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A STUDY OF THE PAPER ELECTROPHORETIC SERUM PROTEIN PATTERNS OF THE SUBSPECIES OF RANA PIPIENS

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ABSTRACT

Blood serum of the subspecies of *Rana pipiens* was separated into individual serum protein fractions by paper electrophoresis to determine relative difference in serum protein patterns between subspecies, and to indicate the value of paper electrophoresis in taxonomical classification on the subspecies level.

The variations between serum protein fractions of different subspecies of *Rana pipiens* were used as a basis for producing a key to taxonomical classification of the subspecies and established paper electrophoresis as a useful tool in taxonomical classification on the subspecies level.

INTRODUCTION

The purpose of this investigation was to determine the value of paper electrophoresis in taxonomical differentiation on the subspecies level by comparing the resulting serum protein fractions of different subspecies as to the number of fractions and the relative concentrations of the individual fractions.

Immunological methods to determine phylogenetic relationships have been used for many years. The introduction of electrophoresis has offered another useful technique for these investigations. Such data as presence or absence of specific fractions and speed of migrations toward the anode or cathode have been found useful as taxonomic characteristics. Dessauer and Fox (1956) used such data in their analysis of plasma proteins of taxonomical orders of Mammalia in which they noted basic similarities and differences in the plasma protein patterns.

Crenshaw and Zweig (1957) undertook a comparison of serum protein patterns of related turtle species. Paper electrophoresis was employed and found useful in intrageneric comparison of this particular animal group.

Ashton (1958), in beginning his work on several related species of horses, noted the widespread serum protein variations which had been obtained from mammals. Complex serum protein patterns were introduced and electrophoresis was proclaimed as a useful tool for revealing variations in closely related horse species.

Previous investigations of related forms by electrophoresis have been limited to vertebrates. However, Sande and Karcher (1960), developed a technique for microelectrophoresis and studied the hemolymph proteins of different species of Triatomidae (Class Insecta) and the family Ixodidae and Argosidae (Class Arachnida). Their results indicated the value of electrophoresis in confirming the findings of taxonomy as each species exhibited a specific electrophoretic pattern.

Another variation of protein material was obtained in skeletal muscle extracts from ten species of a fresh water sunfish family by Lillevik and Schloemer (1961). Their electrophoretic separations indicated the value of electrophoretic methods in analysis of the muscle proteins of individual species of sunfish and they suggested electrophoresis as a possible means of "fingerprinting" any species. At the same time a suggestion was made as to the possible value of electrophoresis in separations of animals on the subspecies level.

MATERIALS AND METHODS

This investigation was limited to the five subspecies of *Rana pipiens: berlandieri, burnsi, kandiyohi, pipiens,* and *sphenocephala.* Twelve male and female representatives of each subspecies of *Rana pipiens* were forced to hibernate in a water-laden tray in a refrigerator at a temperature of ten degrees centigrade for a period of at least forty-eight hours. Upon removal from hibernation, the frogs were exposed to temperatures varying from twenty-three degrees centigrade to twenty-five degrees centigrade for three hours. Following the three hour warm-up period the frogs were double pithed, reducing muscular activity to a minimum. Blood for study was obtained by making a mid-ventral incision at the posterior level of the sternum. This incision exposed the heart from which the pericardium was removed. Samples of blood were collected by severing the ventricle of the heart and allowing drainage from the ventricle of the heart into an eight milliliter test tube. Coagulation of the blood was accomplished in three hours at a temperature of twenty-three degrees centigrade. A fifteen minute period of centrifugation of the blood at fifteen hundred revolutions per minute produced a clear sample of serum, which was transferred to another eight milliliter test tube by a dropper pipette. All serum samples were refrigerated for twenty-four hours to seventy-two hours at a temperature of minus eight degrees centigrade.

The frog serum was separated into protein fractions within the electrophoretic cell developed by Williams (1955), and manufactured by the Spinco Division of Beckman Laboratories. The current for the electrophoresis of the serum was regulated by a Model R D-2 Duostat manufactured by Beckman Laboratories.

The electrophoretic separation of the serum protein fractions was accomplished by following procedure B in the RIM-5 Spinco Model R, Paper Electrophoresis System Instruction Manual furnished by Beckman Laboratories. The procedure was the same as for the separation of protein fractions of human serum, with

the following exceptions. First, moisture pads were installed within the electrophoretic cell to insure against changes in constant water vapor equilibrium due to temperature changes outside the electrophoretic cell. Distilled water was applied to the moisture pads prior to each electrophoretic separation according to instructions in the Beckman Technical Bulletin, Number 6119. Second, the equilibrium time of the phosphate buffer within the electrophoretic cell was continued for two hours to reduce the possibility of migration of the serum before the current was applied to the electrophoretic cell. Fresh phosphate buffer was used in each electrophoretic separation and the pH of the buffer was checked for accuracy on a pH meter. Third, serum samples of 0.01 milliliter were used in each electrophoretic serum protein separation to insure a greater concentration of protein within each fraction, and to facilitate identification of the fractions containing low concentrations of protein. Fourth, the serum of each subspecies was separated twice to substantiate the protein patterns and to obtain an average percentage of each protein fraction. Fifth, electrophoresis was continued for sixteen hours at a constant current of five milliamperes. This change in current was due to the operation of two electrophoretic cells at the same time and from the same power supply. Sixth, the polarity of both electrophoretic cells was reversed thirty minutes prior to the completion of each electrophoretic separation. The reversal in polarity reduced the possibility of ragged edges within each protein fraction, and thus, allowed for a more accurate analytrol reading.

A normal sample of human serum was separated by paper electrophoresis to serve as a basis for the identification of the frog serum protein fractions. The procedures for separation of the human serum and of the frog serum were identical in every aspect.

The recording of the serum protein fractions was accomplished by use of an integrating densitometer manufactured by Spinco Division of Beckman Laboratories and sold commercially as the Model R B Analytrol. Each paper strip was prepared for scanning on the analytrol according to the procedure listed in the RIM-5, Spinco Model R, Paper Electrophoresis System Instruction Manual. The serum protein fractions separated by electrophoresis were identified by comparing migration locations of the fractions of frog serum with the corresponding migration locations of the fractions of human serum. Following identification of each individual serum protein fraction, the density of the serum protein fractions of frog serum was recorded on analytrol graph paper according to the procedure in the instruction manual.

During each electrophoretic separation precautions were taken to insure constant conditions as to temperature, light and drafts. An electrophoretic separation was continued overnight between the hours of five o'clock in the afternoon and eleven o'clock the next morning. A temperature variation of two degrees (twenty-two degrees centigrade to twenty-four degrees centigrade) was not exceeded during each electrophoretic separation. During the scanning of the paper strips, the analytrol was protected from all outside light by black shades. Indoor lighting was provided by a single one-hundred watt incandescent light bulb placed to the side of the analytrol. The photoelectric cell was protected from direct rays of the light at all times, thus, insuring a constant serum protein fractional curve due to the light source within the analytrol.

RESULTS

Identifications of the serum protein fractions of *Rana pipiens* and the serum protein fractions of the human are found in Fig. 1. Comparison of the *Rana pipiens*

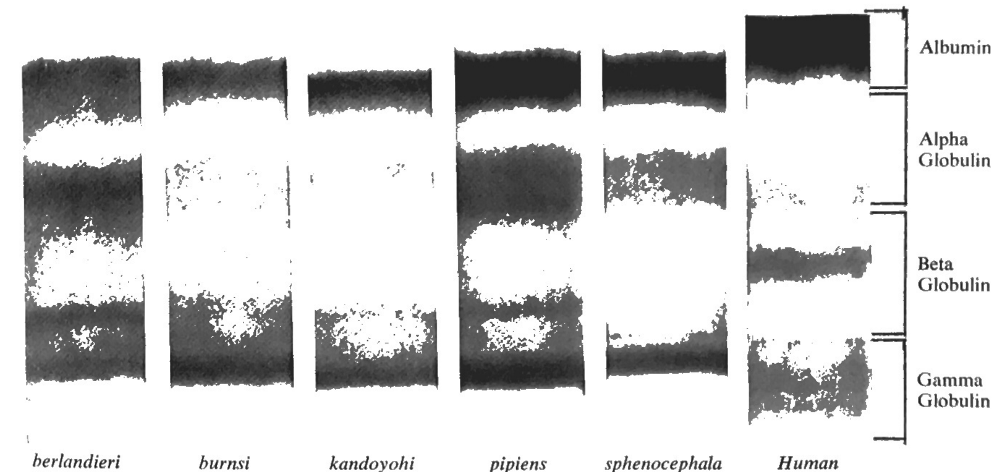

Fig. 1. Typical paper electrophoretic serum protein patterns of the human and the subspecies of **Rana pipiens.**

serum protein migrations with the human serum protein migrations indicates the *Rana pipiens* contains serum protein fractions which migrate at the same speed as the human serum protein fractions.

The data presented in Table I illustrates the percentage concentration variations of each serum protein fraction within the subspecies of *Rana pipiens*. Variations

TABLE I

Minimum and Maximum Serum Protein Fractional Concentrations of the Subspecies of Rana pipiens

Subspecies	% Gamma Globulin of Total Area	% Beta Globulin of Total Area	% Alpha Globulin of Total Area	% Albumin of Total Area
burnsi	11.3-25.3	23.1-35.9	17.2-26.2	28.7-35.0
kandikyohi	9.0-25.3	17.0-29.6	18.2-31.4	35.0-39.9
berlandieri	4.7- 8.5	20.6-44.5	18.6-37.3	28.0-40.0
pipiens	9.7-15.9	15.2-19.4	22.1-26.3	40.8-50.4
sphenocephala	13.7-20.4	18.3-23.8	16.8-21.5	41.2-50.1

between each serum protein fraction within a subspecies regardless of sex proved not to be significant. However, the variations between serum protein fractions of different subspecies of *Rana pipiens* did prove to be significant.

Results of this investigation indicate the value of paper electrophoresis in the taxonomical classification of the subspecies of *Rana pipiens* according to the key to subspecies of *Rana pipiens* in Table II.

TABLE II

Key to Subspecies of *Rana pipiens*

1. Albumin serum protein fraction between 28.0 per cent of total area of serum protein and 40.0 per cent of total area of serum protein; gamma globulin serum protein fraction between 4.7 per cent of total area of serum protein and 8.5 per cent of total area of serum protein. .... *berlandieri*
   Albumin serum protein fraction between 28.0 per cent of total area of serum protein and 50.4 per cent of total area of serum protein. 2
2. Albumin serum protein fraction between 28.7 per cent of total area of serum protein and 50.4 per cent of total of serum protein. .... *burnsi*
   Albumin serum protein fraction between 35.0 per cent of total area of serum protein and 50.4 per cent of the total area of serum protein. 3
3. Albumin serum protein fraction between 35.0 per cent of total area of serum protein and 39.9 per cent of total area of serum protein. .... *kandiyohi*
   Albumin serum protein fraction between 40.8 per cent of total area of serum protein and 50.4 per cent of total area of serum protein. 4
4. Albumin serum protein fraction between 40.8 per cent of total area of serum protein and 50.4 per cent of total area of serum protein fraction between 22.1 per cent of total area of serum protein and 26.3 per cent of total area of serum protein. .... *pipiens*
   Albumin serum protein fraction between 41.2 per cent of total area of serum protein and 50.1 per cent of total area of serum protein; alpha globulin serum protein fraction between 16.8 per cent of total area of serum protein and 21.5 per cent of total area of serum protein. ... *sphenocephala*

SUMMARY

Blood samples were taken from the five subspecies of *Rana pipiens* to determine differences in serum protein patterns between subspecies, and to establish the use of paper electrophoresis in taxonomical classification on the subspecies level.

Separation of the blood serum into the protein fractions gamma globulin, beta globulin, alpha globulin, and albumin was accomplished by paper electrophoresis. The analysis of the serum protein fractions was recorded on a graph using the analytrol densitometer.

Comparison of percentages of each serum protein fraction in relation to the total serum protein showed no significaant variation within a subspecies of *Rana pipiens*. However, the variations between serum protein fractions of different subspecies of *Rana pipiens* were significant and from experimental results a key to taxonomical classification on the subspecies level was established.

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