tris-hydrochloric acid system failed to provide sufficient resolution, as was also the case with the lithium hydroxide system.

Results of the staining trials are as follow. Ponceau S was found to be totally unsuitable for staining starch gel electropherograms. The background stain was pronounced, and the number of bands detected was less than the number detected by either amido black or coomassie brilliant blue. (The maximum number of bands detected in squirrel serum using Ponceau S was six; coomassie brilliant blue and amido black both detected a minimum of seven bands and a maximum of 11). Amido black and

coomassie brilliant blue gave good results, both in terms of the number of bands detected and the resolution of those bands. The use of amido black, however, required several destaining baths, while similar results were obtained with coomassie brilliant blue using fewer baths. Thus coomassie brilliant blue was chosen as the stain for use on the squirrel sera of this study.

All of the above procedures, including the method of starch gel preparation are detailed in Appendix I.

Reproducibility

Table 4 presents a comparison of within-run variation vs. among-run variation of the absolute mobilities of squirrel blood serum proteins from a single sample which was electrophoresed on 18 gels (six gels on each of three runs). From Table 4, it is clear that the absolute mobilities varied more across-run than within-run. Table 4 also shows that the coefficients of variation for the mobilities (within-run and across-run) tended to increase in the anodal to cathodal direction.

To determine the extent of variation due to sampling, four animals were multiple-sampled as in Table 2. (Relative mobilities for these animals were calculated using albumen as the standard; the coefficients of variation for these relative mobilities are presented in Appendix Table II.) Fig. 3 presents schematic diagrams of blood serum electropherograms typical of those encountered in this portion of the study.

Within the electropherograms of Fig. 3, there exist both differences in absolute mobilities and in the band patterns. These differences ocurred within-run and across-run. Specifically, sample E13 in run

Table 4. Comparison of within-run variation vs among-run variation of the absolute electromobilities of squirrel blood serum proteins from a single sample.

Band	Within-run 1*			Within-run 2*			Within-run 3*			Among-runs 1, 2, 3		
no.	Mean	St. dev.	C.V.	Mean	St. dev.	C.V.	Mean	St. dev.	C.V.	Mean	St. dev.	C.V.
1	441	0.0	0.00	411	0.1	0.02	390	0.0	0.00	414	21.53	5.23
2	400	0.6	0.15	373	1.8	0.48	361	0.0	0.00	378	16.78	4.44
3	374	1.2	0.32	348	1.5	0.43	325	0.2	0.06	349	20.60	5.90
4	344	1.9	0.55	310	1.1	0.35	296	0.9	0.30	316	20.73	6.56
5	311	1.8	0.58	276	2.0	0.72	258	0.3	0.12	281	22.64	8.06
6	284	2.1	0.74	256	2.3	0.90	225	1.2	0.53	255	24.79	9.72
7	257	0.3	0.12	229	2.1	0.92	198	1.0	0.51	228	24.78	10.87
8	156	0.9	0.58	140	1.8	1.29	152	0.8	0.53	149	6.99	4.69
9	100	1.1	1.10	82	1.6	1.95	105	0.9	0.86	95	10.16	10.69

*The sample was electrophoresed on 6 gels per run.

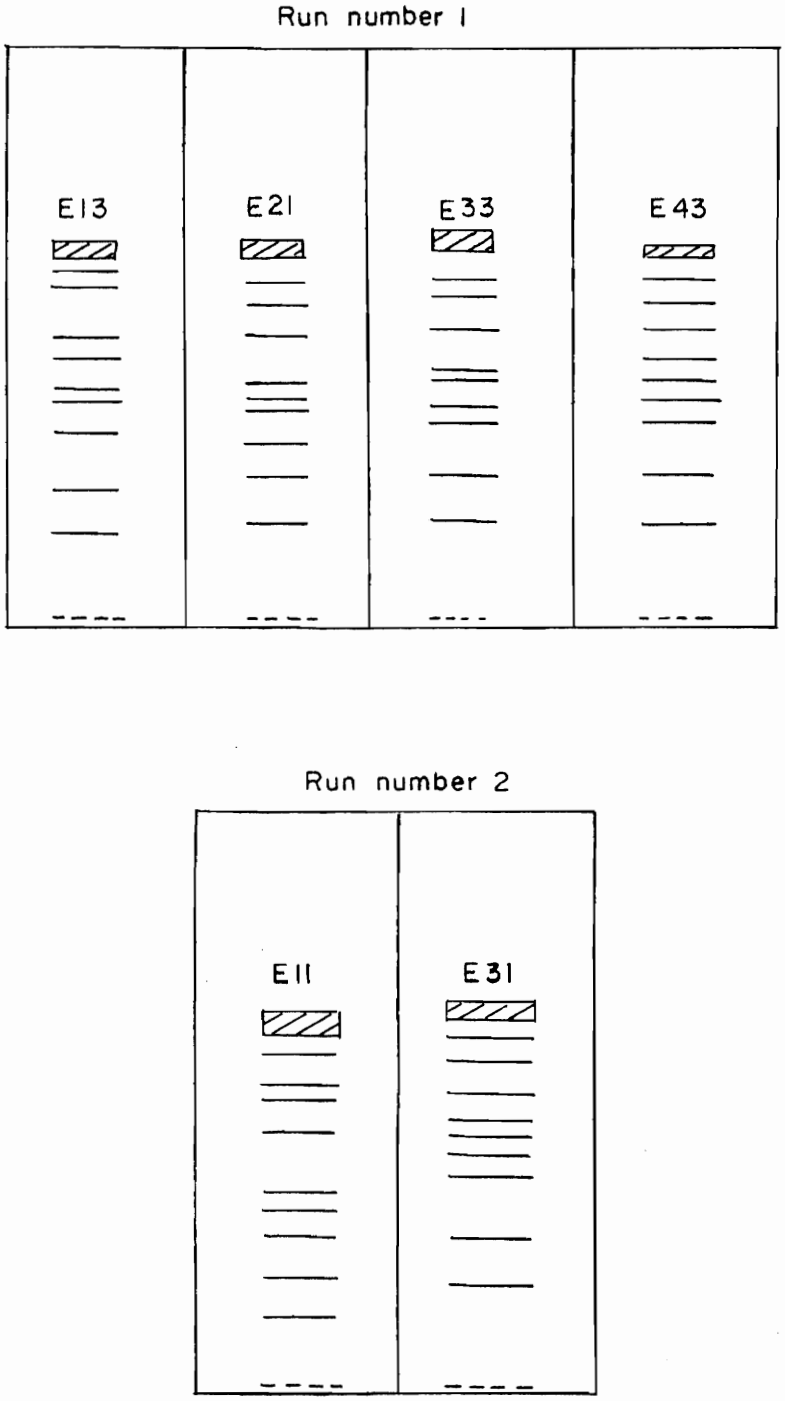

Fig. 3. Schematics of animal E electropherograms demonstrating qualitative differences in electrophoretic patterns within-run. Dashed line indicates origin; cross-hatched bands are albumen.

number one had two bands grouped closely to the albumen, with a distinct separation between this group and the next most anodal band. On the other hand, samples E21 and E43 of the same run had roughly equidistant spacing between the four most anodal bands. Additionally, sample E21 had the fifth, sixth, and seventh most anodal bands tightly grouped, which did not correspond with any of the other E samples of this run. Also, in run two, there were obvious differences between the patterns of samples E11 and E31. Note that all of these samples were from the same animal and still differences occurred within run. Since all of these samples were from the same animal, and since differences occurred within the same run, it follows that there were differences in the samples themselves.

Table 5 presents a comparison of two methods of calculating relative mobilities. The absolute mobilities from which these relative mobilities were calculated are shown in Table 4. The coefficients of variation for both methods of calculating relative mobility were less within-run than across-run, and tended to increase in the anodal to cathodal direction (Table 5). Neither method demonstrated consistently lower coefficients of variation than the other, although the method adapted from Smith et al. (1973) had lower coefficients of variation for the most cathodal band positions.

A comparison of Table 4 and Table 5 indicated that relative mobilities gave greater comparability across-run than did absolute mobilities. Conversely, the absolute mobilities had lower coefficients of variation within-run.

Table 5. Comparison of two methods of calculating mean relative mobilities for 18 electropherograms of gray squirrel blood serum. The sample was electrophoresed on 6 gels at a time over 3 runs. The method, "After Smith et al.", is described in the text; the method, "Albumen", computed mobilities relative to the cathodal edge of the albumen band. The heading "Mob." represents mobility; "C.V." represents coefficient of variation.

Band Position	METHOD															
	After Smith et al.								Albumen							
	Run 1		Run 2		Run 3		Runs 1,2,3		Run 1		Run 2		Run 3		Runs 1,2,3	
	Mob.	C.V.	Mob.	C.V.	Mob.	C.V.	Mob.	C.V.	Mob.	C.V.	Mob.	C.V.	Mob.	C.V.	Mob.	C.V.
1	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-	1.00	-
2	1.00	-	1.00	-	1.00	-	1.00	-	.91	1.0	.91	1.9	.93	0.0	0.92	3.8
3	.94	0.9	.93	2.2	.90	1.0	0.92	1.8	.85	1.4	.84	1.7	.83	0.6	0.84	1.2
4	.86	1.5	.83	1.9	.82	1.6	0.84	2.0	.78	2.1	.75	1.5	.76	1.2	0.76	1.8
5	.78	1.8	.74	3.9	.71	1.4	0.74	4.0	.71	2.2	.67	2.7	.66	1.1	0.68	3.4
6	1.00	-	1.00	-	1.00	-	1.00	-	.64	3.1	.62	3.4	.58	2.1	0.61	4.4
7	.90	0.7	.89	2.9	.88	1.8	0.89	1.1	.58	1.0	.56	3.6	.51	2.0	0.55	5.5
8	1.00	-	1.00	-	1.00	-	1.00	-	.35	2.9	.34	5.0	.39	2.3	0.36	6.4
9	.64	1.7	.59	4.1	.69	1.7	0.64	6.6	.23	5.8	.20	7.5	.27	3.3	0.23	12.9

Study Area Data

To compare the electropherograms of the 46 animals from the study area, relative mobilities were calculated using a method adapted from Smith *et al*. (1973). These relative mobilities are given in Appendix Table XI. Since it was observed (Table 4 and Table 5) that variability was least for the more anodal bands, the most anodal band set was used to make comparisons among woodlots.

The comparisons were made by assigning identities to the bands on the basis of their relative mobilities, and then noting the frequency within woodlot of each of the bands. Independence of woodlot and band frequencies were tested by chi-square contingency tables as shown in Tables 6, 7, and 8. In making these tests, it was assumed that variation within-run and across-run and variation due to sampling was distributed randomly over each of the woodlot population samples. It should be noted that data from four electropherograms were not included in these tests due to the low frequency of their patterns; these were electropherograms from specimens 10, 12, 24, and A4 (Appendix Table VI).

The data were examined to determine: (1) the independence of woodlot and individual bands (Table 6); (2) the independence of woodlot and the seven observed groups of bands (Table 7); and (3) the independence of woodlot and groups of bands composed only of the third, fourth and fifth most anodal bands (Table 8).

In each case, the null hypothesis was: there is no difference between the observed frequencies in the various woodlots and those which would be expected due to random distribution. In other words, it was

Table 6. Chi-square analysis of frequency of individual proteins of most anodal group on electropherograms vs. woodlots of the study.

Unadjusted data (expected/observed)

Protein	Woodlot* PH	TPW	CW	PFW	Other
A	7.62/6	6.00/8	1.62/1	.49/3	0.00/0
B	13.97/17	11.00/9	2.97/3	4.00/2	1.49/2
C	13.97/17	11.00/9	2.97/3	4.00/2	1.49/2
D	11.43/9	9.00/11	2.43/2	2.92/4	1.22/1

Adjusted data (expected/observed)

Cell	Frequencies
1	7.62/6
2	6.00/8
3	6.31/9
4	13.97/17
5	11.00/9
6	6.97/5
7	13.97/15
8	11.0 /9
9	5.43/6
10	11.43/9
11	9.00/11
12	6.92/7

Calculated X^2 = 4.75 (not significant)

Tabular $X^2_{.05}$ = 19.68 (11d. f.)

*PH = President's Hill; TPW = Turkey Penn Woods; CW = Center Woods; PFW = Price's Fork Woods; Other = Off campus.

Table 7. Chi-square analysis of frequency of different groupings of proteins vs. woodlots of the study.

Unadjusted data (expected/observed)

Protein group*	Woodlot				
	PH	TPW	CW	PFW	Other
1	0.90/1	0.62/1	0.19/0	0.19/0	0.10/0
2	2.71/2	1.86/3	0.57/0	0.57/0	0.29/0
3	3.17/2	2.17/3	0.67/0	0.67/0	0.33/0
4	6.33/6	4.33/4	1.33/2	1.33/2	0.67/1
5	0.90/2	0.62/0	0.19/0	0.19/0	0.10/0
6	0.90/0	0.62/1	0.19/1	0.19/1	0.10/0
7	4.07/6	2.79/1	0.86/1	0.86/1	0.43/1
	19	13	4	4	2

Adjusted data (expected/observed)

Cell	Frequencies
1	6.02/6
2	6.48/8
3	5.57/5
4	6.40/4
5	9.50/8
6	5.87/8

Calculated X^2 = 2.325 (not significant)

Tabular $X^2_{.05}$ = 11.07 (5 d.f.)

*Given that the third through sixth proteins are designated A, B, C, and D, respectively, the groups are characterized by having the following proteins: 1 = ABC, 2 = ABD, 3 = ACD, 4 = BCD, 5 = AB, 6 = AC, 7 = BC.

Table 8. Chi-square analysis of different groupings of proteins A, B, and C vs. woodlots of the study.

Unadjusted data (expected/observed)

Group Type*	Woodlot PH	TPW	CW	PFW	Other
1	0.90/1	0.62/1	0.19/0	0.19/0	0.10/0
2	3.62/4	2.48/3	0.76/0	0.76/1	0.38/0
3	4.07/2	2.79/4	0.86/1	0.86/2	0.43/0
4	10.04/12	7.12/5	2.19/3	2.19/1	1.10/2
	19	13	4	4	2

Adjusted data (expected/observed)

Cell	Frequencies
1	6.01/6
2	7.10/8
3	9.91/9
4	8.59/7
5	10.04/12

Calculated X^2 = 0.875 (not significant)

Tabular $X^2_{.05}$ = 9.49 (4 d.f.)

*Given that the third through fifth proteins are designated A, B, and C respectively, the groups are characterized by having the following proteins: 1 = ABC, 2 = AB, 3 = AC, and 4 = BC.

hypothesized that frequencies of bands, either singly or grouped, were totally independent of the woodlots on which they occurred.

In all three of the above cases, tests failed to reject the null hypothesis. Thus, based on these data, there is no evidence to suggest that band frequencies differed among woodlots.

DISCUSSION

The results presented in Table 4 indicate the absolute mobilities for a single sample are highly reproducible within-run, but less reproducible across-run. The across-run reproducibility of a single sample was improved by using relative mobilities (Table 5). Both types of relative mobilities (those based on albumen and those adapted from Smith et al., 1973) possessed lower coefficients of variation across-run than did the absolute mobilities.

Fig. 3 indicates that there was variation due to sampling technique. Multiple samples from the same animal displayed varying electropherograms both within- and among-run. These differences may have arisen from several sources, including the sampling method (cardiac puncture), handling of the samples, or physiological changes in the animal between sampling times. Cardiac puncture may have contributed to variation by introducing body fluids other than blood to the sample. If this is the case, it is a problem which must be accepted, since it is difficult to think of a sampling method that will permit procurement of practical amounts of blood with less chance of including contaminants. The most widely used technique, decapitation, will obviously expose the sample to both respiratory and digestive fluids. In any case, it is desirable to use a non-destructive sampling technique.

Although the samples were handled as identically as possible slight differences in handling may have been present. If, for example, the serum samples were allowed to stand varying lengths of time during clotting, one would expect varying amounts of hemolysis, which might

result in some degree of protein denaturation (Chapman and Morgan, 1973). While every attempt was made to standardize the handling procedure, experimental error during handling cannot be ruled out.

Physical changes in the animal between sampling periods are also a possible source of variation, but the author has little knowledge of the extent to which such changes would alter squirrel serum electropherograms. It is known that such alterations do occur in humans and deer (Kelsey, 1965; Van Tets and Cowan, 1966).

Thus, three sources of variation in the electropherograms of this study were observed: minor variation within-run, variation among-run, and variation among multiple samples from a single individuals. It was assumed that these sources of variation operated randomly and thus affected each population similarly.

Since it had been observed that the more anodal bands exhibited the least variation, considerable reliability was attributed to the identification of these bands. Consequently, as presented in Tables 6, 7, and 8, the frequencies of these bands were used to compare the woodlot squirrel populations. These frequencies were treated both as individual bands and as groupings of bands. The reason for examining the two types of grouping as well as individual bands was to insure that all possibilities were considered. That is, while the frequency of individual bands might be independent of woodlot, specific groupings of these proteins might, in fact, depend on the woodlot in which they occur. As it turned out, no dependence could be demonstrated on the basis of the data. It is possible that an examination of other biochemical systems,

or of the more cathodal bands of blood serum (once they have been properly identified), might reveal differences. Given the failure of the present data to come even close to rejecting the null hypothesis, it is unlikely larger sample sizes would change the results.

Finally, it should be noted that using the method adapted from Smith et al. (1973) to compute relative mobilities for the study area data was an arbitrary choice. It was employed primarily because it facilitated interpretation of the study area data.

CONCLUSIONS AND RECOMMENDATIONS

The use of horizontal starch gel electrophoresis to separate gray squirrel blood serum was investigated. Three sources of variation in the technique were identified: (1) minor variation within-run; (2) variation across-run; (3) variation due to the sampling technique. On the assumption that these sources of variation act randomly, the frequencies of the most anodal bands were compared among several woodlots using chi-square contingency tables. Based on these frequencies, no differences were found among woodlot squirrel populations of this study. It was pointed out, however, that such differences might be present in other biochemical systems.

With regard to technique, it was concluded that:

1) coomassie brilliant blue is a suitable general protein stain;

2) variability in band mobilities tends to increase moving from the anodal to the cathodal bands; consequently, it is recommended that the greatest confidence be placed in the more anodal bands of squirrel starch gel serum protein electropherograms.

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Appendix I. Some Starch Gel Electrophoretic Techniques

Starch Gel Preparation

Materials Required: Hydrolyzed starch (available from Connaught Medical Laboratories, Toronto, Canada), flat bottomed vacuum flask with stopper, gel buffer (described below), Bunsen burner, and gel tray.

Procedure: This procedure will prepare enough gel for six micro gels (75mm x 25mm x 5mm). Measure out 100 ml of gel buffer; measure 12.5 g of starch powder. Pour approximately 75 ml of gel buffer into the flask, which should be connected to a vacuum source, but without actual evacuation at this point. Next, while gently swirling the flask, slowly add the starch powder, observing closely to ensure that all the starch dissolves. Use the remaining 25 ml of gel buffer to wash down starch which may be clinging to the sides of the flask; continue swirling the flask.

While still swirling the flask, heat it evenly over an open flame. After several minutes, the opaque white solution will become translucent and decidedly viscous. Continue swirling over the flame until the viscosity diminishes to the same level as before heating. Once this has occurred, apply the vacuum immediately and observe the starch gel closely; boiling will commence, and after a few seconds the gel will become crystal clear with large bubbles; disconnect the vacuum immediately and pour the gel into the gel tray.

Comments: Feret (personal communication) suggested that placing the gel-laden gel trays in a closed chamber containing moist filter pads

would prevent the gel surface from rippling. The present study confirms this.

Practice in the preparation of these gels will accomplish two goals. First, it will increase the efficiency of preparation; second, it will form habits for the researcher which will contribute to the reproducibility of the experiments.

Buffer Preparation

Tris-glycine (Marsh and Jolliff, 1968): Electrode buffer (pH 8.3) -- glycine 22.6 g; tris 4.7 g; dilute to 1 liter with distilled water. Gel buffer (pH 8.7): tris 36.25 g; edta 1.0 g; HCL (1.0N) 480 ml; distilled water to 1 liter. Dilute with 4 parts distilled water to 1 part buffer solution.

Tris-hydrochloric acid (Selander et al., 1971): gel buffer (pH 8.5) -- 1.21 g tris, diluted to 1 liter. pH adjusted with concentrated HCL. Electrode buffer (pH 8.2): 18.55 g boric acid; 2.40 g sodium hydroxide, diluted to 1 liter.

Lithium-hydroxide (Selander et al., 1971): 1 to 9 mixture of stock solutions A and B, as described below.
Electrode buffer: stock solution A, as described below.

Stock solution A (pH 8.1): 1.20 g monohydrate lithium hydroxide; 11.89 g boric acid; dilute to 1 liter.

Stock solution B (pH 8.4): 6.2 g tris; 1.6 g monohydrate citric acid; dilute to 1 liter.

Comments: The electrode buffer must be changed after each run. Examination of the anode will demonstrate the reason for this; during electrophoresis, decomposition occurs in the buffer, and salts are deposited on the anode. This buffer breakdown, of course, will cause variability from run to run, and thus should be avoided.

Staining Procedures

Ponceau S: Ponceau S is not recommended for use in starch gels. Amido black (Selander, 1971): A fixing solution is prepared from acetic acid: distilled water:methanol in a 1:5:5 ratio. The staining solution is prepared as a 1 percent (W/V) solution of amido black in the fixing solution. The sliced gel to be stained is placed in 50 ml (in the case of micro gels; in a tenfold volume otherwise) of the staining solution for 30-60 minutes. The gels are then destained in 50 ml of fixing solution with agitation; the present study found that a minimum of three changes of fixing solution was necessary for adequate destaining.

Coomassie Brilliant Blue: Prepare large volumes of 10 percent and 12.5 percent Trichloroacetic acid (TCA). Prepare a 1 percent (W/V) aqueous solution of coomassie brilliant blue. After slicing, the gel is placed in 50 ml of 12.5 percent TCA (50 ml for micro gels; a tenfold volume otherwise) for 30-60 minutes. Following this, 2.5 ml of the 1 percent coomassie brilliant blue solution is added directly to the 12.5 percent TCA in which the gel is immersed; the stain is thoroughly mixed in with the TCA solution. After 60 minutes,the gel is transferred to 50 ml of 10 percent TCA where it may be observed for bands immediately. The

bands become slightly more intense if the gels are left in 10 percent TCA for up to 24 hours.

Appendix Table I. Animals caught and sampled, with date of sampling, area, and sex.

Sample No.	Date Blood Drawn	Area[1]	Sex	Comments
1	Apr. 1	ARSENAL	M	Died from shock; high red blood cell vol.
2	2	PH	M	
3	2	PH	M	
4	3	PH	M	
5	4	PH	M	
6	4	PH	M	
7	4	PH	M	
8	5	PH	M	
9	5	PH	F	Serum sample cloudy, not suitable for electrophoresis
10	5	PH	M	
11	9	PFW	M	
12	9	PH	M	
13	9	PH	M	
14	9	PFW	M	
15	16	PH	M	
16	18	PFW	F	
17	19	PH	F	
18	19	PH	F	
19	19	PH	F	
20	19	PH	F	
21	19	BLACKSBURG	F	
22	19	PH	F	
23	19	PH	F	
24	19	PH	F	
25	19	PH	F	
26	19	PH	F	
27	19	PH	F	
28	19	PH	F	
29	24	PFW	F	
30	27	TPW	M	
31	27	TPW	F	
32	27	TPW	M	
33	30	TPW	F	
34	May 1	TPW	F	
35	1	TPW	F	
36	2	TPW	M	
37	2	TPW	M	

Appendix Table I. Animals caught and sampled, with date of sampling, area, and sex. (continued).

Sample No.	Date Blood Drawn	Area[1]	Sex	Comments
A1	May 2	TPW	M	
A2	28	TPW	F	
A3	30	TPW	F	(Pregnant)
A4	30	TPW	F	(Lactating)
A5	30	TPW	M	
A6	30	TPW	F	(Lactating)
A7	Jun 6	CW	M	Serum not suitable for electrophoresis
A8	7	CW	M	
A9	9	CW	F	
A10	10	CW	M	
A11	10	CW	F	Serum not suitable for electrophoresis
A12	11	CW	M	

1/ PH = President's Hill, TPW = Turkey Pen Woods, PFW = Prices Fork Woods, CW = Center Woods, Arsenal or Blacksburg = caught off campus.

Appendix Table II. Coefficients of variation (percent) for absolute mobilities and 3 different computations of relative mobilities of bands from gray squirrel blood serum electropherograms. Abs. = absolute mobility, AC = mobility relative to cathodal albumen edge, AA = mobility relative to anodal albumen edge, and AM = mobility relative to middle of albumen band.

Band No.	Squirrel A(n=5)				Squirrel C (n=4)				Squirrel D (n=7)				Squirrel E (n=8)			
	Abs. (%)	AC (%)	AA (%)	AM (%)	Abs. (%)	AC (%)	AA (%)	AM (%)	Abs. (%)	AC (%)	AA (%)	AM (%)	Abs. (%)	AC (%)	AA (%)	AM (%)
1	5.89	0.00	0.00	0.00	6.97	0.00	0.00	0.00	4.50	0.00	0.00	0.00	2.67	0.00	0.00	0.00
2	8.97	4.22	2.57	3.25	7.65	0.79	3.59	2.32	3.94	3.63	4.86	4.09	3.26	1.97	2.16	1.81
3	9.72	4.16	2.77	3.31	10.18	4.10	5.99	4.94	4.19	4.32	5.67	4.91	4.90	3.17	3.92	3.49
4	18.51	3.91	2.63	3.16	11.40	5.25	7.46	6.39	4.33	14.50	15.64	15.06	3.76	1.83	2.80	2.10
5	13.75	6.59	4.90	5.63	15.28	14.89	15.48	15.18	5.65	9.02	10.75	9.90	6.17	4.32	4.82	4.47
6	14.45	6.20	4.59	5.26	13.50	7.81	9.55	8.59	5.27	9.67	11.34	10.47	5.96	6.77	8.55	7.70
7	25.15	12.51	11.58	12.01	14.60	9.24	10.83	9.97	12.23	20.33	21.87	21.15	6.07	8.07	9.47	8.96
8	29.79	6.40	7.99	7.16	26.42	24.62	25.11	25.06	10.39	25.61	26.61	26.13	9.47	10.54	12.16	11.44
9	28.57	11.29	13.10	12.21	33.70	27.63	29.02	28.18	15.46	25.56	26.34	25.92	8.43	10.76	12.25	11.56
10													4.09	24.89	25.52	25.37

Appendix Table III. Absolute and relative mobilities of reproducibility trial electropherograms arranged by animal. Relative mobilities calculated using cathodal albumen edge as standard.

Absolute

Animal	A					C			
Run No.	1		3			2		4	
Sample No.	A23	A43	A12	A22	A32	C11	C12	C13	C22
	490	490	475	453	444	418	440	489	471
	400	433	430	404	402	388	410	462	439
	370	407	392	374	372	337	351	408	411
	344	378	360	343	344	296	328	374	378
	294	325	340	307	309	159	232	196	206
	266	302	300	280	282	128	134	160	169
	216	277	228	208	257	80	84	100	109
	182	200	172	159	156	39	55	44	70
	129	138	110	99	99	17	20	30	35

Animal	D							E							
Run No.	2		3			4		1				2		4	
Sample No.	D23	D43	D12	D32	D42	D13	D21	E13	E21	E33	E43	E11	E31	E22	E42
	440	472	462	462	479	496	527	491	500	506	496	460	490	517	526
	372	388	401	400	402	442	481	480	462	466	463	439	461	493	500
	345	361	380	378	386	421	457	460	428	440	332	399	432	458	484
	246	278	368	352	360	398	428	396	394	397	400	376	389	404	431
	222	255	288	285	293	296	349	360	323	350	353	335	352	361	391
	196	227	256	257	260	269	315	232	308	330	332	256	332	331	368
	132	158	209	216	229	232	293	305	292	296	298	228	310	295	343
	82	99	158	153	163	179	208	270	250	274	270	196	274	270	328
	49	63	105	101	100	111	120	192	206	204	202	140	190	218	238

Appendix Table III. Absolute and relative mobilities of reproducibility trial electropherograms arranged by animal. Relative mobilities calculated using cathodal albumen edge as standard (cont)

Animal	A					C			
Run No.	1		3			2		4	
Sample No.	A23	243	A12	A22	A32	C11	C12	C13	C22
	816	884	9-5	892	905	928	932	945	932
	755	831	825	826	838	806	798	834	873
	702	771	758	757	775	708	745	765	803
	600	663	716	678	696	380	527	401	437
	543	616	632	618	635	306	305	327	359
	441	565	480	459	579	191	191	204	231
	371	408	362	351	351	93	125	90	149
	263	282	232	219	223	41	45	61	74

Animal	D							D							
Run No.	2		3			4		1				2		4	
Sample No.	D23	D43	D12	D32	D42	D13	D21	E13	E21	E33	E43	E11	E31	E22	E42
	845	822	868	866	839	891	913	978	924	921	933	954	941	954	951
	784	765	823	818	806	849	867	937	856	870	871	867	882	886	920
	559	589	797	762	752	802	812	807	788	785	806	817	794	781	819
	505	540	623	617	612	597	662	733	646	692	712	728	718	698	743
	445	481	554	556	543	542	598	658	616	652	669	557	678	640	699
	308	335	452	468	478	468	556	621	584	585	601	496	633	571	652
	186	210	342	331	340	361	395	550	500	542	544	426	559	522	625
	111	133	227	219	209	224	228	391	412	403	407	304	388	422	452

Appendix Table IV. Absolute and relative mobilities of reproducibility trial electropherograms arranged by bleeding times. Relative mobilities calculated using cathodal albumen edge as standard.

Absolute

Animal	A		C			D		E							
Bleeding No.	2		1			4		1		2		3		4	
Sample No.	A22	A23	C11	C12	C13	D42	D43	E11	E13	E21	E22	E31	E33	E42	E43
	453	490	418	440	489	419	472	460	491	500	517	490	506	526	496
	404	400	388	410	462	402	388	439	480	462	493	461	466	500	463
	874	370	337	351	408	386	361	399	460	428	458	432	440	484	432
	343	344	296	328	374	360	278	376	396	394	404	389	397	431	400
	307	294	159	232	196	293	255	335	360	323	361	352	350	391	353
	280	266	128	134	160	260	227	256	323	308	331	332	330	368	332
	208	216	80	84	100	229	158	228	305	292	295	310	296	343	298
	159	182	39	55	44	163	99	196	270	250	270	274	274	328	270
	99	129	17	20	30	100	63	140	192	206	218	190	204	238	202
								45	131	144	141	134	140	144	134

Relative

Animal	A		C			D		E							
Bleeding No.	2		1			4		1		2		3		4	
Sample No.	A22	A23	C11	C12	C13	D42	D43	E11	E13	E21	E22	E31	E33	E42	E43
	892	816	928	932	945	839	822	954	978	921	954	941	921	951	933
	826	755	806	798	834	806	765	867	937	856	886	882	870	920	871
	757	702	708	745	765	756	589	817	807	788	781	794	785	819	806
	678	600	380	527	401	612	540	728	733	646	698	718	692	743	712
	618	543	306	305	327	543	481	557	658	616	640	678	652	699	669
	459	441	191	191	204	478	335	496	621	584	571	633	585	652	601
	351	371	93	125	90	340	210	426	550	500	522	559	542	624	544
	219	263	41	45	61	209	133	304	391	412	422	388	403	452	407
								98	267	288	273	273	277	274	270

Appendix Table V. Absolute band mobilities of blood serum electropherograms of 46 gray squirrels from woodlots on VPI & SU campus area. Columns read from top to bottom, with top being most anodal. (mobilities given in units of mm X 10).

Specimens

1	2	3	4	5	6	7	8	10	11	12	13
382	366	376	334	360	352	305	269	250	252	260	390
359	336	350	303	319	299	291	257	200	233	210	379
320	325	318	276	286	280	271	230	171	215	160	337
298	280	290	257	262	259	247	213	141	191	138	329
255	249	218	229	232	189	228	185	112	160	106	280
220	204	195	180	189	170	174	163	73	145	90	219
152	169	121	164	170	150	154	141	52	117	73	170
132	109	100	135	108	135	117	110	0	95	0	128
118	72	0	95	77	102	90	89	0	74	0	83
83	0	0	65	0	65	71	67	0	0	0	0

14	15	16	17	18	19	20	21	22	23	24	25
403	391	391	404	409	209	204	203	183	188	207	372
380	377	371	369	392	198	191	186	179	165	179	354
350	336	334	348	358	185	180	160	156	146	159	315
330	314	318	323	330	175	170	145	138	122	132	294
291	290	275	288	298	153	153	126	123	106	107	262
239	225	220	235	220	109	125	100	101	87	87	211
218	200	162	127	172	80	109	71	83	74	64	110
134	123	120	89	120	61	70	57	56	54	0	86
98	89	78	0	79	0	60	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0

26	27	28	29	30	34	35	36	31	32	33	37
384	401	380	395	395	378	385	360	385	369	382	315
369	381	364	381	381	361	343	340	368	341	365	301
330	340	321	345	341	340	322	301	334	325	341	272
310	310	302	318	315	319	291	285	316	298	324	257
272	280	274	279	274	280	224	257	288	260	285	234
232	236	204	228	211	221	182	205	239	215	220	187
111	129	175	175	110	179	118	182	216	183	188	149
80	105	115	119	81	119	80	115	177	118	129	94
0	0	95	96	0	80	0	85	120	75	88	68
0	0	0	0	0	0	0	0	79	0	0	0

A1	A2	A3	A4	A5	A6	A8	A9	A10	A12
318	328	321	362	394	356	349	305	307	369
306	303	276	344	376	339	329	273	275	349
273	278	263	310	348	323	290	249	262	315
257	258	238	295	323	301	271	223	224	297
238	238	183	271	291	285	249	174	168	262
185	196	171	218	226	264	183	138	129	200
174	175	149	198	180	212	141	94	83	163
161	100	98	163	119	191	94	65	55	112
98	71	72	135	85	108	70			78
73	0	0	114	0	78				

Appendix Table VI. Relative mobilities (calculated after Smith et al., 1973) of bands from blood serum electropherograms of 46 gray squirrels. Figures read from top to bottom, with anodal bands at top.

Specimen															
1	2	3	4	5	6	7	8	10	11	12	13	14	15	16	17
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.80	1.00	0.81	1.00	1.00	1.00	1.00	1.00
0.89	0.94	0.91	0.90	0.94	0.93	0.89	0.89	—	0.92	--	0.92	0.92	0.90	0.90	0.94
0.83	0.83	0.83	0.85	0.82	0.87	0.88	0.83	1.00	0.82	1.00	0.87	0.89	0.83	0.86	0.88
0.71	0.74	1.00	0.76	0.73	0.63	0.78	0.72	0.82	0.70	0.86	0.74	0.77	0.77	0.74	1.00
1.00	1.00	0.89	1.00	1.00	1.00	1.00	1.00	0.65	1.00	0.66	1.00	1.00	1.00	1.00	0.82
0.69	0.83	0.56	0.91	0.90	0.88	0.87	0.87	1.00	0.78	1.00	0.78	0.91	0.89	0.74	0.44
0.60	1.00	0.46	0.75	1.00	0.79	0.67	0.67	0.71	1.00	0.81	1.00	1.00	1.00	1.00	0.31
1.00	0.66	--	1.00	0.71	1.00	1.00	1.00	--	0.78	--	0.65	0.73	0.72	0.65	--
0.70	--	--	0.68	--	0.64	0.79	0.75	--	--	--	--	--	--	--	--
18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.00	1.00	1.00	1.00	1.00	1.00	0.87	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.91	0.93	0.94	0.86	0.86	0.88	--	0.89	0.89	0.89	0.88	0.91	0.91	0.91	0.95	0.93
0.84	0.88	0.88	0.68	0.77	0.74	1.00	0.83	0.84	0.81	0.83	0.83	0.83	0.86	0.87	0.89
0.74	1.00	0.80	1.00	1.00	1.00	0.83	1.00	1.00	1.00	0.75	0.73	1.00	0.78	0.76	0.78
1.00	0.71	1.00	0.79	0.82	0.82	0.67	0.81	0.85	0.84	1.00	1.00	0.77	1.00	1.00	1.00
0.78	0.52	0.87	0.56	0.67	0.70	1.00	0.42	0.41	0.46	0.86	0.86	0.40	0.90	0.85	0.85
1.00	0.40	1.00	0.45	0.46	0.51	0.74	0.33	0.29	0.38	1.00	1.00	0.30	0.74	1.00	1.00
0.66	--	0.83	--	—	--	--	--	--	--	0.83	0.81	--	1.00	0.64	0.68

Appendix Table VI. Relative mobilities (calculated after Smith et al., 1973) of bands from blood serum electropherograms of 46 gray squirrels. Figures read from top to bottom, with anodal bands at top. (Continued).

Specimen													
34	35	36	37	A1	A2	A3	A4	A5	A6	A8	A9	A10	A12
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.94	0.94	0.87	0.90	0.89	0.92	0.95	0.90	0.93	0.95	0.88	0.91	0.95	0.90
0.88	0.85	0.84	0.85	0.84	0.85	0.85	0.86	0.86	0.88	0.82	0.82	0.81	0.85
0.78	1.00	0.76	0.78	0.78	0.79	0.67	0.79	0.77	0.84	0.76	1.00	1.00	0.75
1.00	0.81	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.79	0.77	1.00
0.81	0.52	0.89	0.80	0.94	0.90	0.91	0.91	0.91	0.80	0.77	0.54	0.49	0.82
1.00	0.36	1.00	1.00	0.87	1.00	1.00	1.00	1.00	0.72	1.00	0.37	0.33	1.00
0.67	--	0.74	0.72	1.00	0.71	0.72	0.83	0.71	1.00	0.74	--	--	0.70
--	--	--	--	0.74	--	--	0.70	--	0.72	--	--	--	--
--	--	--	--	--	--	--	0.52	--	--	--	--	--	--

VITA

Ralph A. Otto was born January 26, 1946 in Plainfield, New Jersey. He attended public schools in Westfield, New Jersey, and graduated from Westfield High School in 1964. In September of 1964, he entered Rutgers University from which he graduated in 1968 with a B.S. in wildlife biology.

In July 1968, Mr. Otto began Peace Corps Training in Hilo, Hawaii. He was sworn in as a Peace Corps Volunteer in October 1968, and served three years in Malaysia. During this time he worked with rodent control, leatherback turtle conservation, and a fishing survey.

Mr. Otto directed two Peace Corps Training projects. In 1971 he directed a project in Malaysia to prepare volunteers to work with the Malaysian Agricultural Research Development Institute (MARDI); in 1973, Mr. Otto directed a project in Nepal dealing with pond fisheries.

Mr. Otto first enrolled at Virginia Polytechnic Institute and State University in March, 1972, and will complete his M.S. requirements in June, 1975.

Ralph A. Otto

Ralph A. Otto

HORIZONTAL STARCH GEL ELECTROPHORESIS AS A TECHNIQUE FOR EXAMINING GRAY SQUIRREL POPULATION GENETICS

by

Ralph A. Otto

(ABSTRACT)

The use of horizontal starch gel electrophoresis to separate gray squirrel blood serum was investigated. Three sources of variation in the techniques were identified: (1) minor variation within-run; (2) variation across-run; (3) variation due to the sampling technique.

The frequencies of the most anodal bands of 42 gray squirrel blood serum protein electropherograms were compared among several woodlots using chi-square contingency tables. On the basis of these frequencies, no differences were found among the woodlot populations of gray squirrels examined in this study.