COMPARATIVE STUDIES ON THE ELECTROPHORETIC MOBILITY OF NON-SPECIFIC ESTERASES AMONG SELECTED SPECIES OF TICKS (IXODIDAE)

by

James Crish

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

Paul C Peterson 5/31/79

Advisor Date

Dean Rand 6/13/79

Dean of the Graduate School Date

YOUNGSTOWN STATE UNIVERSITY

June, 1979

COMPARATIVE STUDIES ON THE ELECTROPHORETIC MOBILITY OF NON-SPECIFIC ESTERASES AMONG SELECTED SPECIES OF TICKS (IXODIDAE)

by

James Crish

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

Advisory Committee

Paul C Peterson — May 31, 1979
Advisor and Committee Chairman — Date

Paul VanBrandt — May 31, 1979
Date

Marvin Sutkin — May 31, 1979
Date

John J. Iovenna — May 31, 1979
Date

Leon Lund — June 11, 1979
Dean of the Graduate School — Date

YOUNGSTOWN STATE UNIVERSITY

June, 1979

ABSTRACT

COMPARATIVE STUDIES ON THE ELECTROPHORETIC MOBILITY OF NON-SPECIFIC ESTERASES AMONG SELECTED SPECIES OF TICKS (IXODIDAE)

James Crish

Master of Science

Youngstown State University, 1979

The potentiality of the non-specific esterases as biochemical markers to compare the intra- and inter-specific variability of various species of ticks (Ixodidae) is explored. Ontogenic differences in esterase multiplicity, observations on esterase differentiation in various tissues of the adult ticks and the interaction of interspecific genomes in the hybrids are also presented.

The developed methodology employed a commercially prepared cellulose acetate medium and involved conventional histochemical staining procedures coupled to electrophoresis. The study demonstrates the heterogeneity of the esterases in the ticks at various stages and the applicability of the cellulose acetate medium in separating the esterases under the proper electrophoretic conditions.

ACKNOWLEDGMENTS

I wish to express my appreciation and gratitude to Dr. Paul C. Peterson for providing assistance, encouragement, facilities and constructive criticism needed to complete this study. Recognition is also extended to Dr. John J. Yemma for his valuable suggestions. Appreciation is also extended to Dr. Richard Osborn, U.S. Department of Agriculture, Kerrville, Texas, who kindly provided the adult ticks, their developmental stages, and hybrids for study.

Additionally, I would like to thank Mr. Frederick T. Posey, Tri-State Labs Inc., Youngstown, Ohio, for providing his expertise in demonstrating electrophoretic techniques used in the study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (cont.)

INTRODUCTION

The improvement of techniques for enzyme electrophoresis has led to the discovery of multiple molecular forms of enzymes. These multiple molecular forms of an enzyme within the same species have been designated as isoenzymes by the International Commission on Enzymes (Enzyme Nomenclature, 1973). The identification of intra- and inter-specific differences in the structure of homologous enzymes or proteins and of enzyme heterogeneity within tissues of the same species has shown that the different enzyme patterns reflect the changing patterns of gene activation and repression. Consequently, this molecular heterogeneity of enzymes has proved to be a convenient biochemical marker of gene activity and has served as an additional tool besides morphological characteristics for comparative studies in analyzing population differentiation, distinguishing species and the classifying of species.

Esterases are a heterogeneous group of enzymes that have been the subject of extensive investigation and have been used as biochemical markers in a number of organisms such as mice (Markert, et al., 1959) and Drosophila (Ursprung et al., 1968). Esterases have also been useful gene markers for studying mechanisms of insect development (Laufer, 1961). In addition, the esterase polymorphism and inheritance of the allelic isozymes have been studied for several different species of mosquitoes (Simon, 1969). Finally, soluble extracts of housefly heads have been analyzed in an attempt to define the relationship of organophosphate poisoning to the isozyme forms of acetylcholinesterase and other esterases (Eldefrawi, et al., 1970). Other than the above sited

references, little is known regarding the role of esterases and their potential in ticks as genetic markers in development and evolution and indicators of environmental conditions, including chemical resistances.

The heterogeneity of the esterases have also been identified on the basis of their substrate specificity and reaction with specific inhibitors. For example (Markert, 1959), the vertebrate carboxylic esterhydrolases have been characterized according to their substrate and inhibitor properties and are currently recognized as carboxylesterase, arylesterase, acetylesterase, acetylcholinesterase, and cholinesterase.

The wide diversity of these enzymes has made it very difficult to associate the multiple forms of esterases with the term isoenzymes. A desirable prerequisite to the detailed chemical characterization of the esterases and the isoenzyme status of the esterases is a knowledge of the multiplicity and the distribution of the esterase activity. Electrophoresis coupled with specific histochemical staining procedures have proved extremely useful for this purpose and is utilized in this study to examine the multiple forms of esterases in several species of ticks.

The study of the distribution of esterases among species of ticks was selected for a number of reasons. First, virtually nothing is known about esterase polymorphism in the various species of ticks even though it is widely accepted that resistance to organophosphates may be the result of multiple molecular forms of acetylcholinesterase. Secondly, ticks are medically and economically important as a result of their ability to vector certain bacterial, rickettsial, or viral diseases. Some evidence suggests that the degree of developmental success of a pathogen within a host tick may be the result of certain enzyme molecular

forms. Lastly, the availability of interspecific hybrids allows for an analysis of phylogenetic relationships among the species.

Early investigators used zone electrophoresis to separate enzyme preparations into several different components; however, this procedure was time consuming and the degree of resolution and separation of bands was minimal (Beckman, 1966). Hunter and Markert (1959) refined the identification of isoenzymes of many enzymes by coupling the starch gel electrophoresis technique of Smithies (Smithies, 1955) with conventional histochemical staining procedures to identify the separated enzymes. In an effort to give sharp and separated enzyme zones of activity numerous studies have attempted to demonstrate the efficacy of various types of media such as polyacrylamide, agarose, cellogel and agarose-starch in electrophoresis.

The possibility of utilizing a commercially prepared cellulose acetate plate as a medium for assessing esterase heterogeneity in the various tick species was demonstrated. Traditionally, this medium has been successfully used in the clinical laboratory in the separation and identification of the isoenzymes of creatine kinase and of alkaline phosphatase. Recent studies (Kreutzer, et al., 1977) regarding phosphoglucomutase (PGM) variants in mosquitoes have also demonstrated the applicability of the cellulose acetate medium. In this case the technique was demonstrated as a rapid, direct and an efficient means for the electrophoretic separation of the multiple forms of an enzyme.

This study describes the electrophoretic variants of the multiple forms of esterases among several tick species in an effort to illustrate the intra- and interspecific variability of esterase activity, the patterns of esterase multiplicity during tick development and the variable

status of esterase activity in selected tissues.

MATERIALS AND METHODS

The laboratory reared species of Ixodidae ticks used in this study were supplied by Dr. Richard Osborn, U.S. Department of Agriculture, Livestock Insects Laboratory, Kerrville, Texas. The species included were: Amblyomma americana, A. cajennense, A. inornatum, A. maculatum, Boophilus annulatus and B. microplus. Two hybrids, (B. microplus ♂ X B. annulatus ♀) and the reciprocal in addition to a field-collected Boophilus species, heretofore referred to as the Mexican strain, were also examined.

The methodology for the separation of the non-specific esterases was accomplished by electrophoresis on cellulose acetate plate medium coupled with conventional histochemical staining procedures. This electrophoretic procedure employed a Tris-Borate continuous buffer system. The electrode buffer was prepared as follows: 13 g Tris (hydroxymethylaminomethane) and 1 g EDTA (ethylenediamine tetra-acetic acid) were added to 800 ml distilled water. The pH of the solution was then adjusted to pH 8.2 with boric acid crystals and then readjusted to pH 9.1 with 40% NaOH. The final volume was brought to one liter. The gel buffer selected in this study was synonomous to the electrode buffer.

The commercially prepared Helena cellulose acetate plate was selected as the electrophoretic medium. Prior to electrophoresis, the cellulose acetate plate was presoaked in the gel buffer for approximately 20 minutes. Each cellulose acetate plate was slowly immersed in the

buffer to assure complete diffusion within the cellulose acetate matrix. This procedure eliminated the formation of air pockets and enhanced the uniform migration of protein.

Individual ticks were placed in spot wells containing 50 µl of buffer and then hand homogenized with a glass stirring rod. Five microliters of the supernatant was transferred from each of the eight spot wells to the Helena Zip Zone well. The eight samples were simultaneously applied to the blotted cellulose acetate plate by the Zip Zone applicator.

The electrophoretic conditions were maintained by a Vokam Power Supply which was connected to a Helena electrophoretic chamber. A constant current of 3 mamps with a generation of 250 volts was applied for 25 minutes at room temperature. Subsequently, the esterase banding pattern of the zymogram was visualized by a specific histochemical staining procedure developed by Shaw and Prassad (1970). One hundred mg of α-naphthyl acetate and 100 mg β-naphthyl acetate were dissolved in 10 ml of a 50% acetone solution. Two ml of the substrate solution was added to 50 ml of a 0.1M phosphate buffer (pH 6.8) containing 30 mg Fast Blue RR salt.

In order to enhance the resolution of the separated esterase zones, the histochemical stain was applied as an agar overlay. The phosphate buffer solution was added to 50 ml of a warm 2% noble agar medium and transferred to individual petri plates until a thin (@ 2mm), uniform agar layer was formed. After the agar layer solidified, the cellulose acetate plate was placed on the surface of the agar and allowed to incubate at 37°C for approximately 25 minutes. Following visual inspection of the intensity of esterase bands, the cellulose acetate plate was rinsed in distilled water and destained in a 10% glacial acetic acid

solution for approximately 15 minutes. The cellulose acetate plate was rinsed in distilled water and air dryed. The zymograms were kept as permanent records and photographed.

RESULTS

Before the esterase phenotype for a tick species can be selected as a tool for species identification, the degree and nature of esterase variability must be ascertained. In order to substantiate this relationship, esterase variability was examined during the development of B. annulatus, B. microplus, interspecific hybrid (B. annulatus ♂ X B. microplus ♀), and A. americanum. Esterase variants among parental species and their progeny, and the interspecific comparison of esterase activity during developmental stages were also obtained for the above mentioned species. In particular, interspecific comparison of esterase activity among the adults of A. cajennense, A. inornatum, and A. maculatum have been combined and compared with the object of determining whether analysis of esterase electrophoretic mobility alone is sufficient to distinguish any species from any other, even taking into account the possible occurrence of intra-species variants. Finally, the study examined multiple bands of esterase activity within the testes, malpighian tubules, synaptic ganglion/accessory gland, and the salivary gland of A. americanum adults so as to compare the degree of similarity and to see whether individual tissues can also readily be distinguished on this basis. Individual sites of esterase activity were arbitrarily lettered beginning with the band of highest electrophoretic mobility so as to best recognize differences in banding pattern.

Electrophoretic Patterns of Esterases Observed During the Development of Selected Boophilus and Amblyomma Species

During the development of B. annulatus, B. microplus, and the interspecific hybrid (B. annulatus ♂ X B. microplus ♀) twelve esterase variants were observed using cellulose acetate electrophoresis. Individual esterase variants were comprised of three to five esterase bands. The most interesting aspect of esterase variability among the Boophilus species was the fact that only ten electrophoretically distinct esterase bands accounted for the twelve esterase variants. This fact suggests that esterase polymorphism was due to the presence-absence of a selected group of esterase bands. The possibility of additional bands being expressed is valid since adults of B. annulatus and Boophilus Mexican strain were unavailable and not examined at their later stages.

A comparison of the non-specific esterase patterns among the egg and nymphal stages of B. annulatus demonstrated the variability of esterase activity during development (Figs. 1, 2). Three sites of esterase activity were exhibited in each of the egg and nymph stages with the D band being expressed in both stages. However, the G and I bands were characteristic only among B. annulatus eggs. In addition, the expression of new esterase activity was illustrated by the C and J bands in the nymphs; each of which repeatedly had a distinctively different electrophoretic mobility than the G and I bands among the eggs. The C, D and J bands were electrophoretically similar to those bands expressed in the later stages of B. microplus and the Boophilus hybrid (B. annulatus ♂ X B. microplus ♀).

The esterase activity during the development of B. microplus exemplifies the homogeneity of the variants. Among B. microplus eggs,

Fig. 1.--Schematic representation of esterase pattern observed among eggs of B. annulatus. Arrow indicates direction of esterase migration away from cathodal origin (O) and toward anode.

Fig. 2.--Schematic representation of esterase pattern observed among nymphs of B. annulatus.

Fig. 3.--Schematic representation of esterase pattern observed among eggs of B. microplus.

Fig. 4.--Schematic representation of esterase pattern observed among larvae of B. microplus.

FIG. 1

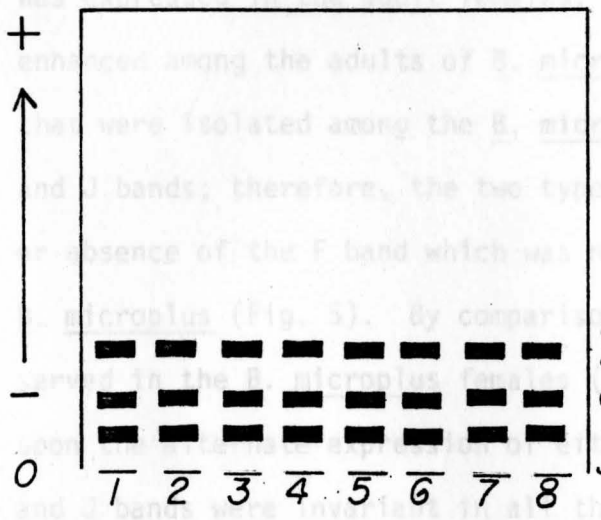


FIG. 2

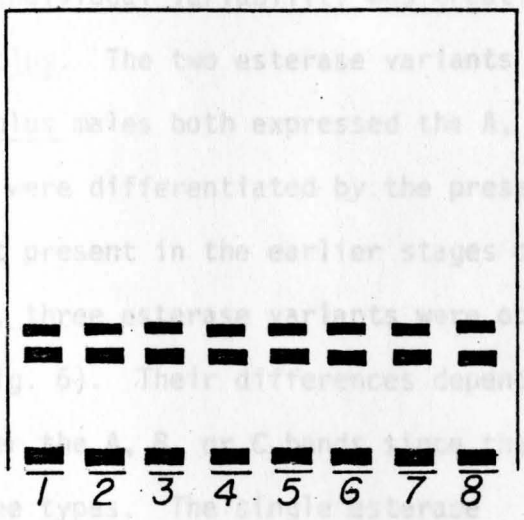


FIG. 3

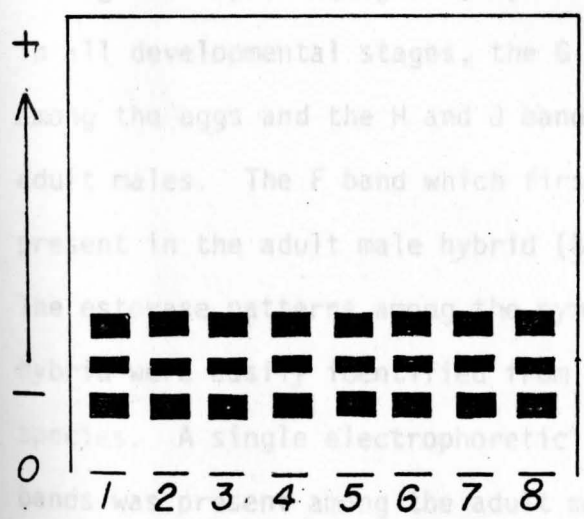
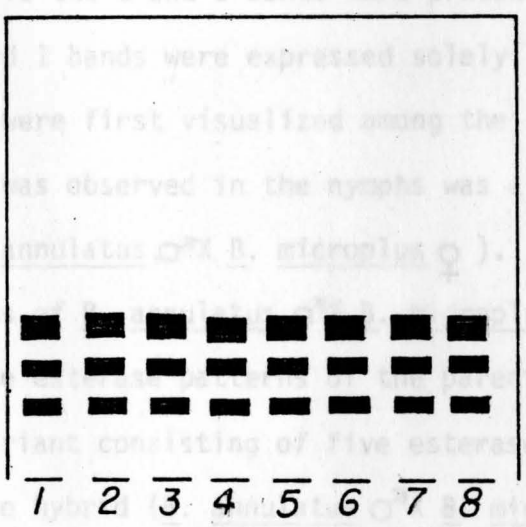


FIG. 4



the electrophoretic pattern was triple banded and corresponded exactly to the larval pattern (Figs. 3, 4). Both the E and H bands were expressed only among the eggs and larvae of B. microplus whereas the C band was expressed in the adult females. Individual variability was greatly enhanced among the adults of B. microplus. The two esterase variants that were isolated among the B. microplus males both expressed the A, D, and J bands; therefore, the two types were differentiated by the presence or absence of the F band which was not present in the earlier stages of B. microplus (Fig. 5). By comparison, three esterase variants were observed in the B. microplus females (Fig. 6). Their differences depended upon the alternate expression of either the A, B, or C bands since the D and J bands were invariant in all three types. The single esterase variant consisting of the A, D and J bands was expressed in both the males and females examined.

The non-specific esterases observed among the Boophilus hybrid (B. annulatus ♂X B. microplus ♀) also demonstrated pattern differences during development (Figs. 7, 8). While the C and D bands were present in all developmental stages, the G and I bands were expressed solely among the eggs and the H and J bands were first visualized among the adult males. The F band which first was observed in the nymphs was also present in the adult male hybrid (B. annulatus ♂X B. microplus ♀). The esterase patterns among the nymphs of B. annulatus ♂X B. microplus ♀ hybrid were easily identified from the esterase patterns of the parental species. A single electrophoretic variant consisting of five esterase bands was present among the adult male hybrid (B. annulatus ♂X B. microplus ♀) (Fig. 8).

Fig. 5.--Electrophoretic patterns of non-specific esterases among adult males of B. microplus.

Fig. 6.--Electrophoretic patterns of non-specific esterases among adult females of B. microplus.

Fig. 7.--Schematic zymogram of esterase patterns found among the eggs (1, 2, 3, 4) of the B. annulatus ♂ X B. microplus ♀ interspecific hybrid and the indistinguishable esterase patterns among the eggs (5, 6, 7, 8) obtained from the reciprocal cross, (B. annulatus ♀ X B. microplus ♂).

Fig. 8.--Esterase pattern obtained from nymphs (1, 2, 3, 4) of B. annnulatus ♂X B. microplus ♀ interspecific hybrid and the electrophoretic esterase pattern observed among the adult males (5, 6, 7, 8) of B. annulatus ♂X B. microplus ♀ interspecific hybrid.

FIG. 5

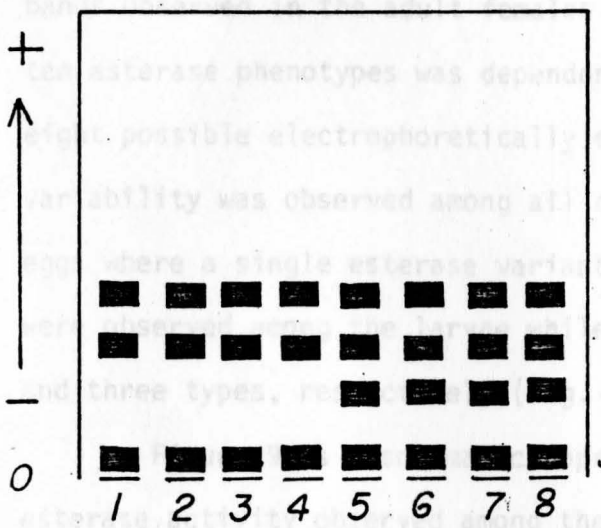


FIG. 6

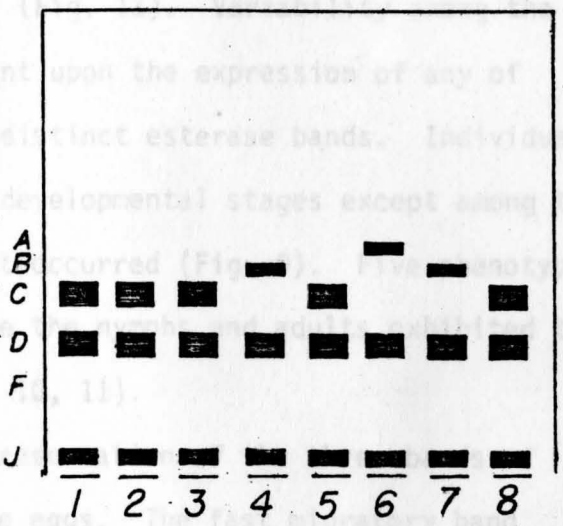


FIG. 7

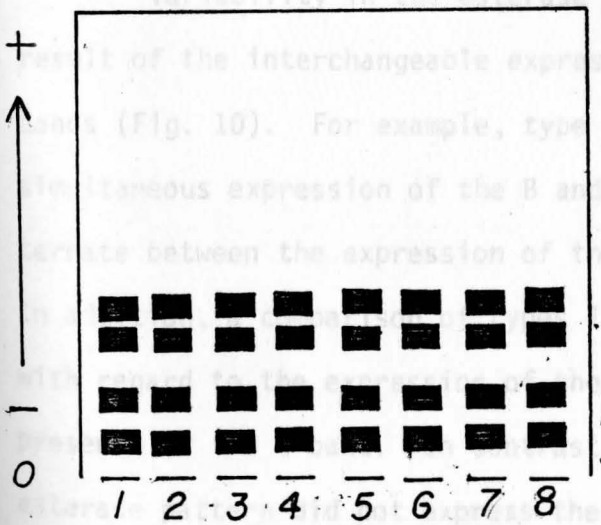
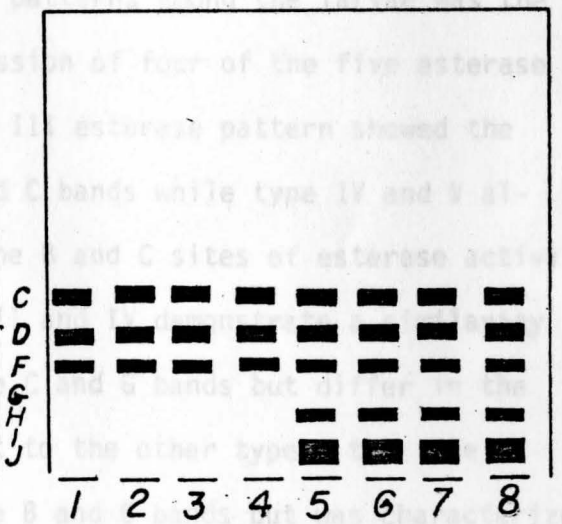


FIG. 8



In comparison, ten electrophoretic variants were observed during the development of A. americanum; each esterase phenotype exhibited either two or three esterase bands except for a single instance of four bands observed in the adult females (Fig. 12). Variability among the ten esterase phenotypes was dependent upon the expression of any of eight possible electrophoretically distinct esterase bands. Individual variability was observed among all developmental stages except among the eggs where a single esterase variant occurred (Fig. 9). Five phenotypes were observed among the larvae while the nymphs and adults exhibited two and three types, respectively (Fig. 10, 11).

Figure 9 is a schematic representation of the three bands of esterase activity observed among the eggs. The fast migratory band labeled B and the slowest migratory band, G, were detected in the later developmental stages while the intermediate band, F, was characteristic of the eggs. Thus, the presence of the F band enabled the B, F, G variant to be egg-specific.

Variability in the esterase patterns among the larvae was the result of the interchangeable expression of four of the five esterase bands (Fig. 10). For example, type III esterase pattern showed the simultaneous expression of the B and C bands while type IV and V alternate between the expression of the B and C sites of esterase activity. In addition, a comparison of types II and IV demonstrate a similarity with regard to the expression of the C and G bands but differ in the presence of the E band. In contrast to the other types, the type I esterase pattern did not express the B and C bands but was characterized instead by the expression of the D band which is not viewed in any of the

Fig. 9.--Diagrammatic representation of the electrophoretic pattern of non-specific esterases on cellulose acetate plate observed among the eggs of A. americanum.

Fig. 10.--Diagrammatic representation of the five esterase variants observed among the larvae of A. americanum.

Fig. 11.--Diagrammatic representation of the esterase variants observed among the nymphs of A. americanum.

Fig. 12.--Schematic summary of non-specific esterase patterns expressed among the adult females of A. americanum.

FIG. 9

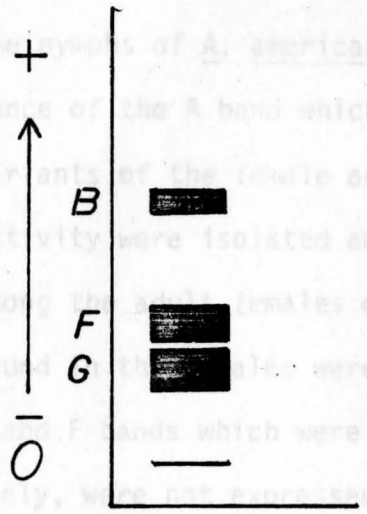


FIG. 10

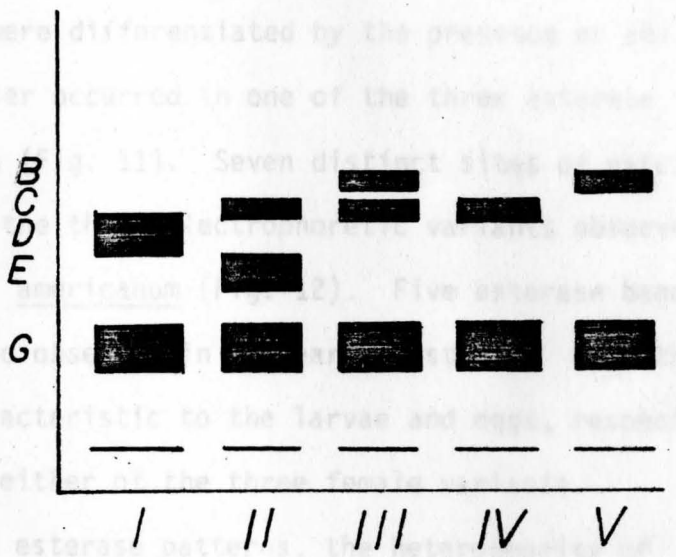


FIG. 11

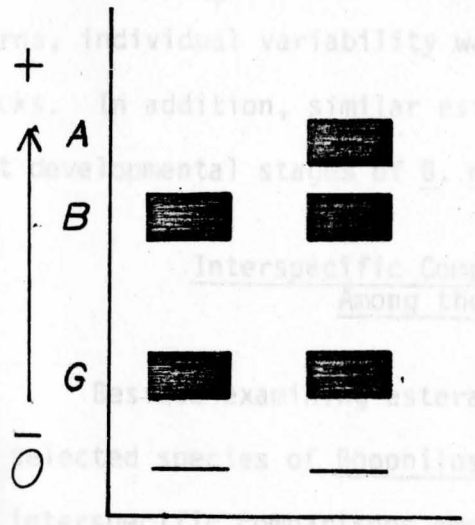
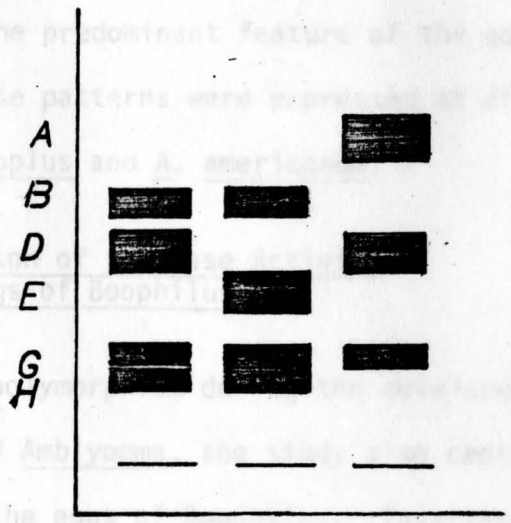


FIG. 12



other larval types. A second feature related to larval development was the fact that the C band was expressed among the larval stage while the B, D, E and G bands were observed in the adults.

The two electrophoretic esterase patterns that occurred among the nymphs of A. americanum were differentiated by the presence or absence of the A band which later occurred in one of the three esterase variants of the female adults (Fig. 11). Seven distinct sites of esterase activity were isolated among the three electrophoretic variants observed among the adult females of A. americanum (Fig. 12). Five esterase bands found in the females were also observed in the earlier stages. Only the C and F bands which were characteristic to the larvae and eggs, respectively, were not expressed in either of the three female variants.

On the basis of these esterase patterns, the heterogeneity of esterase activity was clearly demonstrated during the development of the Boophilus species and in particular that of A. americanum. While the early stages of B. annulatus, B. microplus, Boophilus hybrid (B. annulatus ♂ X B. microplus ♀) and A. americanum demonstrated invariant esterase patterns, individual variability was the predominant feature of the adult ticks. In addition, similar esterase patterns were expressed at different developmental stages of B. microplus and A. americanum.

Interspecific Comparison of Esterase Activity Among the Eggs of Boophilus

Besides examining esterase polymorphism during the development of selected species of Boophilus and Amblyomma, the study also centered on interspecific comparisons among the eggs of Boophilus. Invariant esterase patterns were observed among the eggs of B. annulatus, B. micro-

Fig. 13.--Electrophoretic analysis of esterase activity among the eggs (1, 2, 3, 4) of B. microplus and the eggs (5, 6, 7, 8) of B. annulatus ♂ X B. microplus ♀ interspecific hybrid.

Fig. 14.--Electrophoretic pattern of esterase activity expressed among the eggs of the Mexican strain.

Fig. 15.--Electrophoretic pattern of esterase activity expressed among eggs of B. annulatus ♂ X B. microplus ♀ interspecific hybrid.

Fig. 16.--Electrophoretic pattern of esterase activity observed among eggs of B. annulatus.

FIG. 13

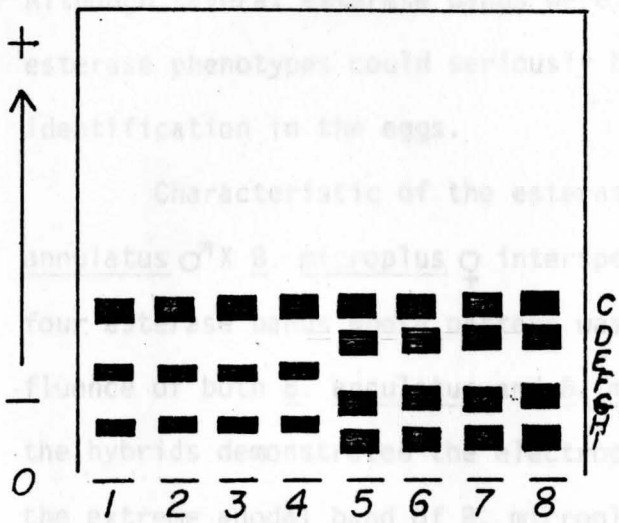


FIG. 14

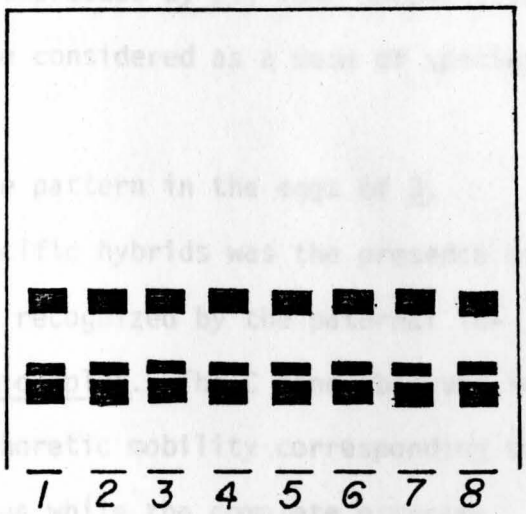


FIG. 15

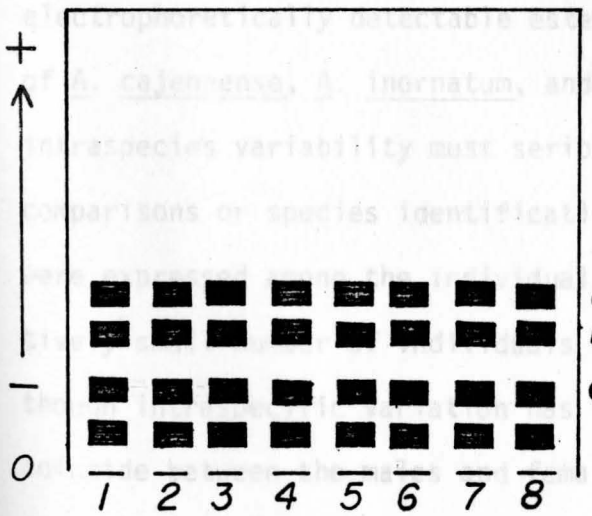
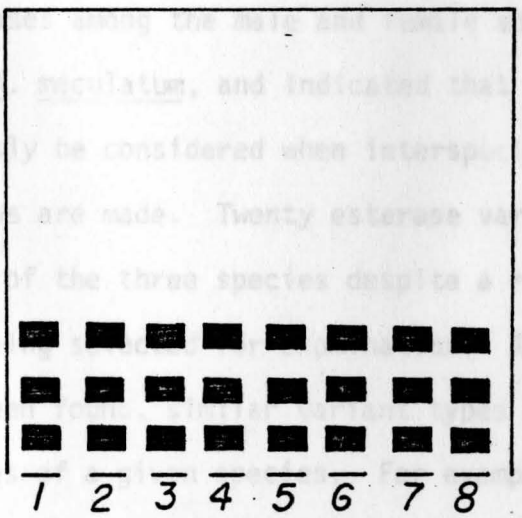


FIG. 16



plus, hybrid (B. annulatus ♂ X B. microplus ♀), and Mexican strain (Figs. 13, 14, 15, 16). The four esterase phenotypes were distinguishable from each other and not observed in later developmental stages. Although several esterase bands were expressed by all four species, the esterase phenotypes could seriously be considered as a mode of species identification in the eggs.

Characteristic of the esterase pattern in the eggs of B. annulatus ♂ X B. microplus ♀ interspecific hybrids was the presence of four esterase bands whose pattern was recognized by the paternal influence of both B. annulatus and B. microplus. The C band observed in the hybrids demonstrated the electrophoretic mobility corresponding to the extreme anodal band of B. microplus while the complete esterase zymogram of B. annulatus was expressed in the hybrid eggs.

Intra- and Interspecies Comparison of Esterase Activity Among Three Adult Species of Amblyomma

The study demonstrated inter- and intraspecies variation in the electrophoretically detectable esterases among the male and female adults of A. cajennense, A. inornatum, and A. maculatum, and indicated that intraspecies variability must seriously be considered when interspecific comparisons or species identifications are made. Twenty esterase variants were expressed among the individuals of the three species despite a relatively small number of individuals being selected for examination. Although intraspecific variation has been found, similar variant types did coincide between the males and females of a given species. For example, the males and females of A. cajennense commonly expressed two esterase phenotypes, and in a similar situation an esterase variant was present in both the males and females of A. maculatum (Figs. 17, 19). In contrast,

Fig. 17.--Comparative schematic variants of non-specific esterases present in the male and female adults of A. cajennense.

FIG. 17

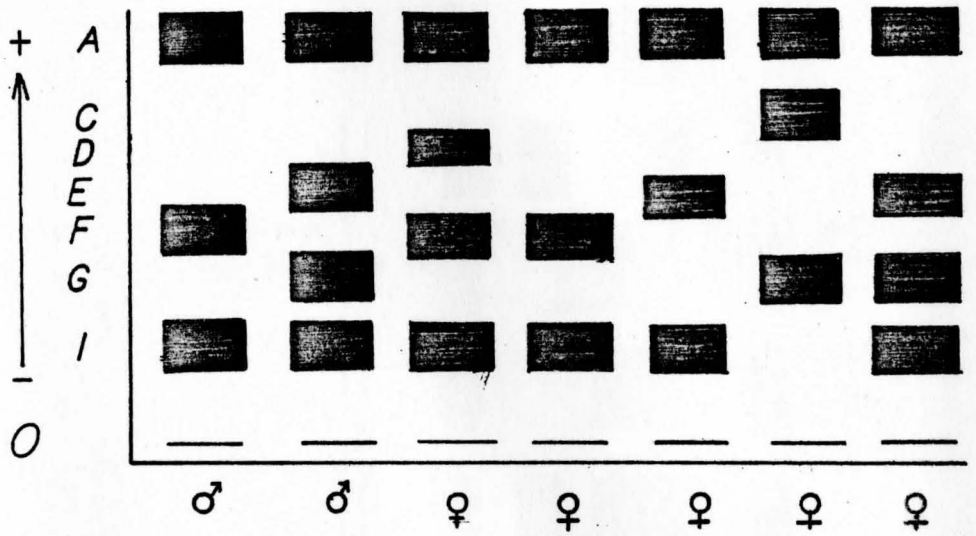


Fig. 18.--Comparative schematic variants of non-specific esterases present in the male and female adults of A. inornatum.

FIG. 18

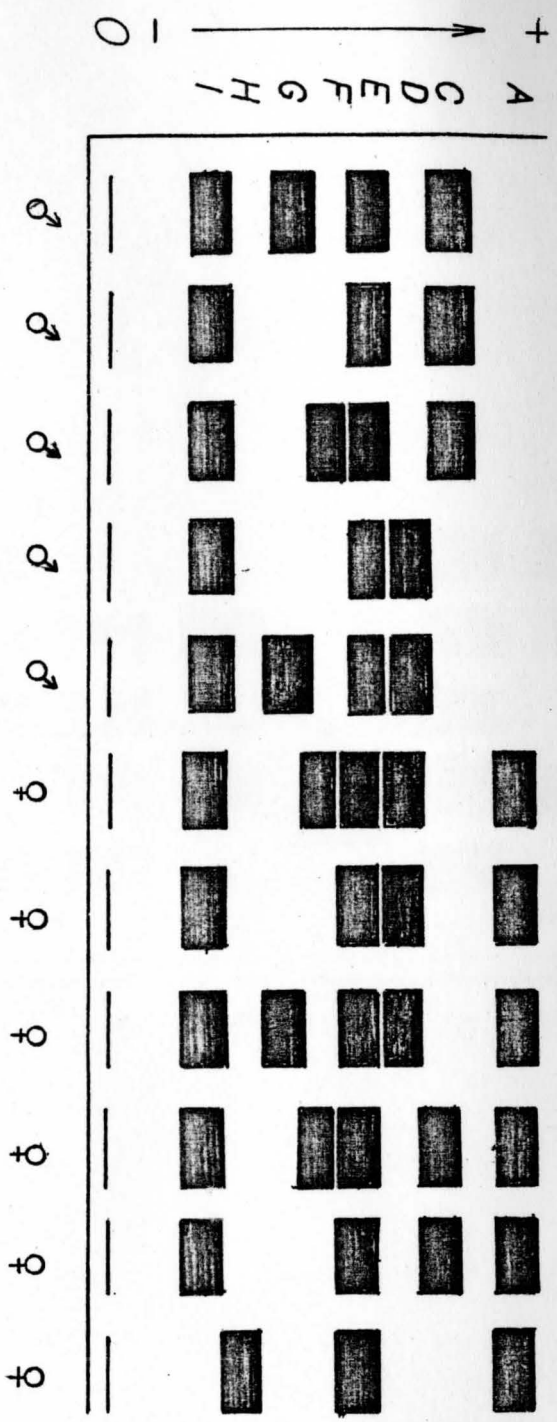
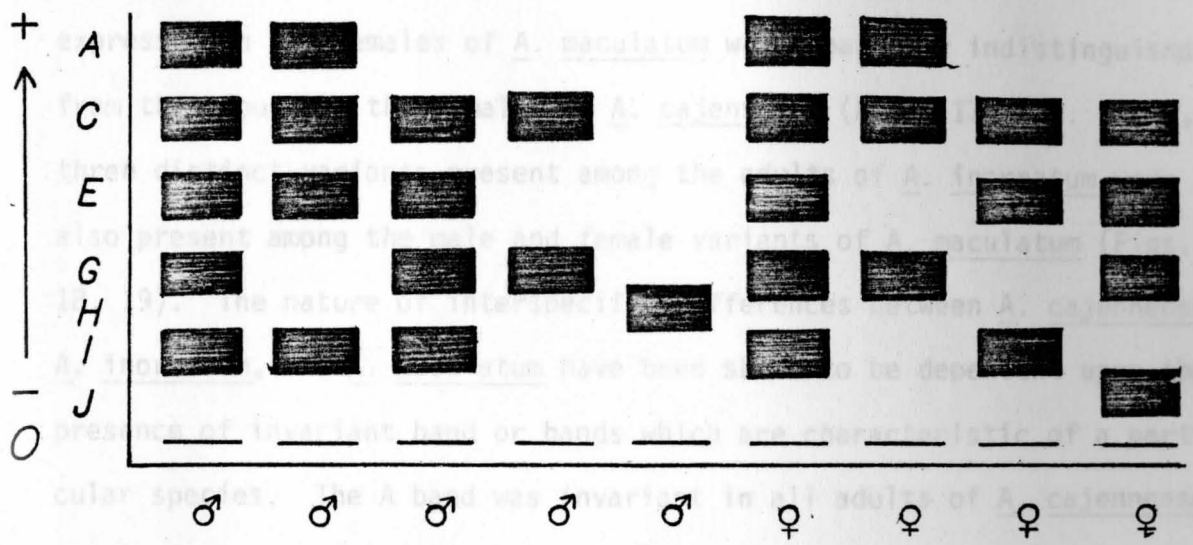


Fig. 19.--Comparative schematic variants of non-specific esterases present in the male and female adults of A. maculatum.

FIG. 19



esterase variants were present that showed possible sexual dimorphism (Figs. 17, 18, 19). The primary determinant of individual heterogeneity within the three adult species was the alternating presence-absence of selected bands. For example, the adults of A. inornatum were differentiated by the presence or absence of the C, D, F, or G bands (Fig. 18). Like A. inornatum, individual heterogeneity among A. maculatum was dependent upon variable expression of the A, E, G and I bands (Fig. 19).

A comparison of the interspecific variants demonstrated that the variants observed within a species were indistinguishable from variants observed in a second species. As an example, a single esterase variant expressed in the females of A. maculatum was apparently indistinguishable from that found in the females of A. cajennense (Figs. 17, 19). Also, three distinct variants present among the adults of A. inornatum were also present among the male and female variants of A. maculatum (Figs. 18, 19). The nature of interspecific differences between A. cajennense, A. inornatum, and A. maculatum have been shown to be dependent upon the presence of invariant band or bands which are characteristic of a particular species. The A band was invariant in all adults of A. cajennense examined while both the A and E bands were invariant in the females of A. inornatum (Figs. 17, 18). In addition, the C band was invariant among the adults of A. maculatum, and the I band was consistently maintained among the adults of A. cajennense and A. inornatum while only occasionally expressed in A. maculatum.

Electrophoretic Heterogeneity of Esterase Activity Among Selected Adult Tissues of A. americanum

The four electrophoretic variants of esterases prepared from testes, malpighian tubules, synaptic ganglion/accessory gland, and sal-

Fig. 20.--Schematic zymogram of esterase variant observed in the testes (1, 2, 3, 4) and the malpighian tubules (5, 6, 7, 8) of the A. americanum adults.

Fig. 21.--Schematic zymogram of esterase variants characteristic to the salivary gland (1, 2, 3, 4) and the synaptic ganglion/accessory gland (5, 6, 7, 8) of the A. americanum adults.

Fig. 22.--Schematic zymogram of the esterase variants characteristic to the salivary gland (1, 2, 3, 4) and the synaptic ganglion/accessory gland (5, 6, 7, 8) of the A. americanum adults.

FIG. 20

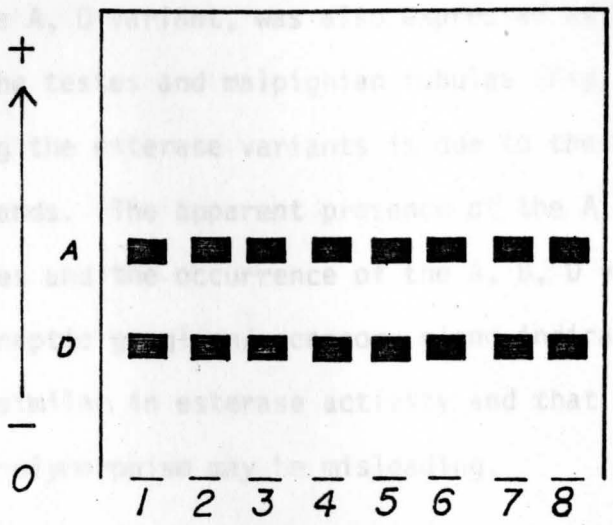


FIG. 21

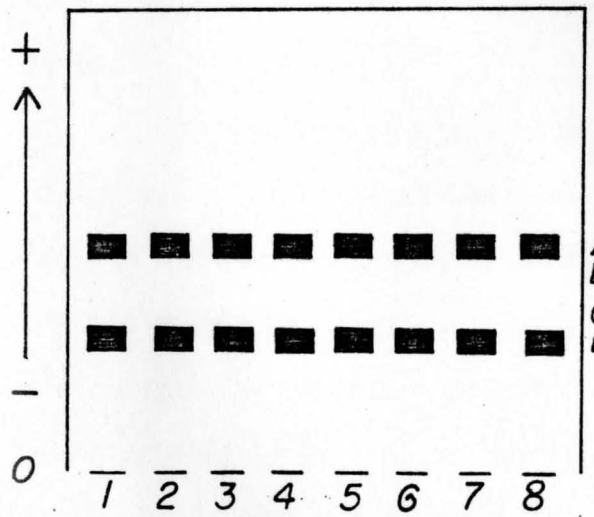
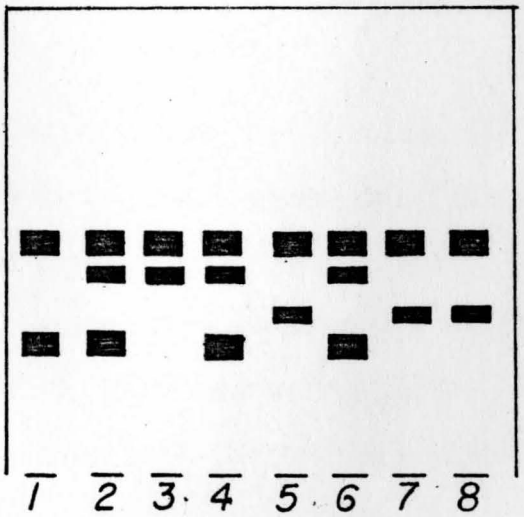


FIG. 22



ivary gland indicated differences in the level of esterase activity among the adult tissues (Figs. 20, 21, 22). Three esterase phenotypes were expressed in the salivary gland and synaptic ganglion/accessory gland; one of which, the A, D variant, was also expressed as the lone esterase variant of the testes and malpighian tubules (Fig. 20). The primary difference among the esterase variants is due to the occurrence of the B or C esterase bands. The apparent presence of the A, D esterase variant in all tissues and the occurrence of the A, B, D variant in the salivary gland and synaptic ganglion/accessory gland indicates that the tissues are closely similar in esterase activity and that tissue identification by esterase polymorphism may be misleading.

DISCUSSION

Esterases are a complex family of hydrolytic enzymes which show a multiplicity of forms within many species. Previously, these forms have been separated on such traditional media as acrylamide or starch gels. This study has demonstrated the feasibility of using commercially prepared cellulose acetate sheets as a substrate in defining electrophoretically the esterase heterogeneity among several species of ticks. Using this procedure, individual bands of esterase activity were distinct, well-defined, and stained quantitatively without excessive background or trailing. These advantages enhanced greatly the reliability of future studies on densitometry. Occasional backgrounds that did develop as the result of overstaining could be lessened by treatment with a mixture containing 95 parts methanol and 5 parts glacial acetic acid which acts as the vehicle. The cellulose fibers are partially dissolved by the action of the methanol and coalesce so that the original air spaces are eliminated.

The movement of the sample molecule across the cellulose acetate medium was closely associated with the pH and ionic strength of the buffer and the current and voltage of the power supply. The cellulose acetate sheets are composed of 80% air spaces that form pockets within the interlacing cellulose acetate fibers. When immersed in buffer for a predetermined time, the spaces fill with buffer. If the electrolyte concentration within the cellulose acetate is too low, the migrating macromolecules conduct a large portion of the current. As a result they are not found in sharp bands but rather spread into diffuse zones and thus

greatly decrease the resolution. Alternatively, if the electrolyte concentration within the cellulose acetate is too high, the amount of current conducted increases while voltage decreases. This usually results in a decreased rate of macromolecular migration and the generation of heat which can denature the enzymes.

Additional advantages of the cellulose acetate sheets have become apparent. For example, the pre-run and experimental operating time are relatively short (25-30 minutes). Thus, the esterase integrity (activity) was assured and the production of artifacts obviated. The sample size employed in the technique was small (5 µl), and the medium did not react with the sample or in any way retard its movement by binding to it. The cellulose acetate sheets demonstrated minimal convection, were easily handled, and were used for future reference. Depending upon the type of commercially available power supply, several cellulose acetate sheets can be practicably run simultaneously. The study lends credence that cellulose acetate electrophoresis can be utilized for other enzyme systems not only in ticks but also in other organisms and/or tissues.

Changes in esterase activity during the course of development of B. microplus, B. annulatus, and the Boophilus hybrid (B. annulatus ♂ X B. microplus ♀), were observed. The early stages of B. annulatus, B. microplus, and the Boophilus hybrid (B. annulatus ♂ X B. microplus ♀) demonstrated invariant esterase patterns which were stage-specific. Furthermore, a comparison of the esterase zymograms from different species reveal patterns which are species-specific. The invariant esterase patterns within each stage and the obvious differences between the species suggest the possibility of cellulose acetate electrophoresis as a tool for the biochemical identification of Boophilus. However, it is requisite

that further studies be done in order to delineate all variability inherent within and among species before taxonomic decisions can be made with certainty.

Variants of esterases observed in the developmental stages of B. microplus were atypical to those esterase patterns observed for B. annulatus. Examination of the electrophoretic variants of the hybrid resulting from crosses between the two species demonstrated the influence of the parental genomes. Alternatively, different esterase phenotypes for the hybrid (B. annulatus ♂ X B. microplus ♀) occurred and absence of certain parental esterase bands in the early stages did not prevent hybrids from further development. The esterase studies allowed information to be obtained only from those elements of the genome that specify esterase structure; however, these may not represent all genetic elements so that their analysis may provide limited information concerning taxonomic or evolutionary relationships in genes and gene pools of the species.

The occurrence of esterase variants within the developmental stages of A. americanum suggests that intraspecies variation depends upon the stage of development of the tick as well as the species involved. A comparison of the esterase phenotypes from the different developmental stages demonstrates that the esterase patterns were irregular and unpredictable; however, instances occur where esterase variants from different stages are indistinguishable. The study recognizes the need to examine the large degree of individual variability of esterase activity in each of the developmental stages before the esterases are selected as a biochemical tool in species identification. Although the results do

not give a clear explanation for the degree of variation in electrophoretic esterase mobility, several explanations are possible. Intraspecies variability within a particular stage suggests a possible change in the rate of esterase synthesis, or in a specific cell type. Also, differences in the intracellular location of the esterases may affect the degree of variation. Finally, the cellulose acetate medium that was selected for the separation of the esterases possibly detected only some of the differences between esterases since many amino acid substitutions do not make a detectable difference in the net charge. It would be difficult to estimate what proportion of substitutions have been detected in this study. Moreover, any change in the amino acid constituents of the esterase that results in a change in the net charge of the molecule should be detected by electrophoresis. Cellulose acetate electrophoresis may detect only a minority of the esterases present in the ticks and those detected may represent a certain class of esterases due to their high concentration. Thus, the degree of esterase differences detected may greatly underestimate the genetic variability of A. americanum.

The large degree of intraspecific variation in electrophoretically detectable esterases among the adults of A. cajennense, A. inornatum, and A. maculatum demonstrated that the esterases are not only variant between species but are also relatively variant within species. The absence of characteristic esterase patterns between the three species clearly shows how different the three adult species need to be in terms of variation of esterase activity to justify considering them as members of separate species. Additionally, several esterase phenotypes within one species were electrophoretically indistinguishable from phenotypes in another species. The three adult species exhibit a wide diversity of esterase

activity and the use of esterase variants alone as a means of species identification can lead to possible underestimating the number of species or possibly to misclassification.

Interspecific variations were expressed as differences in certain types and number of esterase bands. These differences are significant in an attempt to identify gene-enzyme systems between the three species of Amblyomma. The identification of species-specific esterase bands with specific substrates and inhibitors would provide insight into cellular and temporal specificity of gene function. Moreover, this information may elucidate mechanisms responsible for specificity of gene regulation and better explain the selective force that may be involved.

The three esterase variants illustrated in the synaptic ganglion/accessory gland and the salivary gland reflect the different molecular forms necessary to presumably fulfill specialized metabolic requirements. The three variant types of the two tissues possibly reflect the changes in gene expression necessary to catalyze a similar reaction but under different metabolic conditions. Although no planned attempt was made to investigate the mechanisms involved in developing and maintaining different esterase variants in the same tissue, some possibilities may be suggested. It may be possible, for example, that these patterns result from differential gene expression, the differing rates of synthesis and degradation, or differential subcellular localization.

Heterogeneous esterase patterns provide information for understanding intra- and interspecific relationships, for studying the developmental profile of a species and for viewing the regulation of gene expression in differentiated tissues. In addition, the study demonstrates a preparative methodology for the isolation of individual esterase bands. The methodology is not suggestive that all esterases are accurately ex-

pressed or absent on the cellulose acetate zymograms; however, their reproducibility allows for the identification of bands by utilizing specific substrates and inhibitors and thereby determining the adaptive significance of the esterase bands. Finally, it should be stressed that although problems occur from the use of esterase variants as a means of species identification, the method has potential advantages when the proper set of enzymes which are relatively invariant within species and variant between species are selected.

LITERATURE CITED

Allen, R. C. and D. J. Moore. 1966. A vertical flat-bed discontinuous electrophoresis system in polyacrylamide gel. Anal. Biochem., 16: 457-465.

Augustinsson, K. B. 1961. Multiple forms of esterases in vertebrate blood plasma. Ann. N. Y. Acad. Sci., 97: 844-860.

Beckman, L. 1966. Isozyme Variations in Man. John Wiley and Sons, Inc., New York.

Berger, E. 1973. Gene-enzyme variation in three sympatric species of Lifforina. Bio. Bull., 145: 83-90.

Booth, G. M., J. Connor, R. A. Metcalf, and J. R. Larsen. 1973. A comparative study of effects of selective inhibitors on esterase isozymes from the mosquito, Anopholes punctipennis. Comp. Biochem. Physiol., 44B: 1185-1195.

Booth, G. M. and R. L. Metcalf. 1970. Histochemical evidence for localized inhibition of cholinesterase in the housefly. Ann. Entomol. Soc. Amer., 63(1): 197-204.

Brewer, G. J. and C. F. Sing. 1970. An Introduction to Isozyme Technique. Academic Press, Inc., New York. 127p.

Eldefrawi, M. E., R. K. Tipathi, and R. D. O'Brien. 1970. Acetylcholinesterase isozymes from the housefly brain. Biochem. Biophys. Acta., 212: 308-314.

Enzyme Nomenclature. 1973. Recommendations of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry, Elsevier, Amsterdam.

Grafios, M. and D. B. Miller. 1967. Reversible aggregation of acetylcholinesterase II. Interdependence of pH and ionic strength. Biochemistry, 6: 1034-1046.

Hochachaka, P. W. and G. N. Somero. 1973. Strategies of Biochemical Adaptation. W. B. Saunders Co., Philadelphia.

Holmes, R. S. and G. S. Whit. 1970. Developmental Genetics of the esterase isozymes of Fundulus heteroclitus. Biochem. Genet., 4: 471-480.

Johnson, G. B. 1973. Enzyme polymorphism and Biosystematics: The hypothesis of selective neutrality. Ann. Rev. Ecol. Syst., 4: 93-116.

Johnson, G. B. 1974. Enzyme polymorphism and metabolism. Science, 184: 28-37.

Karnovsky, M. J. and L. Roots. 1964. A direct-coloring thiocholine method for cholinesterases. J. Histochem. Cytochem., 12: 219-221.

Kirsch, J. F. 1973. Mechanisms of enzyme action. Annu. Rev. Biochem., 42: 205-234.

Koshland, D. E. Jr., G. Nemethy and D. Filmer. 1966. Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry, 5: 365-385.

Koshland, D. E. Jr. and K. E. Neet. 1968. The catalytic and regulatory properties of enzymes. Annu. Rev. Biochem., 37: 359-410.

Kreutzer, R. D., F. T. Posey, and P. A. Brown. 1977. A fast and sensitive procedure for identifying genetic variants of phosphoglucomutase in certain genera of mosquitoes. Mosquito News, 37: 407-409.

Markert, C. L. and R. L. Hunter. 1959. The distribution of esterases in mouse tissues. J. Histochem. and Cytochem., 7: 42-49.

Markert, C. L. and F. Mooler. 1959. Multiple forms of enzymes: Tissue, ontogenic, and species specific patterns. Proc. Natl. Acad. Sci., U.S.A., 45: 753-763.

Markert, C. L. 1968. The molecular basis for isozymes. Ann. N. Y. Acad. Sci. 151: 14-40.

Markert, C. L. and G. S. Whitt. 1968. Molecular varieties of isozymes. Experientia, 24: 977-991.

Markert, C. L. and H. Ursprung. 1971. Developmental Genetics. Prentice-Hall, Englewood Cliffs, New Jersey.

Markert, C. L. ed., 1975. Isozymes, 4 vols. Academic Press, Inc., New York.

Masters, C. J. and R. S. Holmes. 1972. Isozymes and ontogeny. Biol. Rev., 47: 309-361.

Meera Khan, P. 1971. Enzyme electrophoresis on cellulose acetate gel: zymogram patterns in man-mouse and man-Chinese hamster somatic cell hybrids. Arch. Biochem. Biophys., 145: 470-483.

Shaw, C. R. and R. Prasad. 1970. Starch gel electrophoresis of enzymes--A compilation of Recipes. Biochem. Genet., 4: 297-320.

Smithies, O. 1955. Zone electrophoresis in starch gels: Group variation in the serum proteins of normal human adults. Biochem. J., 61: 629-641.

Smithies, O. 1959. An improved procedure for starch gel electrophoresis: Further variations in the serum proteins of normal individuals. Biochem. J., 71: 585-589.

Tripathi, R. K., Y. C. Chiu, and R. D. O'Brien. 1973. Reactivity in vitro toward substrate and inhibitors of acetylcholinesterase isozymes from electric eel Electroplax and housefly brain. Pestic. Biochem. Physiol., 3(1): 55-60.

Umbarger, H. E. 1971. Control Mechanisms in Cellular Processes. Ronald Press Co., New York.

Whitmore, D. Jr., E. Whitmore, and L. I. Gilbert. 1972. Juvenile hormone induction of esterases: A mechanism for the regulation of juvenile hormone titer. Proc. Nat. Acad. Sci. U.S.A., 69: 1592-1595.