IMMUNOELECTROPHORETIC CHARACTERIZATION OF THE HEMOLYMPH OF THE CRAYFISH, *PROCAMBARUS SIMULANS* AND *PROCAMBARUS CLARKI*

by

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A THESIS

IN

ZOOLOGY

Submitted to the Graduate Faculty
of Texas Technological College
in Partial Fulfillment of
the Requirements for
the Degree of

MASTER OF SCIENCE

Approved

ACKNOWLEDGMENTS

I am deeply indebted to M. Eileen Lowe for her direction of this thesis and to the other members of my committee, John A. Anderson, Robert L. Packard, Chester M. Rowell, and Russell W. Strandtmann for their helpful criticism.

CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF FIGURES	iv
I. INTRODUCTION	1
II. MATERIAL AND METHODS	3
III. RESULTS	8
IV. DISCUSSION	12
V. SUMMARY	17
LIST OF REFERENCES	18

LIST OF FIGURES

Figure		Page
1.	Photograph of Immunoelectrophoretic Protein Pattern of Hemolymph from *P. simulans*	21
2.	Schematic Illustration of the Five Protein Arcs Seen in Figure 1 of Hemolymph from *P. simulans*	21
3.	Schematic Illustration of the Five Protein Arcs of Hemolymph from *P. clarki*	23
4.	Schematic Illustration of Comparison of Hemolymph from *P. simulans* and from *P. clarki*	23
5.	Schematic Illustration of Comparison of Hemolymph from *P. simulans* Occurring in Lubbock and from *P. simulans* Occurring in Austin	25

CHAPTER I

INTRODUCTION

Characterization of crustacean hemolymph protein composition has been attempted using ultracentrifugation (Eriksson-Quensel and Svedberg, 1936) and one-dimensional electrophoretic methods of starch gel (Woods and Paulsen, 1958) and cellulose acetate (Crowley, 1963). Crowley (1963) states that the number of protein components present in crayfish plasma is ". . . at the most five electrophoretically discernible bands." By using immunoelectrophoresis, a two-dimensional method, more decisive results may be obtained.

Immunoelectrophoresis was originally described by Grabar and Williams in 1953. Proteins suspended in a gel migrate at rates dependent upon the sizes and charges of individual protein components when subjected to an electrical field. After electrophoresis, the protein components diffuse through the gel toward diffusing antibodies placed in a trough parallel to the path of the electrophoretic migration. The indicator for the position of the protein diffused in the second dimension is the precipitin arc formed by the reaction of the protein with its specific antibody (Crowle, 1961). By this method, 40 protein components showing as separate arcs have been demonstrated in human plasma (Rogers, 1964) as compared with only six components obtained by paper electrophoresis (Hirschfeld,

1961).

This work reports the characterization of hemolymph proteins and, also, a degree of hemolymph protein variability within the genus Procambarus by examining Procambarus clarki (Girard) and Procambarus simulans simulans (Faxon). P. clarki was collected from the Austin, Texas, area while P. simulans was collected both in Austin and near Lubbock, Texas. In addition to facilitating biochemical characterization, the knowledge of variation of blood proteins and their cross reactions within a particular group will make possible the future use of immunoelectrophoresis in taxonomic work.

CHAPTER II

MATERIALS AND METHODS

Animals

Male and female *P. clarki* and *P. simulans* from the Austin area were provided by Dr. James L. Larimer of the University of Texas. These animal groups had previously been identified with the assistance of Dr. G. H. Penn of Tulane University. *P. simulans* from the Lubbock area were collected from a pond at the intersection of Loop 289 and Buffalo Lake Road east of Lubbock. Bovine plasma was obtained through the courtesy of Dr. F. G. Harbaugh of the Texas Technological School of Agriculture.

Antigen

The hemolymph was obtained from the crayfish by heart puncture using a syringe with 18 gauge needle. From an animal 10 cm in length, 1.5 to 2.0 ml of hemolymph could be collected. Since many of the crayfish survived this procedure, multiple bleedings could be made. Quantities of 2.0 ml were needed for injection into rabbits; however, only 0.5 ml of hemolymph was collected for electrophoretic runs. The antigen was never pooled; blood from each animal was handled separately.

When serum was desired from a crayfish, the hemolymph was transferred to a test tube and allowed to clot. The

tubes were placed at -4° C until frozen, thus hastening the expression of the serum. This was followed by centrifugation at 1500 X g for 25 minutes at 4 ± 2° C in a Lourdes Refrigerated Centrifuge Model LR or a Sorval RC-2 Centrifuge with an angle centrifuge rotor. The serum was stored at 4 ± 2° C and used within 24 hours.

To obtain plasma, crayfish and syringe were packed in ice for 10 to 15 minutes prior to heart puncture. The blood was withdrawn with the crayfish, needle, and syringe remaining on the ice. The hemolymph was immediately transferred to a cooled polyethylene tube, placed directly into the cold centrifuge, and centrifuged at 9500 X g for 30 minutes at 0 ± 2° C (Manwell and Baker, 1962). Immediately after centrifugation the plasma was decanted from the hemolymph cells and stored at 4 ± 2° C.

Bovine plasma was obtained by withdrawing blood from the jugular vein into a 30 ml syringe which contained 3 ml of 2.5% sodium oxalate as anticoagulant. The blood cells were removed by centrifugation at 9500 X g for 30 minutes at 4 ± 2° C.

Antisera

All antibodies were produced in rabbits. Intraperitoneal injections were given with the antigens in Freund's incomplete adjuvant to enhance antibody formation (Crowle, 1961). The adjuvant consisted of three parts light

mineral oil and one part anhydrous lanolin mixed equally with antigen diluted one to one with distilled water (Crowle, 1961). This water-in-oil emulsion was obtained by high speed stirring with a tissue homogenizer. In initial injections of hemolymph from P. simulans no adjuvant was used. Due to the lower antibody concentration obtained, tests using this antiserum did not yield the distinct arcs seen in the later tests where serum or plasma plus adjuvant was used to stimulate antibody production. Each rabbit received four injections of 1.0 to 1.5 ml of serum plus adjuvant at three day intervals. The animals were bled a week to 10 days after the last injection, either from the marginal ear vein or by direct heart puncture. The rabbit blood was allowed to clot and was centrifuged at 9000 X g for 30 minutes at 4 $\pm$ 2° C.

Method of Testing

Components from the LKB immunoelectrophoresis apparatus #3276BN were used with a Hoyt D.C. constant voltage power supply. Ionagar No. 2 of Consolidated Laboratories, Inc. which had been washed for four weeks and dried by lyophilization was found to be the best medium due to its transparency and only slight polarity.

Molten 1% agar containing either phosphate or barbital buffer was pipetted onto 2.5 x 7.5 cm glass slides. Troughs and wells were cut into the solidified agar using

the LKB die #6811 B. The trough, 6.5 by 2.0 cm wide, was cut down the center of the agar, and a well 1.0 mm in diameter was punched 5.0 mm on each side of the trough and 2.5 cm from one end of the microscope slide. Five-thousandths (0.005) ml of the hemolymph to be tested was placed in a well, each well containing hemolymph from a different crayfish. The electrolytic buffer was greater in ionic strength by one tenth and one pH unit lower than the agar buffer. Tests were run with agar buffer solutions of pH 8.2, 7.4, and 6.4. After rayon wicks were placed in position between slides and electrolyte reservoir, a potential of 75 to 85 volts was applied across each of the three bridges with the circuit conducting 50 milliamperes as registered on the power supply. Each bridge was 22.5 cm long and 5.0 cm wide and consisted of six microscope slides arranged end to end to form two rows.

After electrophoresis the agar was removed from the troughs and these were charged with 0.3 ml antiserum. While the antibodies were diffusing through the agar gel, the electrophoretically separated proteins were also diffusing throughout the gel. Precipitin arcs were formed in the equivalence zone of antigen and homologous antibody.

To obtain high contrast between the precipitin arcs and the agar background, unreacted protein was removed by washing in 0.5% saline. The slides were placed in an open wooden slide rack in horizontal position within a 2000 ml

beaker. Air was bubbled through it continuously for 48 hours to facilitate washing. Fresh saline was added once during the washing procedure.

After washing the slides, wet Curtin #7775 filter paper was placed on the slides. Wet Whatman #1 filter paper in double thickness was put over the fine filter paper, and the slides dried at 100° C for 45 minutes to produce permanent records and optimal staining. The slides were taken from the oven and the coarse filter paper removed. They were then flooded with tap water to remove the fine filter paper. The slides were stained for 30 minutes in a triple stain consisting of Thiazine Red R, Amido Black 10 B, and Light Green SF Yellowish, each made to 0.25% in 5.0% acetic acid (Rogers, 1964).

CHAPTER III

RESULTS

Immunoelectrophoresis gives a clear quantitative evaluation of hemolymph protein in the crayfish *P. simulans* and *P. clarki* and shows that there are variations between the two species. Figure 1 is a photograph of a stained slide showing the plasma protein arcs which develop upon immunoelectrophoresis of hemolymph from *P. simulans*. Figure 2 is a line diagram of Figure 1 numbering the five arcs. The clear circle indicated with an arrow is the point of origin of antigen for the electrophoresis, and the long trough is the position where antiserum is placed after electrophoresis.

Characterization of plasma protein for *P. clarki* also shows five arcs (Figure 3). Particularly interesting is the pattern variation between the two species. In *P. clarki* three of the arcs appear approximately in the same position as seen for *P. simulans*, while arcs 1 and 4 migrate slower (Figure 4). Figures 2 and 3 are samples taken from 164 such runs of hemolymph from 17 *P. simulans* and 13 *P. clarki*, each animal tested at least five times. The animals were tested a multiple number of times as each crayfish was compared with other members of its species as well as tested against the opposite species.

All members of *P. simulans* tested, whether from

Austin or Lubbock, show an extremely consistent hemolymph protein separation pattern (Figure 5). Likewise the hemolymph protein pattern of P. clarki is consistent for all animals tested.

Comparisons were also conducted using antisera from three rabbits immunized with hemolymph from P. simulans and two rabbits immunized with hemolymph from P. clarki. There was no apparent difference between the two types of antisera; in both cases the characteristic pattern appeared for hemolymph of P. simulans and for that of P. clarki.

One crayfish sample was compared with another on a single microscope slide to eliminate protein pattern differences due to variations in concentration and thickness of agar gel. Such variations affect electrical conductance and diffusion mobility. Another parameter affecting the protein separation is that of pH. Optimal separation occurred when a pH of 7.4 was used in the agar medium. At pH 6.4 migration was extremely slow; one run requiring three and one-half hours for the same separation obtained in two hours at pH 7.4. The higher pH value of 8.2 offered no advantage over pH 7.4; migration was more rapid but definition was not improved.

In the protein patterns, identification of arcs one and five (Figures 2 and 3) is possible with the identity of the other arcs remaining unknown. Because of its

bluish color, hemocyanin can be seen in the agar gel. Hemocyanin is the component with the fastest migration, and therefore the precipitin arc formed by this antigen and its antibody is the arc most distant from the point of origin. Hemocyanin is always characterized by a strong and usually broad band.

The slowest moving protein demonstrated by the arc nearest the origin (arc 5) is considered to be fibrinogen since this arc was absent in determinations using serum instead of plasma. Preliminary investigations into the nature of this fibrinogen revealed that it differs in both structure and mode of action from mammalian fibrinogen. In 18 determinations no precipitin arc appeared when using crayfish plasma as the antigen and bovine plasma antiserum. If a precipitin arc had occurred, it would most likely have formed by precipitation of crayfish fibrinogen. This would have demonstrated a cross reaction between proteins of similar structure. To confirm the dissimilarity of crayfish fibrinogen and mammalian fibrinogen, 0.1 ml crayfish plasma was mixed with 0.1 ml of 50 units of human thrombin (Ortho Pharmaceutical Corporation, lot #11Z72). No clot was demonstrated using crayfish plasma and human thrombin mixture; as a control normal crayfish blood formed a solid clot. Identical results were seen when mixing crayfish plasma with human prothrombin (Ortho Pharmaceutical Corporation, lot #DD). Since clotting of crayfish

hemolymph is initiated by substances released from hemolymph cells and acting on crayfish fibrinogen (George and Nichols, 1948), a test was conducted using crayfish hemolymph cells mixed with standardized normal human plasma (Dade Reagents, lot #SNP-572). If the substance released from hemolymph cells is a thrombin, then the substance from the hemolymph cells could initiate clotting in human plasma. The human plasma did not clot, indicating again the different modes of action of crayfish fibrinogen and human fibrinogen.

CHAPTER IV

DISCUSSION

Twenty-five years ago Allison and Cole (1940) concluded that in Crustacea only one blood protein, hemocyanin, existed in the serum. This conclusion was based on finding that the copper to protein nitrogen ratio was similar to that for purified hemocyanin. Four years prior to the work of Allison and Cole, Ericksson-Quensel and Svedberg (1936) using ultracentrifugation reported that 80 - 85% of crayfish hemolymph protein was hemocyanin. With Tiselius electrophoresis, Tyler and Metz (1945) revealed that the serum of the spiny lobster, Panulirus interruptus, contained three protein components. In 1958 Woods and Paulson extended hemolymph studies to other invertebrates, including the crayfish Cambarus limosus. Employing starch gel electrophoresis they showed definitely that more than one protein component occurred in crayfish hemolymph. However, the number of proteins present was not reported. Using cellulose acetate electrophoresis, Crowley (1963) studied the plasma proteins of five crayfish, Astacus nigresens being the most extensively studied. He reported the electrophoretic results as bar graphs and made the tentative statement that no more than five protein components were present in the plasma. In the present study five distinct proteins were separated.

The identity and functions of the plasma proteins are poorly known. Two of the five proteins shown in the present study can be functionally identified. Arc #1 is hemocyanin (Figure 2). Hemocyanin serves as the respiratory pigment in transporting oxygen and serves also as a peroxidase (Ghiretti, 1956). Another protein may be a fibrinogen, the coagulating substance of the blood (Florkin, 1960). This is probably represented by arc #5 in the present study (Figure 2). If this coagulating substance is a fibrinogen it differs markedly from mammalian fibrinogen as no cross reactions occur between crayfish fibrinogen and mammalian fibrinogen antibody.

Arcs 2, 3, or 4 cannot yet be assigned specific functions. Crowley (1963) reports that one hemolymph protein is probably associated with the process of molting, but its specific function has not been determined. Tyrosinase is present in crustacean hemolymph cells and may be released into the plasma under certain conditions (Pinhey, 1930). Tyronsinase and phenoloxidase in the epicuticle (Dennell, 1947) act together to harden the newly formed cuticle after the molt. While amylase activity by the plasma of Astacus has been reported, no oxidase, protease, or carbonic anhydrase activity could be detected (Florkin, 1960). Teague and Frion (1964) were unable to demonstrate precipitating antibodies in the blood or any anaphalaxis by the crayfish Cambarus virilis after injection of foreign protein.

Clark and Burnet (1942) were the first to use serological methods in studying interrelationships in Crustacea. They recorded appearance or absence of the precipitin reaction between antigens and antibodies as detected by the ring test and by visual estimation of turbidity. This idea of relationship between biochemistry and animal systematics originated from work with mammals by Nuttall in 1901 (Boyden, 1943). Leone (1949) conducted non-morphological tests in his comparison of some brachyuran Crustacea. His method utilized the photonreflectometer in measuring turbidities developing as a result of the interaction of antigens and antibodies. This gave a more quantitative estimate than did the visual observations of Clark and Burnet. The main criticism of turbidity measurements is that only a gross quantitative estimate can be made. The cross reactions between several pairs of animals may give identical values; however, the same turbidity may be produced by reactions between different proteins. As Leone (1949) assumed that hemocyanin was the only protein present, he might be in error in estimating the degree of correspondence between the Brachyura studied. Leone chose to pool the crayfish sera, hiding subtle differences important for taxonomic studies. Another variable was that Leone used some stored hemolymph from which bacterial growth had been removed by centrifugation.

With development of starch gel electrophoresis more

discriminating comparisons can be performed. Woods and Paulsen (1958) first used this technique to survey patterns produced by several Crustacea. They reported that three species of Uca had nearly identical serum electrophoretic patterns. Two species of Pagurus also gave patterns which were quite alike but differed from those of Uca. In the next application of electrophoresis to crustacean sera, Manwell and Baker (1963) showed distinct differences in electrophoretic patterns produced by the crabs, Callinectes sapidus, Emerita talpoida, and Uca pugilator. They tested many specimens of each species and reported some qualitative and quantitative variation, but individual variation never obscured the species specific patterns.

This paper reports the first use of immunoelectrophoretic techniques on crustacean blood, and is apparently the first report of such application to any invertebrate. Five distinct protein arcs were differentiated. While Crowley (1963) reports the presence in crayfish plasma of "at most not more than five electrophoretically discernable bands" using a one dimensional cellulose acetate technique, his results are reported as bar graphs and degree of band distinctness is not shown. In any electrophoretic technique, absorption of proteins by supporting medium may produce a streaked pattern; however, this streaking is considerably less using an agar medium than when using cellulose acetate or starch gel.

In immunoelectrophoresis the protein is separated by electrophoretic fractionation in one dimension and in the second dimension by diffusion in the absence of an electric field. Using specific antisera to locate the antigens in the gel further increases resolution. This increased resolving power has resulted in the first report of distinct and consistent interspecific pattern differences within a crustacean genus. Other workers using one dimensional techniques have reported clear cut differences only at a generic or family level (Woods and Paulsen, 1958).

As in any study, sample populations must be of a size so one may recognize an erratic pattern variation due to a minor gene change not necessarily representing speciation or permanent change. In this study slight variations were seen between individuals of the same species but these in no case obscured the distinctness of the species specific pattern.

Immunoelectrophoresis requires only small amounts of test sera, produces consistent and readily reproducible results, and allows easy comparison between different sera. These things coupled with a sensitivity which can demonstrate protein pattern variation at species level, makes immunoelectrophoresis a promising tool for many biological investigations.

CHAPTER V

SUMMARY

1. Characterization of hemolymph protein was determined by immunoelectrophoresis of the hemolymph of *P. simulans* and *P. clarki*.
2. Consistent protein patterns were observed for each of the two species. In both cases five protein components were demonstrated; however, the pattern for hemolymph of *P. simulans* showed consistent differences from that of *P. clarki*.
3. Comparison of hemolymph from *P. simulans* occurring in Austin and in Lubbock was conducted. Results agreed with morphological findings that the crayfish from both areas are *P. simulans* since the protein patterns were very similar.
4. Of the five proteins found, one was identified as hemocyanin and a second tentatively identified as crayfish fibrinogen. This fibrinogen showed no cross reactions with mammalian material.

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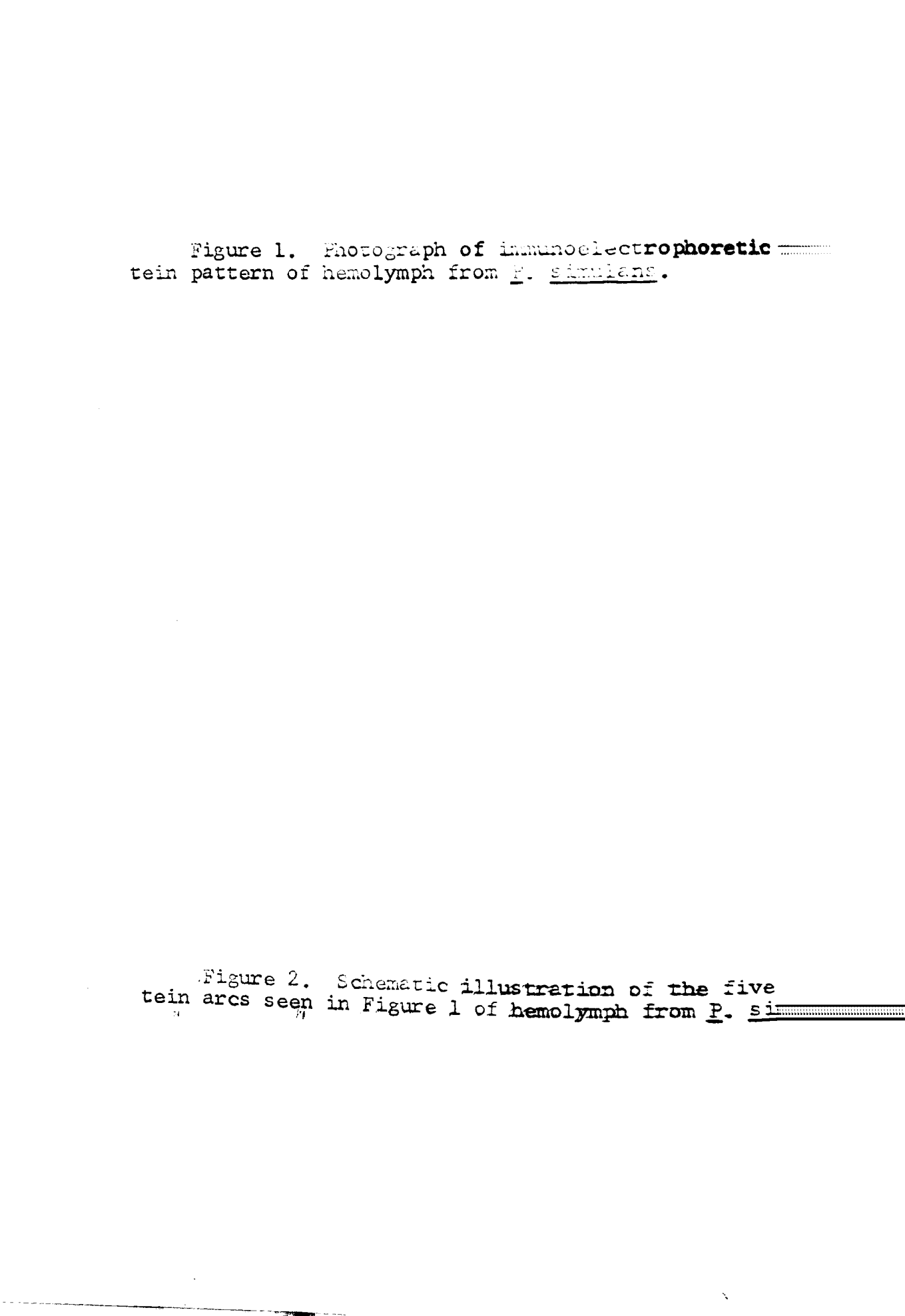
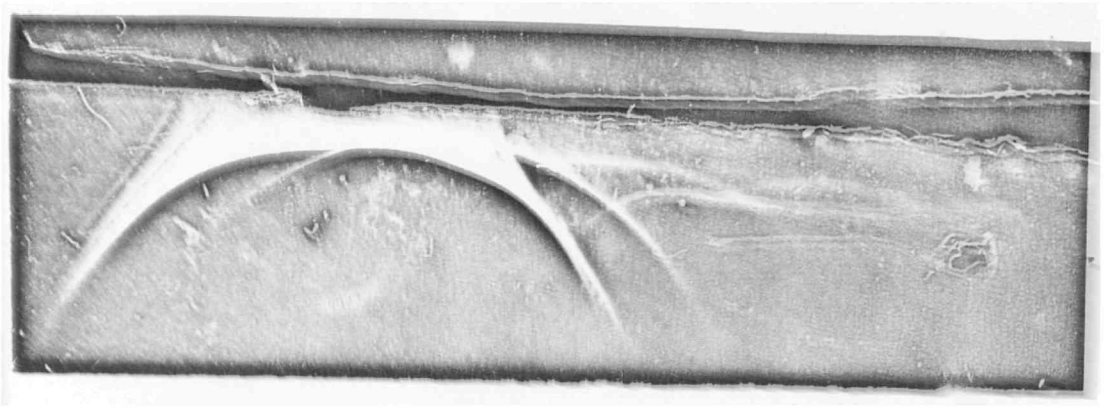


Figure 1. Photograph of immunoelectrophoretic pattern of hemolymph from P. simulans.

Figure 2. Schematic illustration of the five tein arcs seen in Figure 1 of hemolymph from P. si

P. simulans



P. simulans

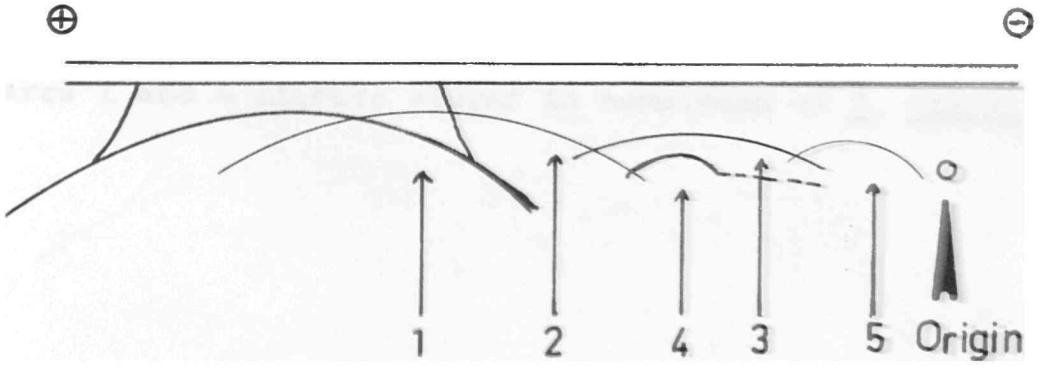
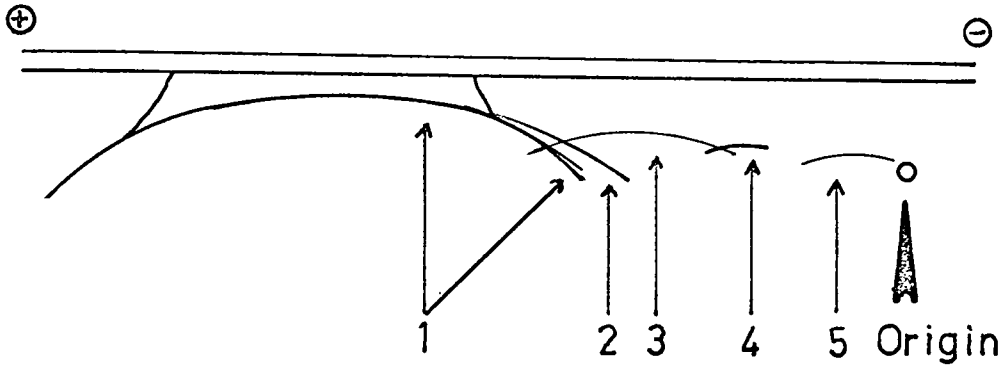


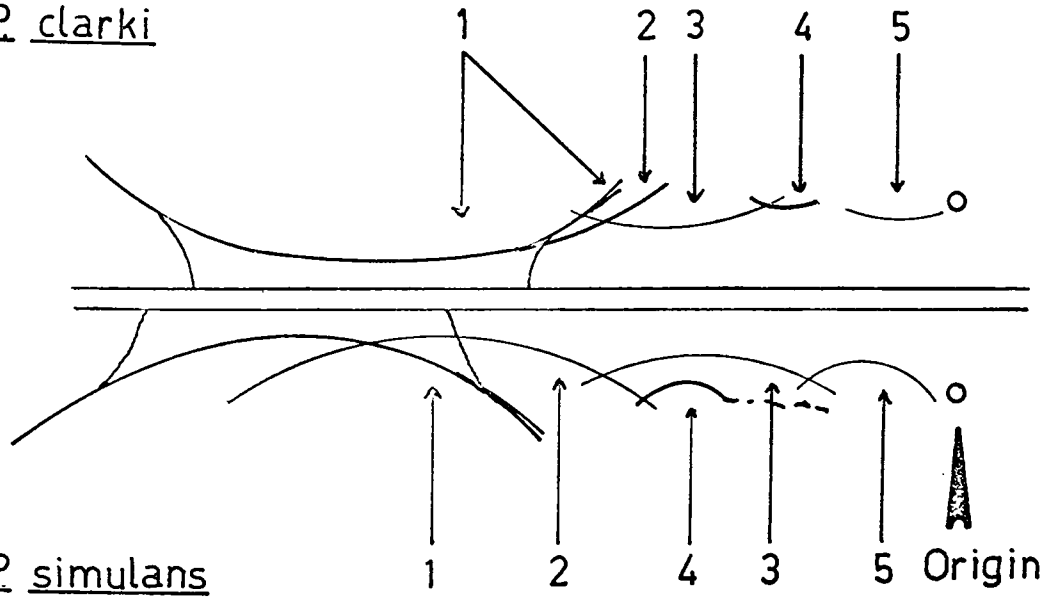
Figure 3. Schematic illustration of the five protein arcs of hemolymph from P. clarki.

Figure 4. Schematic illustration of comparison of hemolymph from P. simulans and from P. clarki. Arcs 2, 3, and 5 are approximately in the same position; however, arcs 1 and 4 migrate slower in hemolymph of P. clarki.

P. clarki

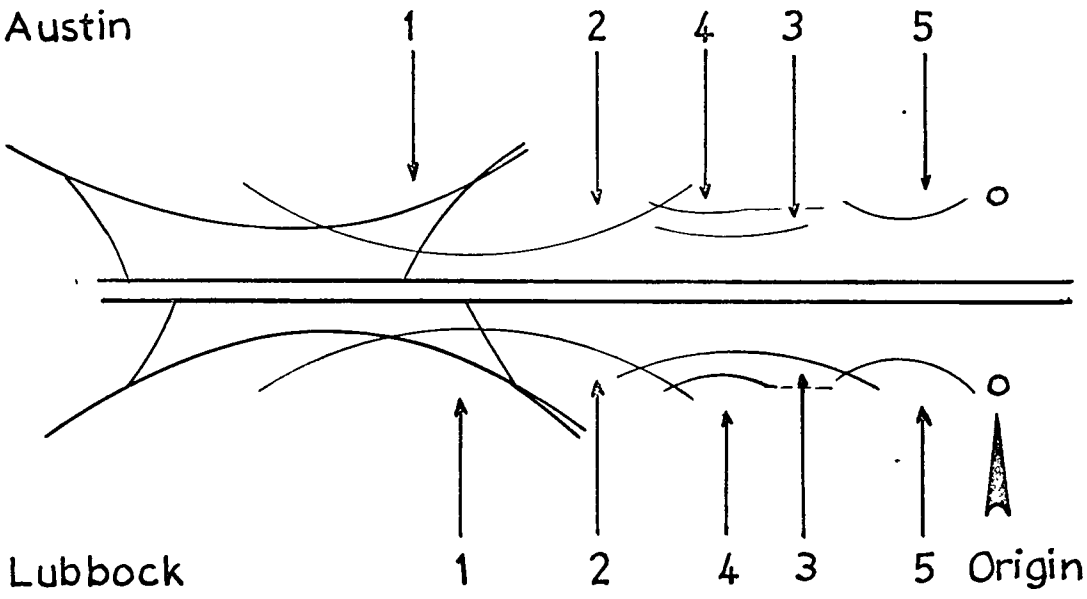


P. clarki



P. simulans

Figure 5. Schematic illustration of co
hemolymph from P. simulans occurring in Lubb
from P. simulans occurring in Austin.


Austin
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Origin