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Serum Proteins as an Indication of Species Specificity in Larval *Curculio*

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SUMMARY

After finding that external morphology alone could not be used to separate larval *Curculio*, research was begun on the possibility of using characters of a biochemical and physiological nature. It has been adequately demonstrated by many investigators that serological reactions are species-specific for many vertebrate and invertebrate animals. Since these serological reactions are dependent on certain proteins found in the blood, it was hypothesized that analysis of the proteins of the blood could also demonstrate these species differences.

Due to the small volume of blood available from an individual insect (.0005-.500 ml), the technique of electrophoresis was used to separate these protein fractions. Samples of blood, 0.001 ml in volume, were obtained from mature *Curculio* larvae by means of a ventral thoracic puncture.

Sixty larvae were tapped and 50 yielded usable patterns. Five protein fractions were found, three migrating to the positive pole (+) and two to the negative pole (–). Seven patterns made up of various combinations of these fractions were found. Approximately 44 percent of the patterns were of the type +2 –1.

The number of patterns corresponds exactly to the number of adult species identified in this study. The preponderance of the +2 –1 pattern compares favorably to the number of adults; over 41 percent identified as *C. pardalis,* or near. The sample size was too small to permit an adequate species identification. Also, no *Curculio* could be identified in the larval stage.

It is concluded that definite evidence exists that *Curculio* blood proteins are species-specific.

Serum Proteins as an Indication of Species Specificity in Larval *Curculio*

Jerome Brezner

(*Insecta-Coleoptera-Curculionidae*)

By Jerome Brezner

Biochemistry is basic to the science of animate objects. An individual organism cannot be defined merely as a simple morphological unit, for this unit appears only as the end product of a vast number of biochemical systems. It follows, then, that if phenotypes may be aggregated into taxonomic units on the basis of morphological characters alone, e.g., size, color, shape, it should be equally justifiable to group these individuals at a level more basic than the morphological shell.

The comparative biochemist attempts to demonstrate that a biochemical individuality exists, and further, that biochemical individuality is a genotypic response and may be reproduced in accordance with the laws of genetics. Thus, just as there exist specific nucleotide units on the chromosome for morphological characters, there are also nucleotide units in the same DNA (desoxyribose nucleic acid) chain which dictate the biochemistry of the resulting organisms.

In examining individual animals for biochemical specificity, a study must be made of the physiological systems. In effect, a number of the components of a particular system must be screened and tested in the same way that external morphological characters must be screened and tested for significance in relation to taxonomy. A phrase which best applies to the application of physiological traits in taxonomy is "physiological taxonomy" and will be referred to by this name throughout this paper.

Before attempting a taxonomic study of any kind, a number of conditions must be fulfilled, whether external morphological characters or physiological morphology is used. First, an attempt must be made to find a trait which is genetically stable from parent to progeny and which resists changes brought to bear by non-genetic influences. Second, enough traits of taxonomic importance

must be present to permit an evaluation of the phylogenetic position of the organism as completely as possible. And last, but probably most important, taxonomically significant characters must be found.

Applications of these three conditions have been found in most cases where external morphological characters have been used. However, our knowledge of the biochemistry and physiology of insects at the present time is far from complete. One can only draw parallels from other groups whose physiology is better understood and postulate on existing physiological conditions in insects. Concerning physiological characters, Mayr, Linsley, and Usinger (1953) stated:

> Physiological characters have been very unevenly exploited for taxonomic purposes. Yet in constancy, diversity, and significance they probably far exceed morphological characters.

In the application of physiology to taxonomy, one point seems obvious; living material is essential. With the death of the organism, the delicate physiological balances no longer exist and comparative studies can no longer be made. When biochemical studies are pursued, the same problem is encountered, as many of the components of the biochemical system undergo a change of state with the death of the organism. This is one drawback to the use of physiological characters at present. However, it seems possible that in the near future biochemical techniques will allow information to be gained from dried museum specimens, just as this information is procured from living systems. For example, there are techniques whereby coagulated proteins may be made soluble once again (Anson and Mirsky, 1931). Thus it is entirely conceivable that even old museum specimens may yield enough data to consider them as biochemical entities rather than empty morphological shells. Even today, to those taxonomists who are currently engaged in research on population genetics and speciation, and those who are utilizing living material, the use of physiological characters should be regarded with a great deal of favor.

Many physiological systems are present in any living organism. The system of taxonomic significance must be selected. In insects, the hemolymph probably is most suitable for qualitative and quantative analysis of component biochemical constituents, due principally to its liquid state.

The next step is to determine the method of study; in this case, an analysis of the differences in the chemistry of the hemolymph. A number of compounds exist in the hemolymph—proteins, amino acids, sugars, lipids, hormones, etc. A selection of the most "stable" of these compounds must be made. In an examination of adult vertebrate serology, one finds that diet may alter the concentrations of amino acids, sugars, lipids and inorganic constituents for a short time, so that the same species feeding on different nutrient material may show different levels of concentration. A study of these components might reflect a difference in diet rather than one of taxonomic significance. The constancy of serum proteins, however, has been established in a number of ways and by different authors. On the subject of protein specificity, Boyden (1943) stated:

1. The antigenic composition of animals is an important part of their essential natures and must be considered in any sound natural system of classification.
2. Protein antigens are conservative hereditary traits.
3. Good precipitin techniques are well adapted to reveal the relative degree of biochemical similarity of protein antigens.

Dr. Boyden's conclusions were based on data obtained from a wide range of animals including Crustacea. Cumley, Irvin, and Cole (1941) reported on the typical inheritance patterns of serum protein differences and similarities in hybrid doves. Using the precipitin technique on the Pearlneck and the Senegal strains of doves, they performed a number of back crosses and calculated the probable genetic responses. They reported:

> The results of this and previous investigations show beyond reasonable doubt that the species-specific qualities of the serum proteins are determined by gene action and suggest that the total protein complex of the serum is likewise determined by genes.

Other investigators have arrived at the same conclusions, which are discussed in the Review of the Literature.

Since the specificity of serum proteins has been demonstrated in other animals, both vertebrate and invertebrate, there is no reason to doubt its existence in insects.

Determinations of protein constituents of the blood of an individual insect have hampered physiologists and biochemists. This has been due principally to the very small volume of hemolymph available from an individual insect. In past years a "pooling" of material was the general rule, to obtain a sample large enough for an analytical run. This procedure is still followed by some workers. The pooling of material for taxonomic purposes is not only undesirable but it defeats the entire purpose of a systematic study. The systematist must be able to examine one individual and compare that individual with another.

Today a separative technique calletd electrophoresis, is being employed in more and more entomological research. Quantities as small as one-half lambda (.0005 ml.) may be analysed for component parts (Stephen and Steinhauer, 1957). The larvae tested in the present research program, *Curculio* (Coleoptera, Curculionidae), yielded up to five lambdas of hemolymph, which permitted the analysis of a single specimen.

REVIEW OF THE LITERATURE

There are a number of excellent texts which may be consulted for details on the physiochemical basis of the electrophoretic technique, among them being Block, Durrum,and Zweig (1958), and Lederer (1955). These texts adequately cover the theoretical and applied aspects of electrophoresis. A brief resume and studies of the electrophoretic separations of compounds in insect hemolymph will be outlined here.

Systematic serology is a relatively new science, the earliest data on this subject having been recorded by Nuttall (1901). In his many treatises he attempted to categorize some members of the animal kingdom by means of the similarity of their serological reactions. He employed the precipitin test of Kraus (1897) which involved the production of an antiserum in a test animal (rabbit). The antiserum was tested against other experimental animals and the degree of precipitation noted. A correlation was found: the greater the reaction of precipitation of antiserum and serum, the closer the systematic relationship of the experimental animals. Nuttall's reactions in his first four papers are interesting. In the first (Nuttall, 1901a) he examined bloods of different animals for serological distinctions. He stated:

> We have in this test the most delicate means hitherto discovered of detecting and differentiating bloods, and consequently we may hope that it will be put to forensic use.

A little later the same year, he tested a number of additional animals and concluded from 140 specimens that:

> It seems certain that interesting results from the point of view of zoological classification will thus be brought to light. (Nuttall, 1901b)

He then obtained a number of monkeys, representing 18 species, and became even more enthusiastic as to the application of serology to systematics:

> The above experiments, which are being prosecuted on a large scale, the attempt being made to obtain a variety of antisera, indicate with certainty that we possess in this test a most valuable aid in the study of classification of animals. (Nuttall, 1901c)

By the following year, he stated:

> I am inclined to believe that with care we shall perhaps be able to measure species by this method . . . (Nuttall, 1902)

He then summarized his findings and attempted a serological classification in a text (Nuttall, 1904).

Soon after the appearance of Nuttall's articles, a new field of study evolved; one concerned with the biochemical evolution of animals. If, as Nuttall concluded, antigenic responses were species-specific, then it follows that there must have been a biochemical evolution paralleling or preceding morphological evolution. Although this area is not the specific subject of this paper, it is deserving of some attention, brief as it may be. If a biochemical character is employed for systematic appraisal it is necessary to demonstrate that this character follows the laws of genetic inheritance. And if it can be shown that evolution involved a number of specific individual biochemical changes, the idea of species specificity of a biochemical nature may be adequately justified. Haldane (1937) stated: "Our final theory of evolution will see it largely as a biochemical process."

Another biochemical evolutionist, Needham (1929, 1930, 1934, 1936, 1938), published a great deal on this subject. His latest text (1942) reviewed the field of morphogenesis and biochemistry, while his shorter articles dealt with specific

subjects on the associations of the various evolution theories and biochemical parallels.

Other investigators, Florkin (1949) and Florkin and Duchateau (1943) demonstrated the overwhelming evidence in favor of biochemical evolution and subsequent specificity. A review of these articles is excellent for a first hand account of the field of biochemical evolution.

Baldwin (1937) summed up the problem of classical morphology and biochemistry in a short paragraph: viz.,

> In the past, as is undeniable, there has been between classical morphology and biochemistry a great gulf fixed, the precise meaning of which has evaded clear description and definition. Too exclusive an attention to morphological form, as has been said, leads to a tendency to people the empty spaces with ghosts. Reluctance to see the gap bridged, indeed, must often imply a psychological preference for mysticism. But acceptance of continuity involved the interpretation of morphological form as the outward and visible signs of molecular and submicroscopic configuration and opens up a host of problems to experimental investigation.

Although this "gulf" is partially bridged today, a great deal more "building" is needed. Much more could be said concerning the part played by biochemistry in speciation. However, the aforementioned authors have reviewed this field adequately.

The field of systematic serology may be divided into two distinct categories, the first being a systematic appraisal on the basis of precipitin reactions. The second category is protein analysis, *in vitro,* of the blood. Since precipitin reactions were the earliest to be employed in systematic appraisal, an inquiry into this field first would be in order.

Following Nuttall's preliminary investigations, a number of others began to examine serological affinites among animals more critically. Among them Boyden (1926) studied the relationships of a number of common animals and indicated that the precipitin reactions paralleled that of the systematic position of these forms. Additional work led him (Boyden, 1934) to examine the relationship of the serology of animals and their phylogeny. Finally, he reviewed completely the field of systematic serology (Boyden, 1942). A critique of the method was given and salient principles and procedures outlined. He called attention to the morphologist and solicited his active support, for the "true" taxonomist still had little regard for the field of biochemistry. Boyden (1951) also outlined the major advances in the field some nine years later.

Landsteiner is noted for the same interest as Boyden. His revised text (1945) was, and still is, the principal text in serological identity.

Leone, one of Boyden's former students, is actively engaged in serological work at the University of Kansas. He has published a number of articles on serological relationships, principally in the Class Crustacea. From the beginning of serological work in 1901 down to 1941, the evidence that proteins were hereditary traits was based on indirect evidence. This is not to imply that the

evidence was insufficient. On the contrary, the evidence was wholly in favor of genome response. However, Cumley, Irvin, and Cole (1941) attacked the problem from the genetic point of view. Their findings emphasize the fact that proteins are "conservative hereditary traits."

Insect serology is almost as old as serology of other animals, although the impetus has not been great for these small animals. Erhardt (1929) reviewed the work in insect serology, done principally in Europe. The first recorded work in the United States was performed by Brown and Heffron (1928). Antiserum was obtained from guinea pigs after injection of an extract made from a whole male specimen of the butterfly, *Eurymus philodice.* The antiserum was tested against *Papilio troilus, Pieris rapae, Eurymus philodice, E. eurytheme,* and *Argynnis cybele.* Both "papered specimens," 1½ years old, and fresh specimens were tested. Their conclusions were:

> There was a distinct intersubfamily reaction when dried insects were used, and
>
> There was a distinct inter-generic reaction when freshly killed insects were used, and
>
> There was no interspecific reaction when dried, closely allied species of insects were used, as *E. philodice* and *E. eurytheme.*

Twenty species, representing 14 genera in six subfamilies in the Phalaenidae (= Noctuidae) and representatives of Sphingidae and Nymphalidae were examined serologically for phylogenetic relations by Martin and Cotner (1934). Only the dried thorax was used to make the extract and rabbits were used as the test animals. Quoting from their paper:

> It is evident from the reactions of certain proteins from the above mentioned species that the precipitin reaction is useful in determining phylogenetic relationships between genera and sub-families of the family Phalaenidae.

Cumley (1940) compared the serological ranking against the ranking of 14 morphological characters of *Drosophila.* He found serological analysis to be more effective in the third and fourth ranking units. Leone (1947) tested the serological reactions of 11 members of the order Orthoptera for systematic relationships. He found that:

> In general the relative intensities of these precipitin reactions are parallel with the systematic position of the species compared.
>
> Leone also pointed out:
>
> Admittedly, comparisons using insect sera, or extracts of homologous structures or organs in the insect body would theoretically present a more critical basis for comparison. However, the use of sera, or extracts of homologous organs are not always practical when small organisms are being considered.

There are methods available today, however, which may be employed for individual biochemical analysis. This introduces the second category, i.e., *in vitro* analysis of the protein constituents of insects. But before the development of serum proteins *per se,* in taxonomic studies, is traced, it would be advantageous to examine the field of paper chromatography in taxonomic studies.

Paper chromatography may be successfully employed in the separation of the various amino acids and has been used, in the light of biochemical individuality, to distinguish taxa. Among the many investigators in this area of research only a few will be mentioned. The importance of this technique is not discounted, but since no information is presently available on the correlation of amino acid intake and subsequent hemolymph concentration, the question of amino acid specificity in actively feeding insects is open to debate. Micks and Ellis (1951, 1952), Micks and Benedict (1953), Micks (1954, 1956), Micks and Gibson (1957), and Lewallen (1957) have all employed amino acid analysis as a tool in the separation of mosquitoes and, in one case, ticks (Micks and Gibson, 1957). In all cases chromatographic analyses of the free amino acids were employed. Newly emerged and starved adults were treated in such a manner as to precipitate the proteins. A number of adult forms were crushed directly on the filter paper and a one or two dimentional chromatogram was developed. It is significant here to point out that only non-fed individuals were used, thus minimizing a nutritional variable.

Buzzati-Traverso and Reichnitzer (1953), analyzing for amino acids in fish muscle tissue, pointed out that amino acid analysis would become a useful technique in taxonomic and population-genetics studies.

Buzzati-Traverso (1953), working with *Drosophila melanogaster*, analyzed free amino acids from adults crushed directly on filter paper. He demonstrated that paper chromatography could differentiate two different genotypic strains with an identical phenotype. He further concluded that the individual's biochemistry was constant, that biochemistry would demonstrate differences with different genotypes, and that biochemical analysis could uncover genotypic differences masked by dominance.

Lewallen (1958), altering the standard free amino acid analysis technique, analyzed the amino acids of the proteins in whole crushed *Musca domestica* by acid hydrolysis. Although not relating specifically to taxonomic inquiry, the author did go one step further than analysis for free amino acids. As this author showed, it was possible by a number of indirect methods to analyze for the presence of simple peptides.

It is also possible to analyze for proteins directly by means of paper chromatography. However, the paper chromatography of proteins has not been reduced to a routine procedure due to the fact that, "every protein system studied may require a new system of chromatographic development;" also, "electrophoretic separation of proteins has been so successful" (Block, Durrum, and Zweig, 1958). The use of electrophoresis as a tool in studying animal protein systems is relatively new. Jameson and Alvarea-Tostato (1939) tested the changes in rat serum proteins after colostrum injection in an effort to find correlations in changes of proteinaceous anti-bodies. Applying electrophoretic patterns of serum proteins to taxonomic endeavors is also quite recent. Deutsch and Goodloe

(1945), working with a Tiselius cell, examined the protein patterns of a number of vertebrates. They stated:

> The electrophoretic patterns of plasma from 20 species of animals obtained under identical conditions reveal distinct species variation in mobility, amount, and number of protein components.

Moore (1945) applied the same separative techniques to a number of mammals, some hens, and a fish (carp). He examined the differences with emphasis on strain and sex differentiation. He pointed out that:

> . . . It is evident that many species under the conditions studied here present serum protein patterns which are so characteristic that it is possible to name the species and in some cases even the strain (rat) and sex (chicken) from a glance at the pattern.

Many other investigators have used electrophoresis to separate serum proteins of various animals, and each has added comments on the specificity of the electrophoretic patterns obtained. Among them may be mentioned Common, McKinley, and Maw (1953) who worked with bird serum; Gleason and Friedberg (1953) who demonstrated the specificity of serum proteins obtained from rats, frogs, opossums, turtles, *Necturus,* and a salamander; Berg and Scheiffarth (1954) who worked with various animals; and Morris and Courtice (1955) who investigated lipoproteins from various animals.

Electrophoresis of the blood obtained from insects, however, has not been studied to any great extent.Due to the scarcity of literature dealing with protein separations, it is rather difficult to determine when the first electrophoretic separation of insect blood was made. One of the earliest, if not the earliest, investigations was made by Bowen and Kilby (1953). By electrophoretic mobility patterns, they showed the changes in locust blood protein concentration caused by clotting of the hemolymph.

Wunderly and Gloor (1953), employing the Tiselius apparatus, examined the proteins and lipids from whole crushed larvae of *Drosophila melanogaster* and *D. hydei.* They demonstrated that in the lethal genotypes a distinct protein fraction difference existed and that the lethal mutant could be demonstrated in the larval form by blood protein patterns. These authors came to the same conclusions as Buzzati-Traverso (1953), who worked with amino acids. Blood proteins were thus apparently genetically linked characteristics.

Drilhon (1954) demonstrated that the different stages of silkworms exhibited different protein patterns. She used the Elphor II paper electrophoresis apparatus, a veronal buffer and Whatman #1 paper. Proteins were detected by both ultra-violet flourescence and by amido black stain.

Clark and Ball (1956) demonstrated a number of protein fractions in *Drosophila, Periplaneta,* and *Blattela* using the Antweiler micro-electrophoresis apparatus for determination of the proteins present. Stephen and Steinhauer (1957) examined the blood of five species of insects and compared the differences in protein patterns in connection with various developmental stages of the insects. As

Drilhon (1954) previously demonstrated for the silkworm, these authors found marked differences in different developmental stages and in the sexes. Quoting two conclusions from their paper:

> 1. Species specificity of proteins in insect blood is indicated. The specificity is of a qualitative nature and may be represented by from two to five fractions, depending upon the species studied.
>
> . . . 5. The size of the blood sample required for analysis, which ranges from .0005 to 0.01 ml., permits a broader application of this method to an analysis of individual and population variation.

Micks (1953) employed infra-red spectrum analysis for determination of species of mosquitoes. This is a new approach, and although nothing has appeared in the literature since, it appears worthy of further consideration.

METHODS AND MATERIALS

Preparation of the Sample

Acorns were collected from various sections of the state and returned to Columbia for rearing larvae. (Rearing procedures are outlined in Missouri Experiment Station Research Bulletin 747.) Mature larvae, naturally emerged from their acorn hosts, were used for blood analysis. Each larva was held manually while the point of a sharp, hooked scalpel blade pierced the ventral thoracic integument. Great care had to be exercised during the tapping operation, for if too deep an incision was made the fat body and part of the proventriculus would prevent the accumulation of a clear sample of hemolymph. The hemolymph was then drawn into a capillary tube, which had been sterilized in alcohol and flamed, and expelled into the polished bottom of a depression slide. A calibrated ultramicro pipette was filled from the slide to the one lambda mark and applied as a spot to the paper strip. Following the blood tap, the larvae were preserved in KAA for subsequent dissection and embedding.

All dissections were accomplished with the aid of a binocular dissecting microscope at a magnification of 20 diameters. The head was severed from the body and turned ventral side up. The labium and associated maxillae were removed and placed in 10 percent potassium hydroxide. The adductor mandibular muscles were severed and the mandibles spread so that the labrum could be seen clearly. The head was also placed in the macerating solution overnight. After maceration was completed, the parts were embedded on microslides in Hoyer's medium. Before the coverslip was affixed to the labial preparation, the left maxilla was separated and turned dorsal side upward so that the setation on the dorsal face of the mala might be viewed.

Due to the thickness of the head capsule, a mounting cell had to be made and was constructed of ordinary modelling clay. (See Figure 1.) The clay was

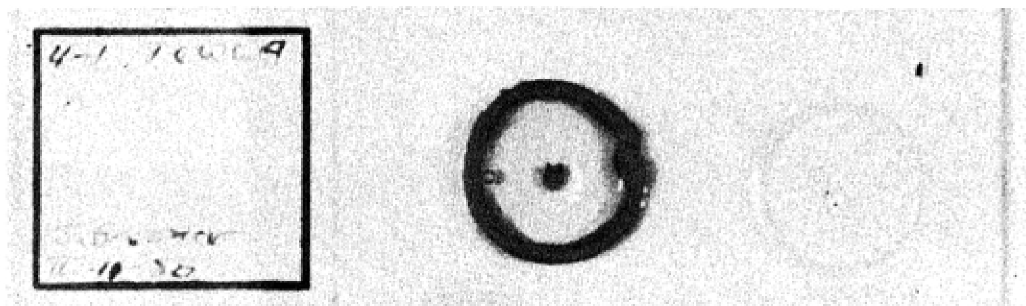

Fig. 1—A slide mount of the head capsule (left) and associated mouth parts (right) of a *Curculio* larva. The ring surrounding the head capsule is modeling clay.

rolled into long strips which were circled on the slide to a diameter of about 10 millimeters. The slide was filled with Hoyer's, the head capsule inserted, and the coverslip affixed.

The slide material was examined with a compound microscope at magnifications of both 100 and 440 diameters. A special table was constructed and the images of the larvae projected directly on tracing paper. This method was followed for all larval drawings to assure accuracy in retaining the proportionality of component parts. The drawings were examined for salient morphological structures; findings were entered on punch cards. These cards were coded for serological and morphological correlations.

Electrophoresis Preparations

A horizontal electrophoresis cell was used in these experiments, following the technique of Weiland and Fischer (1948). The apparatus was of commercial manufacture, made by the Ivan Sorval Company, of Stockholm, Sweden. The buffer compartments of the apparatus were filled with Veronal (sodium barbital-acetate solution) buffer having a pH of 8.6 and ionic strength of 0.05. Filter paper strips, type S&S 2043A, 12 inches long and 1 inch wide, were marked at the center and placed between the buffer compartments. Six such strips were used for each run. The papers were then wetted with an atomizer filled with buffer solution. The area containing the strips was sealed under a heavy glass plate and an electrical potential was applied. This blank run permitted the apparatus to arrive at a stable operating temperature and aided in the saturation of the area between the glass plate and the paper (Fig. 2).

After the initial warm-up run, a sample of hemolymph was applied to the center spot. Six individual larvae were run at one time. Each run lasted nine hours at a current of seven milliamperes. No potentials were recorded with this apparatus as there was no power supply voltmeter. At the conclusion of a run, the papers were dried in a hot air oven at from 130° to 140° C. for 30 minutes. The strips were then stained in bromphenol blue for six hours. After two rinses

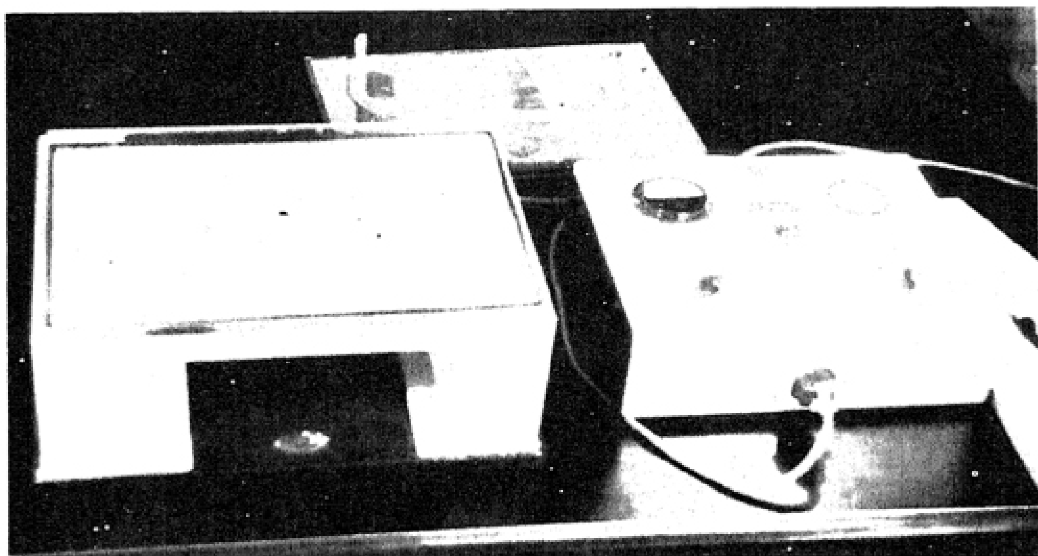

Fig. 2—An L. K. B. horizontal electrophoresis unit, manufactured by the Ivan Sorvall Company, Stockholm. Right, power supply. Left, cell for the paper strips.

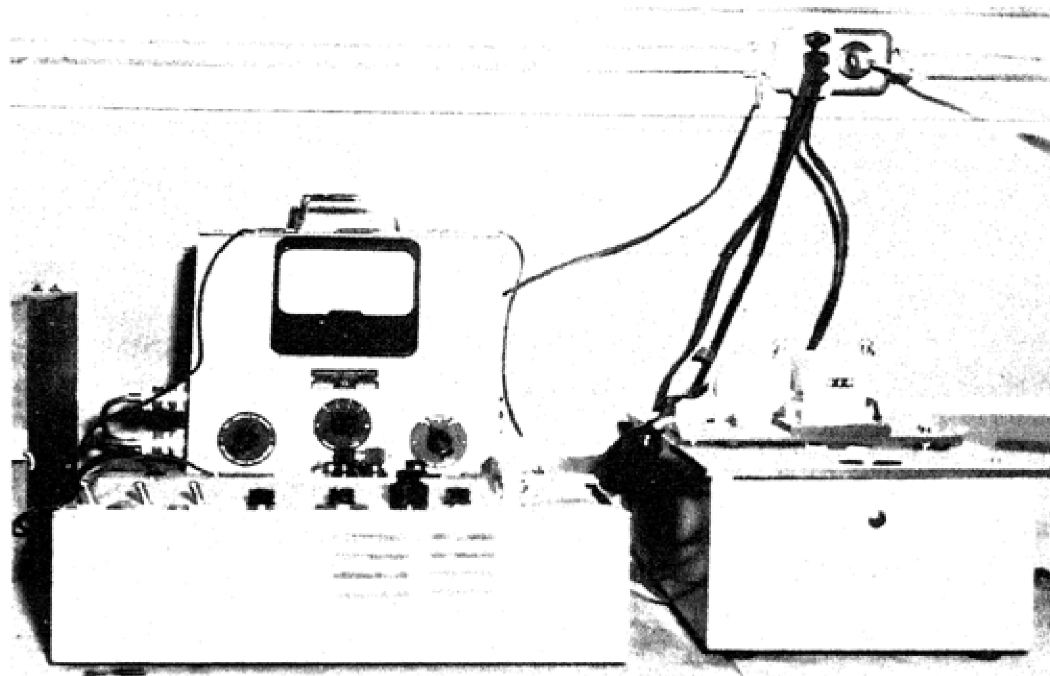

Fig. 3—A densitometer for evaluation of the stained protein spots on filter paper following electrophoretic separation. This unit is manufactured by Photovolt Corp., New York City.

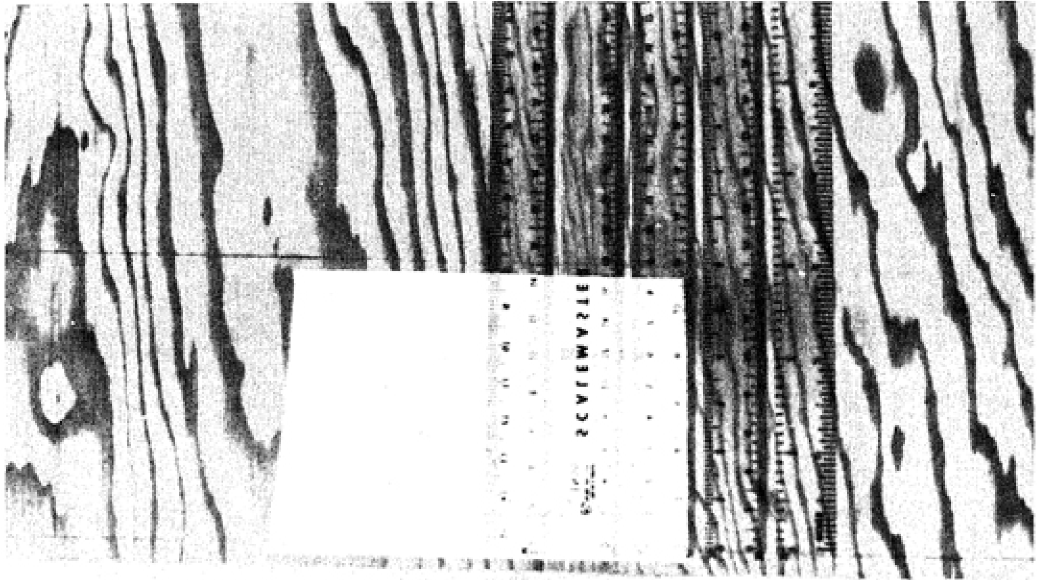

Fig. 4—Measuring device used for evaluating the graphs obtained from densitometric graphs of electrophoresis strips. The transparent millimeter rule may be seen at the center over a graph.

in 5 percent acetic acid and a fixing bath, a mixture of 5 percent acetic acid and 3 percent sodium acetate (50-50 v/v), each for six minutes, the papers were air dried at room temperature for 24 hours. Scanning of the strips was accomplished with a Photovolt transmission densitometer (Fig. 3). A graphic record was made of the number of spots and the densities of each. The distance separating peaks of the graph and the heights of the peaks were recorded in millimeters (Fig. 4). Length-frequency distributions of the resulting spots were plotted for the various protein fractions.

All information obtained from the serological analysis was entered on punch cards and correlations of the morphological data and serological data were begun.

RESULTS AND DISCUSSION

Before proceeding with the results of the systematic phase, a discussion of some of the information obtained from the electrophoresis trials may be of value.

The protein patterns obtained by the methods previously described were quite satisfactory although a number of modifications markedly improved strip patterns. Although the horizontal strip electrophoresis apparatus gave acceptable results, a newly acquired Durrum type cell (Fig. 5) seemed far superior. In preliminary tests with blood of a number of insects, patterns appeared to be a good deal sharper. There was also much less risk of water condensation on paper strips during the run and the migration rate of the fractions was much more rapid.

A different filter paper, Whatman #54, proved far superior to S&S 2043A. This finding is in accord with findings of other investigators (Block, Durrum, and Zweig, 1958; Lederer, 1955). The barbital buffer remained essentially unchanged, except that for a more stable pH range and economy, a home-made buffer was used. This home made buffer contained reagent grade sodium barbital buffered with acetic acid to the desired pH. It was noted that with the commercially-prepared buffers, a salting out occurred on the anode (positive electrode) which could be removed easily by reversing the polarity of the electrodes

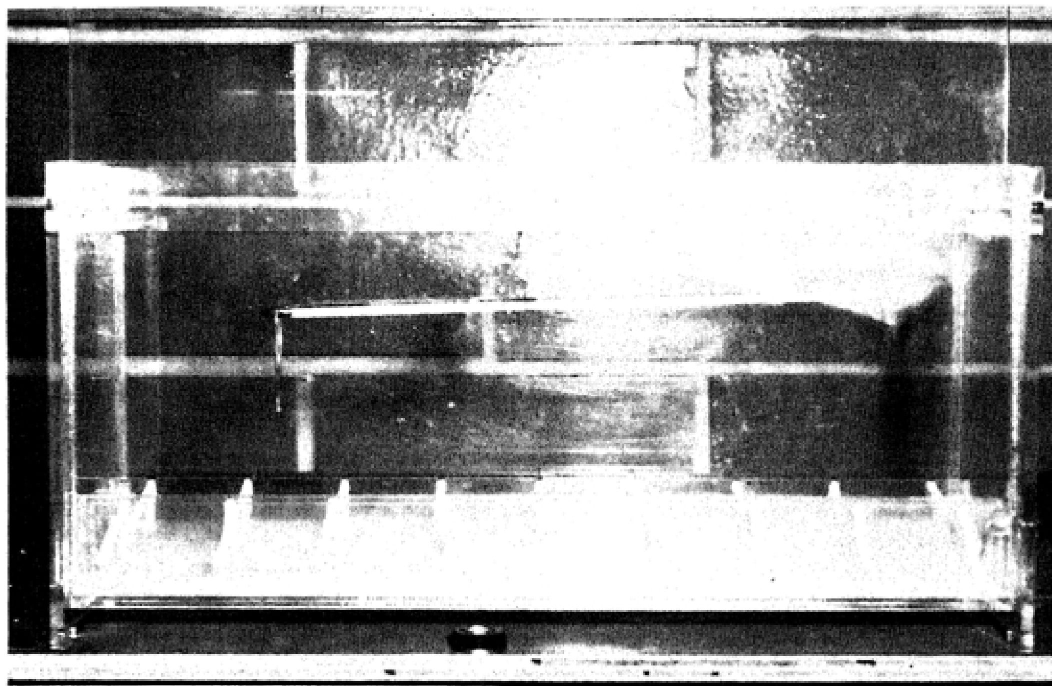

Fig. 5—A hanging strip, Durrum type electrophoresis cell, manufactured by the Shandon Scientific Co., London.

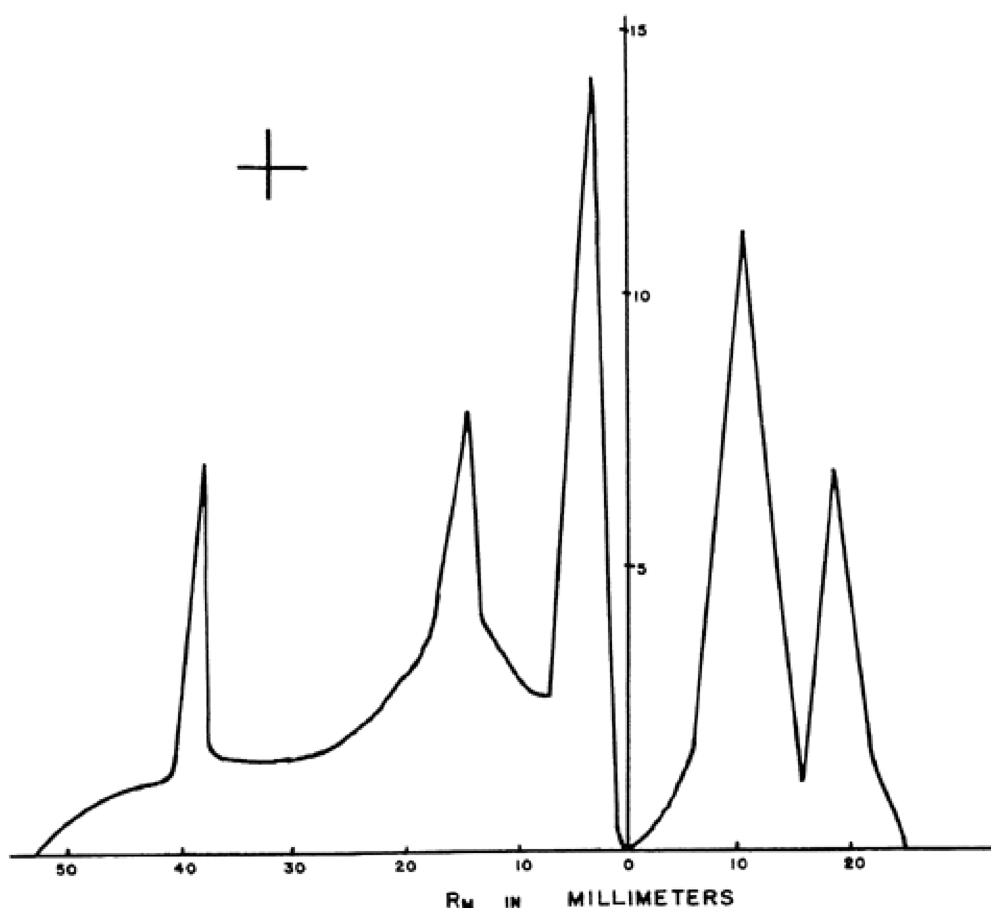

Fig. 6—Frequency distribution of the hemolymph proteins of 54 *Curculio* larvae exhibiting the relative migration (Rm) in millimeters from the origin (0) to the positive (+) and negative (-) fields. Five protein fractions are present.

for a few minutes. The home-made buffer, however, did not precipitate, and could be used indefinitely if the pH was checked and adjusted after each run. This procedure required the emptying of both buffer compartments after each run and thoroughly mixing the used barbital.

Sixty larvae were analyzed for blood proteins. Of these, 50 larvae yielded usable patterns. A number of factors eliminated the 10 from the sample, including the active condensation of water on the strip during the run, which resulted in a streaked protein pattern. One strip got too close to the heating element and was charred; another strip dropped to the floor.

A frequency distribution of the resulting spots clearly indicated five distinct fractions, three migrating anodically (positively) and two cathodically (negatively) (Fig. 6). The strips were examined qualitatively and individual patterns were found, made up of various combinations of the five-spot total.

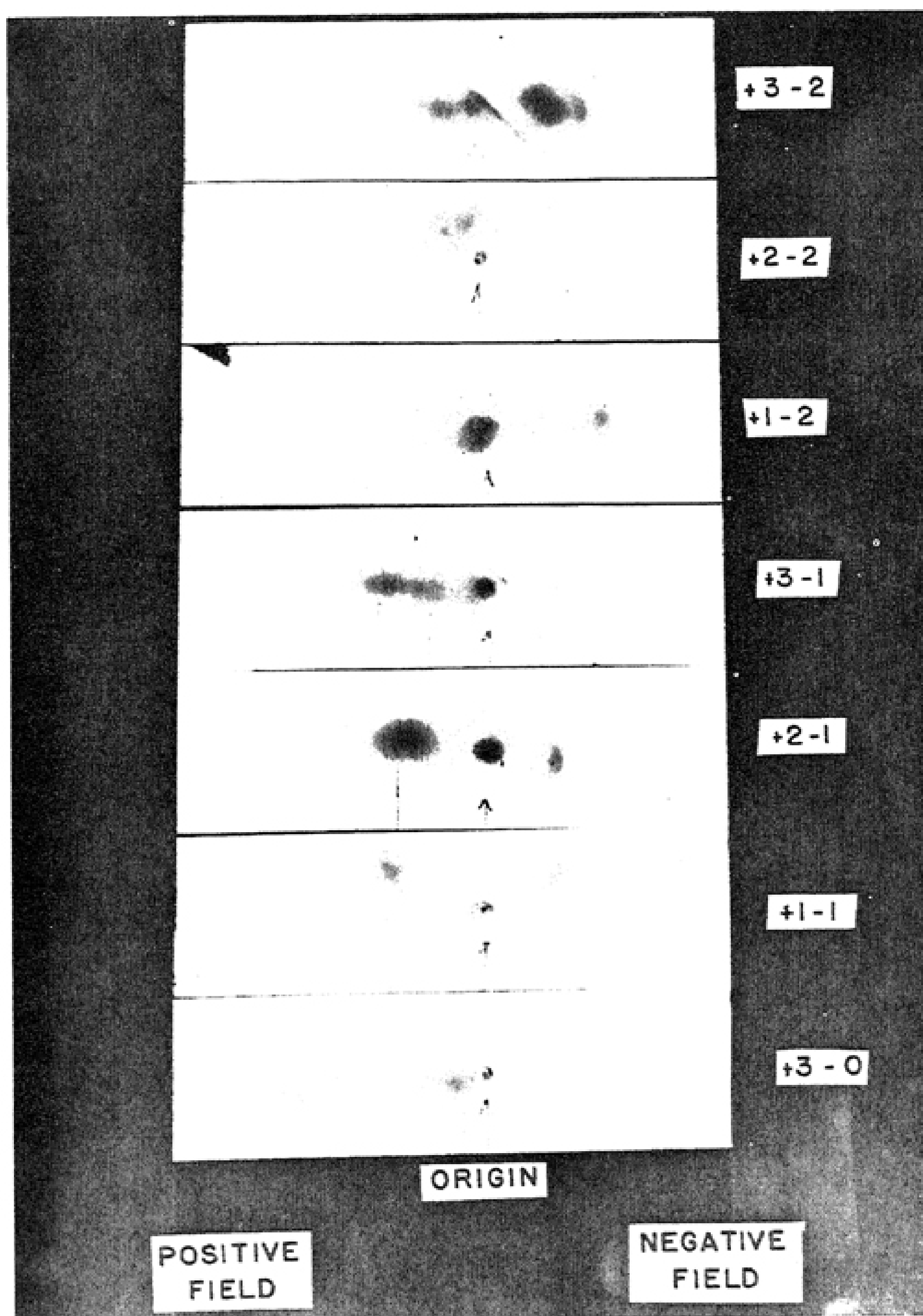

Fig. 7—Stained electrophoresis strips of the hemolymph proteins of *Curculio* larvae showing seven patterns as indicated at the right. The origins are indicated by the arrows.

They were grouped as follows (Fig. 7):

3 positive spots and 2 negative spots (+3-2)
2 positive spots and 2 negative spots (+2-2)
1 positive spot and 2 negative spots (+1-2)
3 positive spots and 1 negative spot (+3-1)
2 positive spots and 1 negative spot (+2-1)
1 positive spot and 1 negative spot (+1-1)
3 positive spots and 0 negative spots (+3-0)

This fact is evident: seven patterns were obtained from randomly selected larvae. This number compares exactly with the number of species of adults obtained in the rearing operation. Table 1 shows the marked preponderance of the +2-1 pattern, 44 percent of the samples exhibiting this configuration. Of the

TABLE 1--FREQUENCY DISTRIBUTION OF MATURE LARVAL CURCULIO SERUM PROTEIN PATTERNS, FOLLOWING ELECTROPHORESTIC SEPARATIONS

Pattern Number	Number of Spots	Number of Individuals	Percent
1	+3-2	2	4
2	+2-2	8	16
3	+1-2	2	4
4	+3-1	7	14
5	+2-1	22	44
6	+1-1	8	16
7	+3-0	1	2
Total		50	

280 adults reared and identified, 41 percent were members of the *C. pardalis* complex. It seems probable that with the proportions so close, this pattern represents the complex.

This is certainly an excellent indication that the morphology of larval *Curculio* is quite variable. The characters which have been chosen are relative rather than absolute, with size, shape, and angles being the only differences found in the frontal arms and the epipharyngeal rods, and the number of setae on the mala. With the low sample size, a significant coefficient of correlation between morphological and serological characters was difficult to obtain, although indications of this correlation were evident.

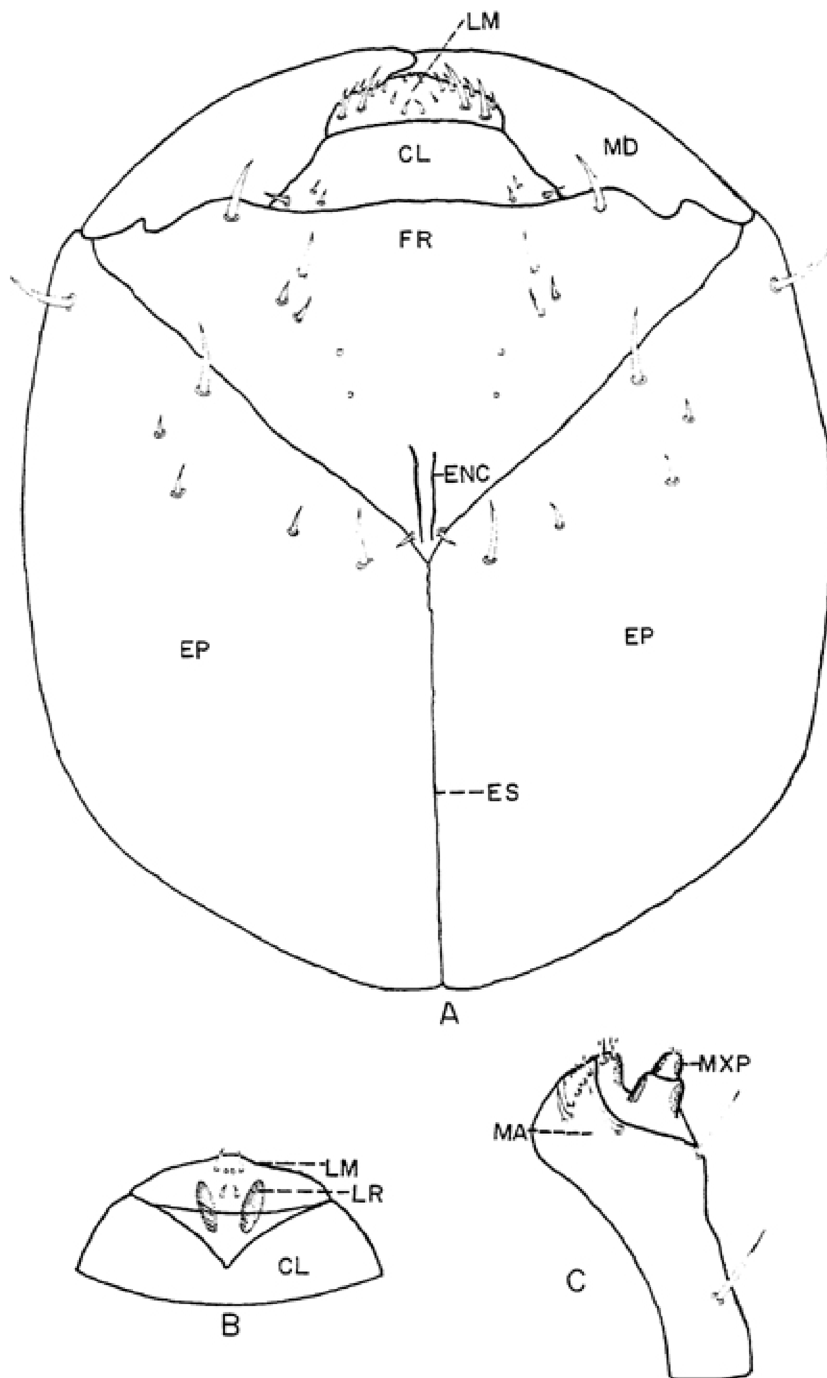

Fig. 8—(A) Diagram of the head capsule of a *Curculio* larva, dorsal view. (B) Labrum and clypeus, ventral view. (C) Dorsal aspect of the right maxilla. CL, Clypeus; ENC, endocarina; EP, epicranium; ES, epicranial suture; FR, front; LM, labrum; LR labral rods; MA, maxillary mala: MD, mandible; MXP, maxillary palpus.

The frontal arms meet in an angle at the junction of the epicranial suture. These were divided into categories as follows (Fig. 8):

1. angle acute, sharp.
2. angle acute, round.
3. angle obtuse, round.

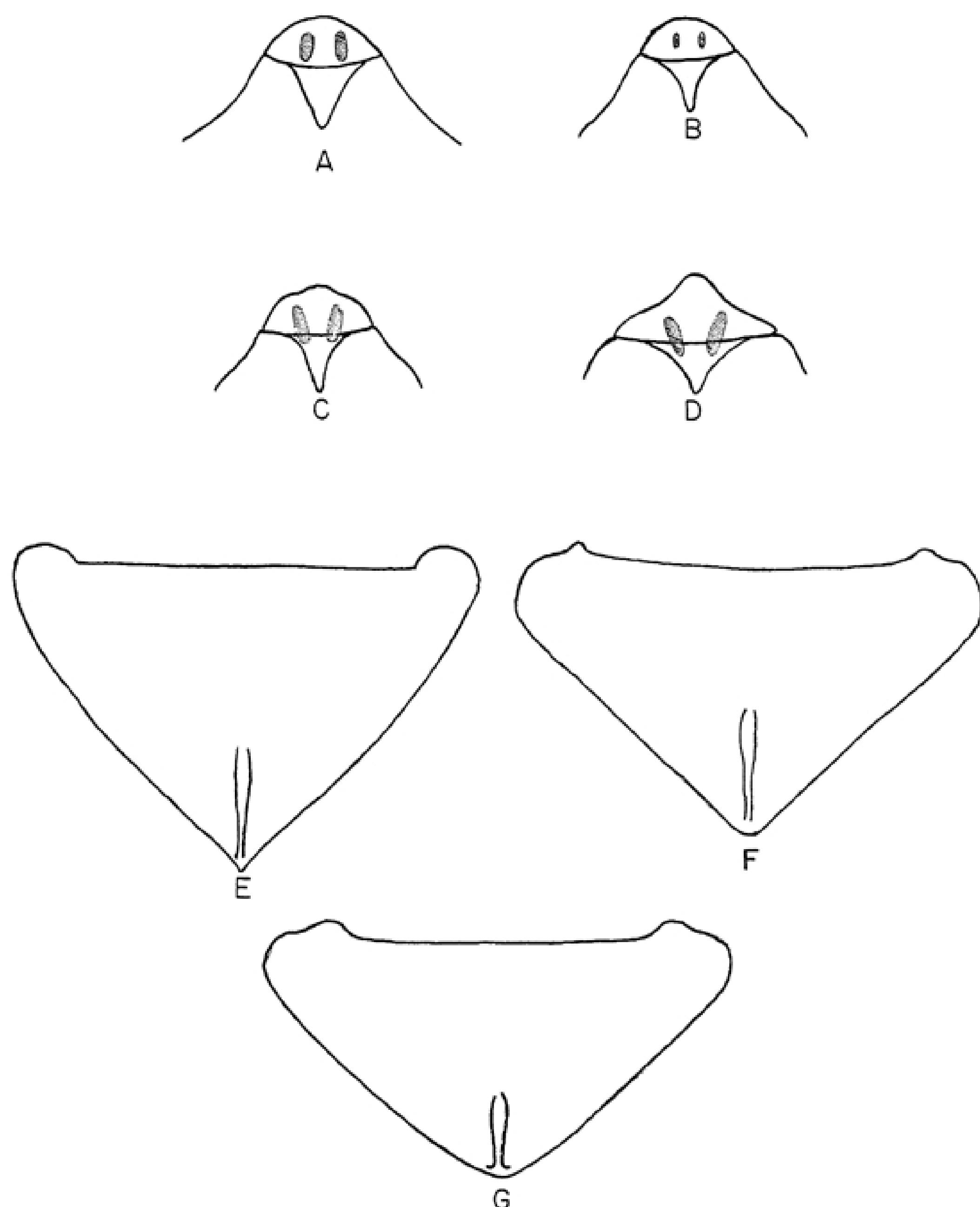

Fig. 9—A through D, ventral view of the labrum of *Curculio* exhibiting the labral rods. (A) rods short and parallel; (B) rods short and convergent; (C) rods long and parallel; (D) rods long and convergent. E through G, dorsal aspect of the front to emphasize the frontal triangle. (E) Angle acute and sharp; (F) angle acute and rounded; (G) angle obtuse.

The labral rods were either parallel or met at an angle, were thick or thin, and long or short (Fig. 9). The ratio of width to length of the thick rods was over 0.6, that of the thin rods was under 0.5. Long rods reached the middle

margin of the clypeus; short rods reached no farther than the anterior margin of the clypeus. Malar setae varied from four to nine on the dorsal face, but proved quite variable, even on the same individual.

The best that might be accomplished on the basis of the information at hand is an examination of the comparisons of serological data with morphological units.

Serum Pattern #1 (+3-2)

Two individuals are represented in this pattern with a strong single group indication. Both individuals had acute frontal angles and possessed long diverging labral rods. The number of setae on the dorsal side of the mala was five in one case and six in the other.

Serum Pattern #2 (+2-2)

Eight individuals are represented in this category. Two groups appear from the data, separation based on the acute frontal angle associated with long parallel labral rods and an obtuse frontal angle with diverging short labral rods.

Serum Pattern #3 (+1-2)

Two forms show totally different characteristics so that no conclusion could be reached.

Serum Pattern #4 (+3-1)

All seven individuals possessed acute frontal angles, but three had long parallel labral rods and three had long diverging rods. One individual had short diverging rods. Two strong groups, on the basis of the labral rods, can be differentiated from this category. One form appears abberant as it is distinct from all others in this group.

Serum Pattern #5 (+2-1)

This group constituted the pattern best represented in this study, 22 forms. The grouping of this pattern is as yet indistinct, with only slight indication of a double grouping system. From the diversity of forms, it appears that, serologically, this represents a complex which may be fairly close to the *C. pardalis* complex.

Serum Pattern #6 (+1-1)

Two strong categories were indicated by these eight individuals; good association was noted between acute angle of the frontal and parallel rods, and obtuse and diverging rods.

Serum Pattern #7 (+3-0)

This is the only specimen which was totally different from the rest, the head area being ¾ the size of the other individuals. Morphologically, it was distinct and serologically it followed the same pattern.

The exact position of the serological patterns in relation to the morphology of these larvae is still somewhat obscure. At present it is not entirely possible to separate these forms from the data at hand. The reasons are obvious. First, the sample size is much too small to cope with the great variability of morphological characters found in this study. Second, the application of physiological taxonomy is a new tool; consequently, adequate appraisal may be obtained only from known, morphologically distinct, larvae.

The one great difficulty encountered in this study was the fact that an attempt was made to determine species by blood protein analysis of unknown insects. Since controlled oviposition could not be obtained, the larvae did not represent known species. Could they have been reared to adulthood and the adults identified, definite correlations of species and blood patterns might have been found. Even today, these larvae cannot be identified by species. It is axiomatic that specific distinctions are not necessarily represented by morphological characters, as evidenced by our knowledge of sibling species. Even as Cumley (1940) found for *Drosophila,* and as Buzzati-Traverso (1953) for the same genus, genotypic differences may not be indicated by varying phenotypes. Thus it is possible that the variable morphology of the *Curculio* larvae may be of little value in taxonomic distinction, whereas biochemical differences may offer the first step in breaking the species complex.

The biochemical phase of this investigation has proven to be of great potential value to taxonomy. As discussed earlier, many larvae were examined morphologically with no distinct differences noted. Yet only 50 larvae, by blood protein determination, were required to give the best separation when compared with identified adults. Two things, requiring many years of additional work, have yet to be accomplished to complete the project. First, a much larger sample size must be obtained, and second, but most important, blood taps must be small enough to permit the larva to remain alive and complete its life cycle. Thus the final accumulation of data will include blood protein patterns, larval head capsules, and their corresponding adult forms.

BIBLIOGRAPHY

Anson, M. L. and A. E. Mirsky. 1931. The reversibility of protein coagulation. Jour. Phys. Chem., 35:185-193.

Babers, F. H. 1938. An analysis of the blood of the sixth instar southern armyworm *(Prodenia eridania)*. Jour. Agr. Research, 57:697-706.

Baldwin, E. 1937. Rigidification in phylogeny, *in:* Perspectives of Biochemistry. J. Needham and D. E. Green, Ed., Cambridge Univ. Press, London, Pp. 99-107.

Barratt, J. O. W. and G. Arnold. 1911. A study of the blood of certain Coleoptera: *Dytiscus marginalis* and *Hydrophilus piceus.* Quart. Jour. Mic. Sci., 56:149—165.

Berg, G. and F. Scheiffarth. 1954. Zur frage der lagebeziehung verschiedener protein-fraktionen auf grund von beweglichkeitsmessungen mit der papierelektrophorese. Klin. Wochschr. 32:472-474.

Bishop, G. H., A. P. Briggs, and E. Ronzoni. 1925. Body fluids of the honey bee larva. II. Chemical constituents of the blood and their osmotic effects. Jour. Biol. Chem., 66:77-88.

Block, R. J., E. L. Durrum, and G. Zweig. 1958. A manual of paper chromatography and electrophoresis. Academic Press, Inc., New York, pp. 710.

Bowen, T. J. and B. A. Kilby. 1953. Electrophoresis of locust hemolymph. Arch. Internat. de Physiol., 61:413-416.

Boyden, A. A. 1926. The precipitin reaction in the study of animal relationships. Biol. Bull., 50:73-107.

Boyden, A. A. 1934. Precipitins and phylogeny in animals. American Nat., 68:516-536.

Boyden, A. A. 1942. Systematic serology: A critical appreciation. Phys. Zool., 15:109-145.

Boyden, A. A. 1943. Serology and Animal Systematics. American Nat., 77:234-255.

Boyden A.A. 1951. A half century of systematic serology. Ser. Mus. Bull. No. 6, Pp. 1-3.

Brezner, J. and W. R. Enns. 1958. Preliminary studies utilizing paper electrophoresis as a tool in insect systematics. Jour. Kansas Ent. Soc., 31(4):241-246.

Brezner, J., R. A. Hart and W. R. Enns. 1959. A simple method for *in vitro* storage of insect hemolymph. Jour. Kansas Ent. Soc., 32(2):92-94.

Brown, F. M. and H. M. Heffron. 1928. Serum diagnosis and Rhopalocera. Jour. New York Ent. Soc., 36:165-168.

Buzzati-Traverso, A. A. 1953. Paper chromatographic patterns of genetically different tissues: a contribution to the biochemical study of individuality. Proc. Nat. Acad. Sci., 39(5):376-391.

Buzzati-Traverso, A.A., and A. B. Reichnitzer. 1953. Paper partition chromatography in taxonomic studies. Science, 117(3029):58-59.

Clark, E. W. and B. H. Ball. 1956. Preliminary microelectrophoretic studies of insect proteins. Phys. Zool. 28:206-212.

Common, R. H., W. P. McKinley and W. A. Maw. 1953. Filter paper electrophoresis of avian serum proteins. Science, 118:86-89.

Cremer, H. D. and A. Tiselius. 1950. Electrophorese von Eiweiss in Filterpapier. Biochem. Zeit., 320:273-283.

Cumley, R. W. 1940. Comparison of serological and taxonomic relationships of *Drosophila* sp. Jour. New York Ent. Soc., 48:265-274.

Cumley, R.W., M. R. Irwin, and L. J. Cole. 1941. Genetic effects on serum proteins. Proc. Nat. Acad. Sci., 27:565-570.

Deutsch, H. F. and M. B. Goodloe. 1945. An electrophoretic survey of various animal plasmas. Jour. Biol. Chem., 161:1-20.

Drilhon, A. 1954. Etude electrophoretique des proteines de l'hemolymphe du *Bombyx mori* au cours de son cycle de croissance. Comptes. Rend. Acad. Sci., 238:2452-2454.

Edstrom, G. E. 1958. Determination of organic compounds below the microgram range. Microchem. Jour., 2:71-82.

Erhardt, A. 1929. Die Verwandtschaftsbestimmung mittels der Immunitatsreacktionen in der Zoologie und ihr Wert fur Phylogenetische Untersuchungen. Ergebnisse und Fortschritte der Zoologie, 7:279-377.

Florkin, M. 1949. Biochemical Evolution. Academic Press, Inc., New York, pp. 157.

Florkin, M. and G. Duchateau. 1943. Le graphique de precipitation par les phosphates du plasma sanguin d'un insecte *(Hydrophilus piceus).* Acta. Biol. Belg., 3:1-2.

Gleason, T. L. and F. Friedberg. 1953. Filter paper electrophoresis of serum proteins from small animals. Phys. Zool., 26:95-100.

Goldschmidt, R. 1938. Physiological Genetics. McGraw Hill, New York, pp. 375.

Hadorn, E. and H. K. Mitchell. 1951. Properties of mutants of *Drosophila melanogaster* and changes during development as revealed by paper chromatography. Proc. Nat. Acad. Sci., 37:650-665.

Haldane, J. B. S. 1937. The biochemistry of the individual, *in,* Perspectives in Biochemistry. J. Needham and D. E. Green, Ed. Cambridge Univ. Press, London. Pp. 1-10.

Jameson, E. and C. Alarez-Tostato. 1939. A study of blood serum proteins by electrophoresis. Jour. Phys. Chem., 43:1165-1172.

Jenks, W. R., E. R. B. Smith, and E. L. Durrum. 1956. Clinical significance of the analysis of serum protein distribution by filter paper electrophoresis. Research Dept. WRAIR-45-56. Walter Reed Army Institute of Research., pp. 32.

Kraus, R. 1897. Uber specifische Reaktionen in keimfreien Filtraten aus Cholera-Typhus Pestabacillenkulturen, erzeug durch hologes serum. Wien. Klin. Wchnschr., 10(32):736-738.

Kunkel, H. G. and A. Tiselius. 1951. Electrophoresis of proteins on filter paper. Jour. Gen. Physiol., 35:89-118.

Kutscher, F. and D. Ackermann. 1933. The comparative biochemistry of the vertebrates and invertebrates. Ann. Rev. Biochem., 2:355-376.

Kutscher, F. and D. Ackermann. 1936. The comparative biochemistry of the vertebrates and invertebrates. Ann. Rev. Biochem., 5:453-462.

Landsteiner, K. 1945. The specificity of serological reactions. (Rev. Ed.) Harvard Univ. Press, Cambridge, Massachusetts, pp. 310.

Lederer, M. 1955. An introduction to paper electrophoresis and related methods. Elsevier Pub. Co., New York, pp. 206.

Leone, C. A. 1947. A serological study of some Orthoptera. Ann. Ent. Soc. America, 40(3):417-433.

Leone, C. A. 1954. Serological studies of some arachnids, other arthropods, and molluscs. Phys. Zool., 27(4):317-325.

Luetscher, J. A. 1947. Biological and medical applications of electrophoresis. Physiol. Rev., 27:621-642.

Lewallen, Lawrence L. 1957. Paper chromatography studies of the *Anopheles maculipennis* complex in California (Diptera, Culicidae). Ann. Ent. Soc. America, 50(6):602-606.

Lewallen, Lawrence L. 1958. Paper chromatographic analysis of certain protein constituents from the house fly, *Musca domestica* Linneaus (Diptera, Muscidae). Ann. Ent. Soc. America, 51(2):167-173.

Lewis, C. E. and B. N. Smallman. 1956. Estimation of acetyl choline in insects. Jour. Physiol., 134:241-256.

Martin, S. and F. B. Cotner. 1934. Serological studies of moth proteins with special reference to their phylogenetic significance. Ann. Ent. Soc. Amer, 27:372-383.

McDonald, H. J. 1955. Ionography, electrophoresis in stabilized media. Yearbook Publishers, Chicago. pp. 268.

Micks, D. W. 1954. Paper chromatography as a tool for mosquito taxonomy: the *Culex pipiens* complex. Nature, 174:217-218.

Micks, D. W. 1956. Paper chromatography in insect taxonomy. Ann. Ent. Soc. America, 49:576-581.

Micks, D. W., and A. A. Benedict. 1953. Infrared spectrophotometry as a means for identification of mosquitoes. Proc. Soc. Expt. Biol. and Med., 84:12-14.

Micks, D. W., and J. P. Ellis. 1951. Free amino acids in mosquitoes. Proc. Soc. Expt. Biol. and Med., 78:69-72.

Micks, D. W. 1952. Free amino acids in the developmental stages of the mosquito. Proc. Soc. Expt. Biol. and Med., 79:191-193.

Micks, D. W. and F. J. Gibson. 1957. The characterization of insects and ticks by their free amino acid patterns. Ann. Ent. Soc. America, 50(5):500-505.

Moore, D. H. 1945. Species difference in serum protein patterns. Jour. Biol. Chem., 161:21-32.

Morris, B. and F. C. Courtice. 1955. The protein and lipid composition of the plasma of different animal species determined by zone electrophoresis and chemical analysis. Quart. Jour. Expt. Physiol., 40:127-137.

Needham, J. 1929. Protein metabolism and organic evolution. Sci. Prog. 23:633-648.

Needham, J. 1930. The biochemical aspect of the recapitulation theory. Biol. Rev. 5:142-158.

Needham, J. 1934. Chemical heterogony and the ground plan of animal growth. Biol. Rev. 9:79-109.

Needham, J. 1936. Order and Life. Cambridge Univ. Press, London, pp. 175.

Needham, J. 1938. Contributions of chemical physiology to the problem of reversibility in evolution. Biol. Rev. 13:225-251.

Needham, J. 1942. Biochemistry and Morphogenesis. Cambridge Univ. Press, pp. 785.

Nuttal, G. H. F. 1901a. On the formation of specific antibodies in the blood following upon treatment of the sera of different animals together with their use in legal medicine. Jour. Hyg., 1:357-387.

Nuttal, G. H. F. 1901b. A further note of the biological test for blood and its importance in zoological classification. British Med. Jour., 2:669.

Nuttal, G. H. F. 1901c. The new biological test for blood in relation to zoological classification. Proc. Roy. Soc. London, 69:150-153.

Nuttal, G. H. F. 1902. Progress report upon the biological test for blood as applied to over 500 bloods from various sources. British Med. Jour., 1:825-827.

Nuttal. G. H. F. 1904. Blood immunity and blood relationship. Cambridge Univ. Press, London, pp. 444.

Pryor, C. W. and C. A. Leone. 1952. Serological comparisons of Astacuran Crustacea. Biol. Bull. 103(3):433-445.

Stephen, W. P. 1957. Hemolymph proteins and their use in taxonomic studies. Proc. 10th Int. Cong. Ent. (in press).
Stephen, W. P., and A. L. Steinhauer. 1957. Sexual and developmental differences in insect blood proteins. Phys. Zool. 30(2):114-120.
Telfer, W. H. and C. M. Williams. 1953. Immunological studies of insect metamorphosis. I. Qualitative and Quantitative description of the blood antigens of the Cecropia silkworm. Jour. Gen. Phys., 36:389-412.
Wieland T. and E. Fisher. 1948. Uber Electrophorese auf Filtrierpapier. Naturwiss, 35:29-30.
Williams, Roger J. 1956. Biochemical individuality. The basis for the genetotrophic concept. John Wiley and Sons Inc., New York, pp. 214.
Woods, K. R., E. C. Paulsen, R. L. Engle Jr. and J. H. Pert. 1958. Starch gel electrophoresis of some invertebrate sera. Science, 127(3297):519-820.
Wunderly, C. H. and H. Gloor. 1953. Versuche zur charakterisierung der larvalen blutproteine normaler und letaler genotypen von *Drosophila* mittels papierelektrophorese. Protoplasma, 42:273-282.