A CHARACTERIZATION OF THE SALIVARY GLAND PROTEINS OF THE BLOOD-SUCKING BLACKFLIES, *SIMULIUM VITTATUM* AND *SIMULIUM DECORUM* (DIPTERA: SIMULIIDAE).

by

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ABSTRACT

Microscale protein assays, gradient polyacrylamide gel microelectrophoresis, and guinea-pig skin sensitivity tests, were employed to investigate changes occuring in the quantities and certain properties of salivary gland proteins of the females of two species of haematophagous blackflies. These changes in salivary gland protein properties were postulated to reflect the changes occuring in the nature and severity of the host response.

A rapid dissection method was developed for the handling of the large numbers of laboratory-reared larvae, pupae and post-emergence adults.

A progressive increase in protein content of macerated salivary glands was detected, beginning with pupae, and extending through the first 4 days of post-emergent adults. The protein content of glands from S. decorum increased from 1.5 µg in the pupa to 4.5 µg in the day 4 adult, and for the smaller species, S. vittatum, it increased from 0.9 µg to 2.6 µg. The water-soluble protein averaged 64% of the total protein for S. decorum and 76% for S. vittatum, these values being relatively constant throughout all ages.

Polyacrylamide gel extending as a concentration gradient from 1 to 40% in 10 µL capillaries, and acting as a graded sieve, was used to separate the various proteins according to progressively smaller molecular size.

Protein loads of between 0.2 µg and 1.0 µg were resolved into as many as 32 individual bands.

The protein patterns of larval salivary glands differ quantitatively and qualitatively from those of pupae or adults.

Pupal salivary glands are smaller than those of post-emergent stages, but structurally resemble those of adults and display a similar sequence of electrophoretic separation.

Three protein bands from S. decorum in the MW range of 50,000 to 80,000 show a progressive increase in relative predominance from day 0 through day 4, the stage presumed to be the most prepared for blood-feeding. There is also a concentration of more rapidly migrating protein of ca. 10,000 MW in the day 4 salivary glands.

Most of the protein in S. vittatum post-emergence salivary glands occurs in the range of 50,000-100,000 MW. The glands contain a progressively increasing concentration of ca. 90,000 MW protein which culminates in the day 4 glands. Day 1 salivary glands are the only stage to reveal three bands of rapidly migrating low MW protein in the 2,000-5,000 MW range.

Skin tests with S. vittatum salivary gland preparations in guinea-pigs, previously sensitized with whole fly tissue, gave presumptive evidence of a combination of antigenic and toxic factors in the glands.

Salivary glands from all age classes induced delayed reactions, with day 4 yielding the strongest antigenic response. Both control and sensitized guinea-pigs showed a strong cutaneous response to the injection of day 1 salivary glands, implicating a toxic factor in the salivary glands at this stage.

The recognition of two essentially different categories of noxious substances, namely toxins and sensitizers, paves the way for improved prognosis, prophylaxis involving antigens and therapy.

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DEDICATION

THIS THESIS IS DEDICATED TO
THE 2,713 BLACKFLIES WHOSE SALIVARY
GLANDS WERE DECIMATED IN
THE NAME OF SCIENCE.

INTRODUCTION AND LITERATURE REVIEW

Most research on haematophagous insects has been concerned with the transmission of pathogenic organisms and with population control of the insects involved. However, apart from disease transmission, haematophagous feeding can induce a number of adverse reactions in the host. These reactions may result in severe manifestations ranging from pruritus, generalized urticaria and persistent lesions, to anaphylaxis and death (Benjamini and Feingold 1970; Feingold et al. 1968; McKiel and West 1961).

Offending venomous insects cannot always be managed adequately, and individual hosts cannot always be protected from the bites. Accordingly, there is need for additional information and for countermeasures to cope with the noxious salivary substances which the blood-feeding insect introduces into the host during the feeding process.

Many different substances have been identified as being injected into animal hosts by venomous insects either in the act of defense or in their pursuit of food (Davis 1979). Of these substances, relatively few have been cited as occurring in the oral secretion of species which feed on blood. Nevertheless, the salivary substances of haematophagous insects constitute a special problem of great importance because of their severe effects on the host. An understanding of the components of the blood-feeding processes is basic to the development of improved methods of host protection and therapy.

The impacts of the blood-feeding habit are the result of factors in the attack process interacting with factors in the host. Significant features of attack consist of harassment, debilitation of the host from loss of blood, transmission of pathogens, and envenomization (Hocking 1952). Each of these factors contributes to the total impact, not only by direct action, but also by enhancing the conditions for action of the others (Feingold et al. 1968). Determinants originating from factors in the host derive from heritable factors, age, physiological condition, prior sensitization, and nutritional elements (McKiel and West 1961).

Of the various elements contributing to total impact, envenomization is perhaps second in importance only to disease transmission, and ranks similarly to loss of blood (Benjamini and Feingold 1970). The host response to envenomization is complicated by the individual differences in natural susceptibility, the mechanical aspects of wounding, the injected toxic components and the injected allergenic components of the saliva (Beard 1963; Feingold et al. 1968).

It is generally considered that in most blood-sucking Diptera, saliva is important in maintaining the blood in a fluid state for transport to the gut, once the mouthparts pierce the host's tissues (Yang and Davies 1974). Some of the known substances in the saliva of haematophagous insects serve as anti-coagulants to prevent premature coagulation of the blood meal (Fairbairn and Williamson 1956; Hudson 1964; Hutcheon and Chivers-Wilson 1953), or as agglutinins to induce delayed coagulation during digestion (Yang and Davies 1974), or possibly as anaesthetisers to numb the invaded tissues (Frazier 1969; Hudson et al. 1960). The noxious properties of

salivary secretions are hardly surprising when it is realized that they might contain digestive enzymes corrosive to the invaded tissues (Beard 1963). It is not clear whether or not all of the noxious constituents play a functional role for the insect.

The various substances identified in saliva of mosquitoes include agglutinins, acid proteins, anticoagulants, conjugated mucopolysaccharides, PAS positive substances glycoproteins, carbohydrate protein complexes and haptens (Orr et al. 1961). Mosquito saliva has also been shown to contain certain anaesthetic factors (Hudson et al. 1960), and traces of histamine (Wilson and Clements 1965). A sequence of events in host skin reactivity over time has been established in response to mosquito bites, indicating the involvement of antigenic factors in the injected saliva (Mellanby 1946; McKiel 1959).

A definite sequence of skin reactivity has also been recorded for flea bites (Benjamini et al. 1961). It is thought that the saliva of fleas contains a haptenic factor which combines with certain components of the host skin to form a sensitizing antigen (Benjamini and Kartman 1963).

The saliva of certain species of blackflies contains small amounts of histamine (Hutcheon and Chivers-Wilson 1953), an anaesthetic component (Frazier 1969), an unidentified anti-coagulant, and agglutination factors (Yang and Davies 1974). The presence of noxious factors in the saliva is indicated by the reactions of certain individual hosts (Brown and Bernton 1970; Fallis 1964; Frazier 1969; Gudgel and Grauer 1954; Hansford and Ladle 1979; McKiel and West 1961). No definite sequence of skin reactivity has been reported in response to bites of the blackfly.

If more were understood of the identity of factors in insect saliva and their mode of action, it should be possible to predict the outcome of bites under various circumstances. In addition, it may be found whether or not active or passive immunity can be achieved. Furthermore, it may be ascertained what forms of therapy may be appropriate for particular circumstances as to severity of attack and time lapse after attack.

Advancement in our knowledge concerning insect salivary venoms and reactions to them has progressed with difficulty for several reasons. Greater priorities of effort have been given to the study of disease transmission by insects, and to their biologies and ecology. In the realm of countermeasures, priorities have been given to management of the insect populations and to temporary decimation by chemical agents. Some attention has been given to protection of individuals by chemical deterrents, sought largely through empirical testing.

The study of insect venoms has also been hampered by technological limitations of equipment and methods for obtaining, handling and resolving the ultraminute fractions of components in the extremely small quantities that are contained in particular tissues and fluids. New methods are now emerging. Of particular interest are the developments of micro-electrophoresis (Neuhoff 1973; Poehling 1979; Poehling et al. 1976).

There are two main aspects to the study of the impacts of haematophagous feeding on the host. The first, the study of salivary venoms, is confronted by several different kinds of possible variables. Several kinds of noxious substances may be found in any one sample. These may differ in proportions and total amounts with the post-emergence age of

an individual insect (Barrow et al. 1976; Gosbee et al. 1969; Mellink and van Zeben 1976; Poehling 1978), between individuals within a species, and between species (McKiel 1959; Poehling 1979). The second, and complementary aspect, which deals with host reactions, is also challenged by its own complexities. One complicating factor in human and other non-laboratory subjects is the uncertainty of prior exposure to a salivary venom which may have altered the state of reactivity (Feingold et al. 1968; McKiel 1959). The variability of humans in respect to genetics, age, gender, physiological state, environmental influences, individual habits, medications, and nutrients poses further difficulties in this area of study (Benjamini and Feingold; Jamnback 1973; McKiel and West 1961; Shulman 1967). Numerous factors can differ and change between and within individual hosts to influence the kind, intensity and duration of the host response.

The salivary constituents of blackflies of the dipteran family Simuliidae are of particular interest because the blood-feeding of these flies may be followed by unusually severe skin and systemic reactions in both humans and domestic animals. When a blackfly takes a blood meal from a host it first lacerates the skin with its blade-like mandibles. The mandibles, maxillae and hypopharynx then enter the host's skin to a depth of approximately 120-150 microns (Crosskey 1973). The thickness of the human epidermis varies considerably in different body regions, but averages approximately 70 microns (Storer and Usinger 1957). Therefore, the penetration is well into the dermis of the human host. This would correspond to intradermal injection, and thereby defines one of the

conditions for the skin sensitivity tests performed in this study. The trauma associated with the mechanical aspect of the bite is more severe than that caused by arthropods possessing finer piercing mouthparts (Benjamini and Feingold 1970). However, even more important than the mechanical trauma is the injection of saliva into the host's tissues, which can result in severe local and systemic host reactions (Brown and Bernton 1970; Gudgel and Grauer 1954; Hansford and Ladle 1979; Minar and Kubec 1968). Furthermore, the reactions in humans may also involve a condition known as "blackfly fever", which is manifest as a headache, swollen glands, fever and nausea. Deaths of domestic animals following bites from various species of blackflies have been reported: for example, cattle in Saskatchewan following bites of Simulium arcticum (Fredeen 1958; Rempel and Arnason 1947); mules in Arkansas following bites of S. pecuarum (Bradley 1935); horses, pigs and sheep following bites of S. columbaczense (Fallis 1964); and poultry following bites of S. griseicolle (Garside and Darling 1952).

Notwithstanding an extensive literature dealing with blackflies during the past two centuries (reviewed in Watts 1975), very little precise information is available on the nature of the venom or on the mechanisms of action and reaction. No convincing evidence exists, for example, that antibody immunity develops in response to the bites. It is not certain whether or not such immunity is theoretically possible, or, if it is, how it might be manipulated.

In the study of reactions to salivary venoms a distinction must be made between toxins and sensitizers which may occur in the saliva. This

distinction, which is not clear in the literature (Benjamini and Feingold 1970; McKiel and West 1961), is significant in that, in contrast to sensitizers, toxins cause immediate reactions without prior host exposure to the substance, and without causing subsequent enhanced host sensitivity. It follows that the prognosis, prophylaxis and therapy for conditions caused by the two classes of factors must be different.

There are several possible relationships between the whole glandular contents and the products entering the saliva of the fly. It can be postulated that, in addition to the structural material of their protoplasm, glands might also contain substances which are continually being formed to be released unchanged, others which are molecular building blocks for new substances, or proteins with attached haptenic substances which become split away either before or after entering the saliva, or even after entering a host. Some of the glandular secretory products may be derived from uptake and transportation of blood proteins (Laufer 1968). Notwithstanding these uncertainties, it is obviously worthwhile to consider the salivary glands as a primary source of the salivary secretions, and as a source of the noxious substances involved in the host response.

In the present study attention is focussed on the protein constituents of the salivary glands of females of two species of blood-sucking blackflies. This aspect of salivary gland chemistry was chosen because it is generally believed that proteins are the most biologically active

component of venoms (Tu 1977). Although it might be expected that the membrane-bound protein components of the salivary glands are not directly involved in the bite reactions, Laufer (1968) has postulated that some such component might be released into the saliva at the time of biting. Presently, techniques for collection of saliva in the exact same composition as that injected into the host are not well developed. In the present study whole salivary glands, comprising both membrane-bound and water-soluble proteins, were used for all analyses. This emphasis on the total protein content of the glands will form a vital frame-work for future studies which might then be directed towards the saliva known to be injected during the blood-feeding process. Larval and pupal salivary glands were included in the study to complete the picture of post-hatching stages in the ontogenetic sequence.

Based on the evidence that the severity of host reaction varies according to the age of a fly at the time when it attacks (Hocking 1952; Mellink and van Zeben 1976), it can be postulated that corresponding changes must occur in the nature and/or quantity of the glandular substances. As a basis for the pursuit of immunological forms of host protection, it is necessary to investigate the existence and nature of such postulated changes.

The present study was undertaken to test the hypothesis that progressive changes occur in the quantities and certain properties of salivary gland proteins. These properties are postulated here to reflect the changes which occur in the nature and severity of toxic and/or immune reactions in the host.

The experiments herein described used micro-electrophoretic separations to investigate the molecular weights of the salivary glands of different metamorphic stages and different post-emergence ages of two species of haematophagous blackflies. Guinea-pig skin tests with the different salivary gland preparations were conducted to determine the nature and severity of the host response.

1. MATERIALS AND METHODS

1.1 PROCUREMENT OF BIOLOGICAL RESEARCH MATERIAL

The study required the collection and storage of several hundred pairs of salivary glands from haematophagous female blackflies (Diptera: Simuliidae) local to the Vancouver area, British Columbia.

1.1.1 Blackfly species selected for study

Two species of blackfly, namely Simulium decorum and S. vittatum, were chosen from the local area as being known pests of man and relatively easy to collect. Simulium decorum Walker (Figure 1), a grey-brownish fly with banded legs, (wing span 4 mm) has been recorded across Canada (Fredeen 1973). It is known to feed on deer and horses, as well as on man (Davies and Peterson 1956; Fallis 1964; Fredeen 1958). Simulium vittatum Zetterstedt (Figure 2) is abundant throughout British Columbia and has been recorded across Canada (Hearle 1932). It is silver grey in colour, with a wingspan of 3-5 mm. This species is known to feed on nectar, cattle, horses, sheep and man (Fallis 1964; Hearle 1932; Davies and Peterson 1956). Both species breed in small streams in the Vancouver area. S. vittatum overwinters in the larval stage, becoming active in the late spring, and often producing several generations of adults from late April to early August. S. decorum, in contrast, has a short adult life span with only one generation in late April - early May.

FIGURE 1: Simulium decorum female adult. X20

FIGURE 2: Simulium vittatum female adult. X20

1.1.2 Larval collection sites and collection methods

Simulium decorum larvae were collected from a small stream in the Nitobe Gardens on the University of British Columbia campus, Vancouver, British Columbia (Figure 3). The stream is part of a man-made lake system and is mechanically provided with a constant flow rate and water depth. A man-made waterfall in the course of the stream generates the turbulent conditions ideal for a blackfly breeding habitat. Stream depth is maintained at approximately three to five cm throughout the year.

Simulium vittatum larvae were collected from a stream flowing from Deer Lake to Burnaby Lake in Burnaby, British Columbia (Figure 4). This stream has an erratic flow rate, swelling to a depth of one metre, or more, in the winter months and reducing to a five to ten cm deep trickle in the summer months. Both sampling sites abounded with small rocks which served to provide attachment sites for the larvae. The most heavily populated rocks were collected into 20 litre buckets containing local stream water and transported back to the laboratory.

The in-transit time for *S. decorum* larvae was approximately fifteen minutes from stream to the laboratory. *S. vittatum* larvae were subjected to a travelling time of almost one hour from Burnaby back to the laboratory. During that period, special aeration was not required to maintain viability of the larvae.

Field collections of *S. decorum* larvae were made between April 26th and May 4th, 1979, and during the period April 10th to April 24th, 1980. *S. vittatum* larvae were collected from April 29th to May 31st, 1979, and May 6th to May 22nd, 1980.

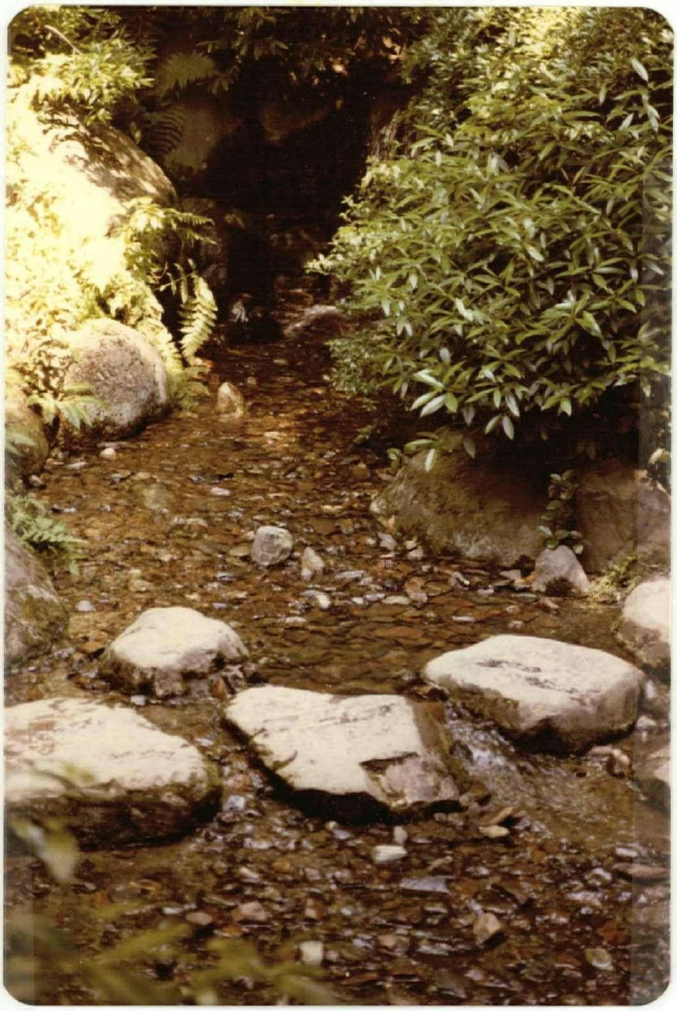


FIGURE 3: Collecting site for S. decorum. Nitobe Gardens, Vancouver, B.C.

FIGURE 4: Collecting site for S. vittatum. Deer Lake stream, Burnaby, B.C.

1.1.3 Laboratory rearing and maintenance

The 20-litre buckets used for stream collections were also used as laboratory rearing containers for the larvae, pupae and newly emerged adult blackflies. The provision of four airstones to each bucket generated sufficient aeration to simulate a turbulent stream environment for the feeding larvae. Powdered yeast was fed to the larvae every two or three days at the rate of 25 mg per litre.

Pupation occurred either on the rocks, the bucket sides or on the airstones themselves. As newly emerged adult flies rose from the water they were trapped at the mouth of the bucket by a layer of plankton screening which had been very tightly secured by bulldog clips. Newly emerged adults were captured with an entomological aspirator. Collections were made several times a day so that the age of the flies could be tallied accurately. The flies were stored in 500 mL glass flasks, approximately 50 flies per flask, in a controlled environment of 13°C and 85% RH until needed for dissection.

1.2 THE EXTIRPATION OF SALIVARY GLANDS FROM LIVE BLACK FLIES

A rapid and efficient technique for salivary gland extraction was needed for this study. Rapidity of technique was of prime concern in order to keep up to date with the different post-emergence age groupings of glands required. Furthermore, the nature of the study required that large quantities of material be handled within the limited activity period of the adult blackflies.

Although the precise location and anatomical description of blackfly salivary glands has been well documented (Cox 1938; Gosbee et al. 1969; Krafchick 1942; Smart 1935; Wachtler et al. 1971; Welsch et al. 1968; Wenk 1962), the technique for removal of these glands has been described in somewhat more general terms (Bennett 1963; Poehling 1976). At the outset of this study many different techniques for gland extraction were tested. The chosen method required the removal of the glands as an intact pair from live flies before the inception of any cellular degradation. The technique finally perfected to meet the specific requirements of this project is detailed fully in the following sections.

1.2.1 Preparation of live flies for gland extraction procedures

It was considered possible that changes might occur in the quantity and composition of the salivary gland proteins during adult life of the female flies. This possibility was investigated by sampling glands from flies of different ages, as well as from larvae and pupae. For S. vittatum the range of ages, included day 0, 1, 2, 3, 4, and 5 respectively. Under

the prevailing laboratory conditions very few adults of S. vittatum survived beyond five days. For S. decorum the limit was four days.

Approximately 50 selected flies were immobilized with a stream of carbon dioxide gas, sorted to sex, and the females transferred to a previously chilled 8 cm x 1 cm glass tube. The glass tube was then dropped into a custom made ice-jacket. The ice-jacket was previously made by freezing a styrofoam mug of water around an empty glass tube. The latter was then removed to leave a cavity in the ice. The flies were kept completely immobilized as long as the glass-tube remained jacketed by ice. It was found that CO_2 immobilization without the transfer to an ice-jacket did not hold the flies for the time needed to complete the dissection procedure.

Pupae and larvae were similarly held on ice prior to use.

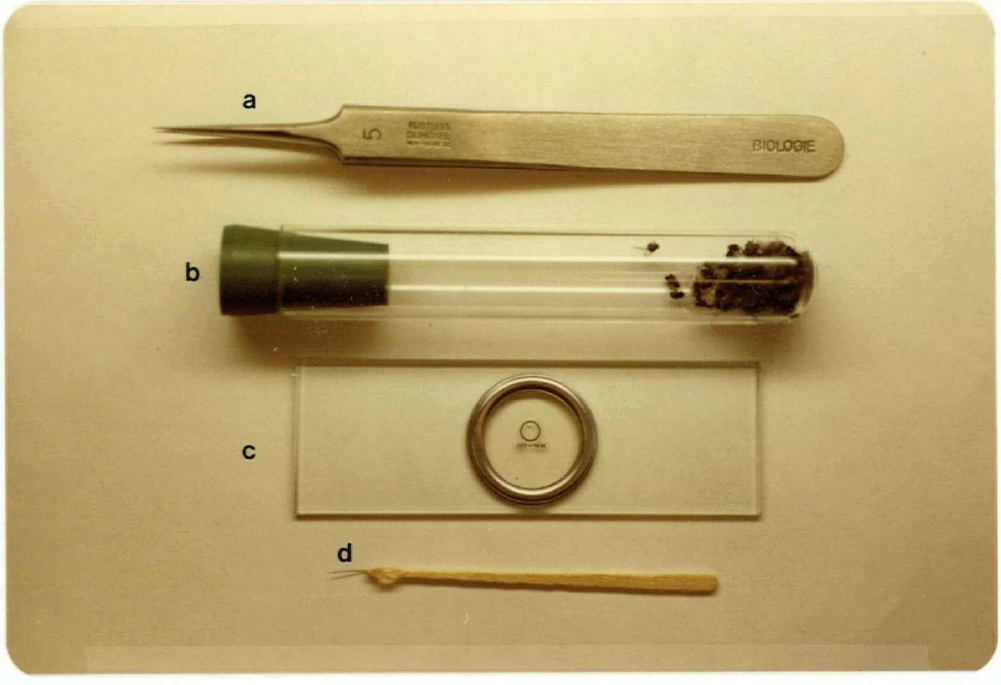


FIGURE 5: Gland extraction equipment: a. forceps; b. holding tube; c. raised-ring calibration slide; d. minutien pin mounted on a toothpick. X0.8

1.2.2 Gland removal techniques from adult, pupal and larval flies

Immobilized flies were handled individually. Each fly was removed with forceps from the holding tube and plunged under insect Ringer's solution (see Apendix for formulation) contained within a raised-ring calibration slide (Figure 5). The slide and contained specimen were then placed under a dissecting microscope at a magnification of approximately x50. Cold light was provided by a fibre-optics ring illuminator mounted between the microscope objective and stage (Figure 6).

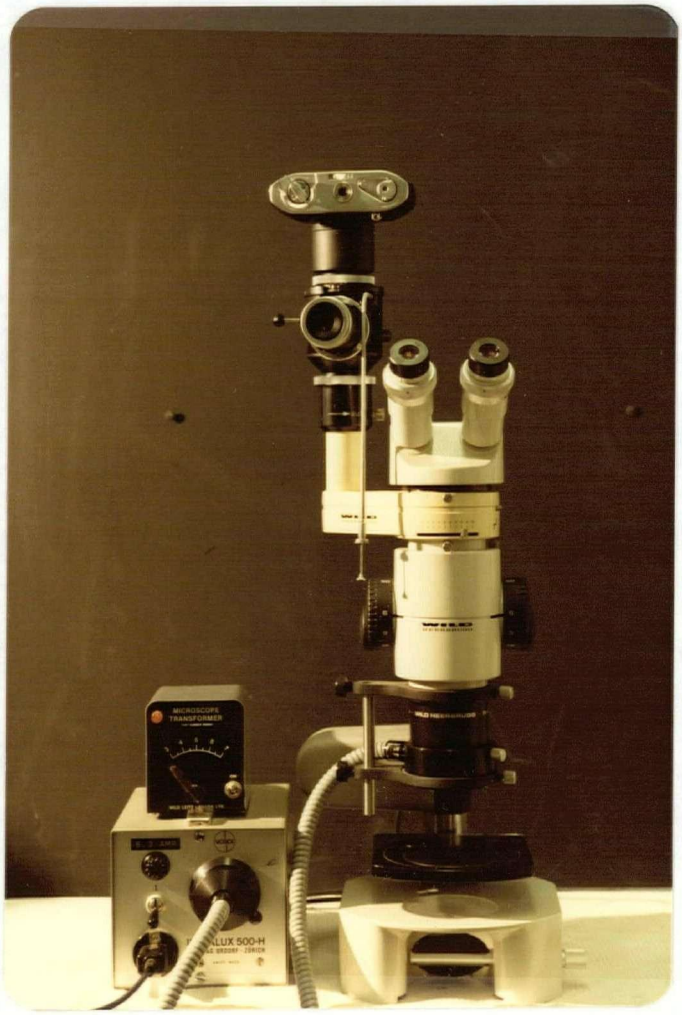


FIGURE 6: Wild M8 dissecting microscope and fibre-optics lighting equipment.

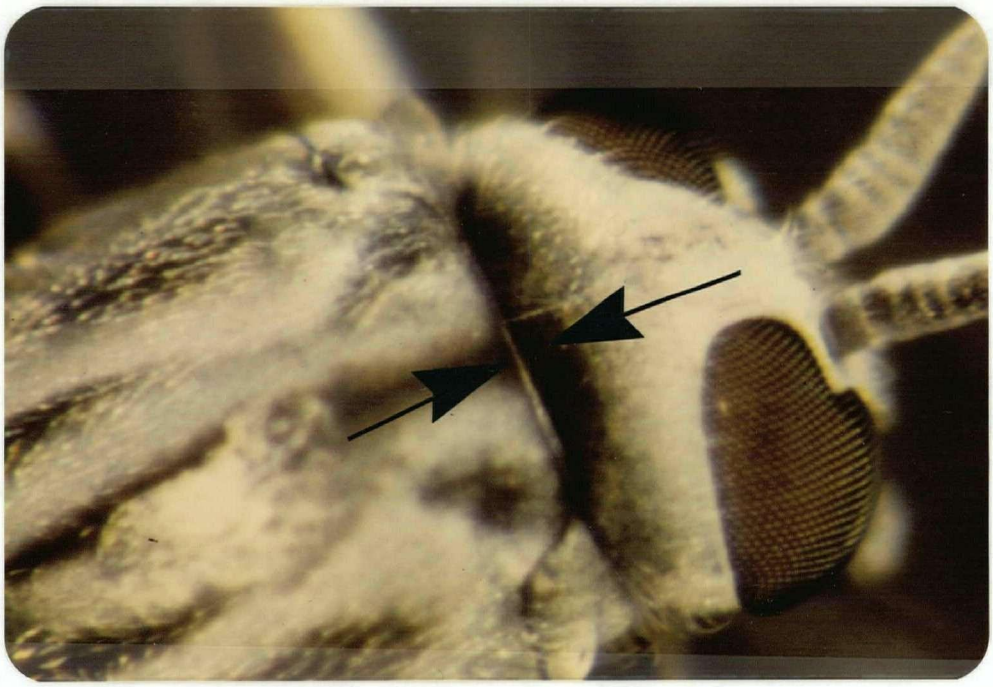


FIGURE 7: Dorsal view of the neck region of S. vittatum. Arrows mark point at which the head is pulled from the thorax. X150

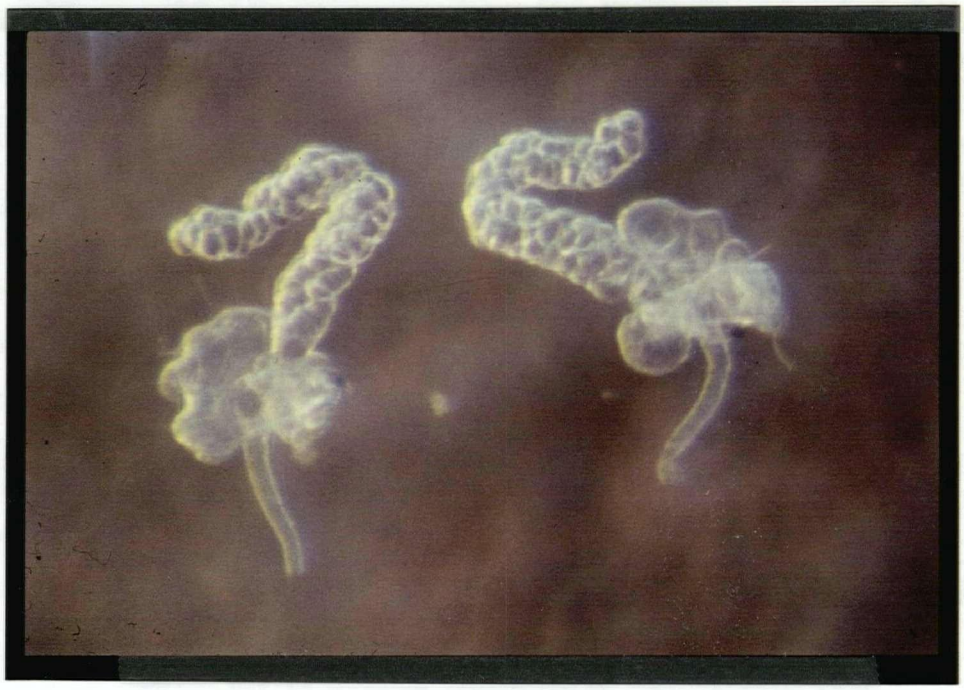


FIGURE 8: Salivary glands of S. vittatum, photographed immediately after detachment from the head. X150

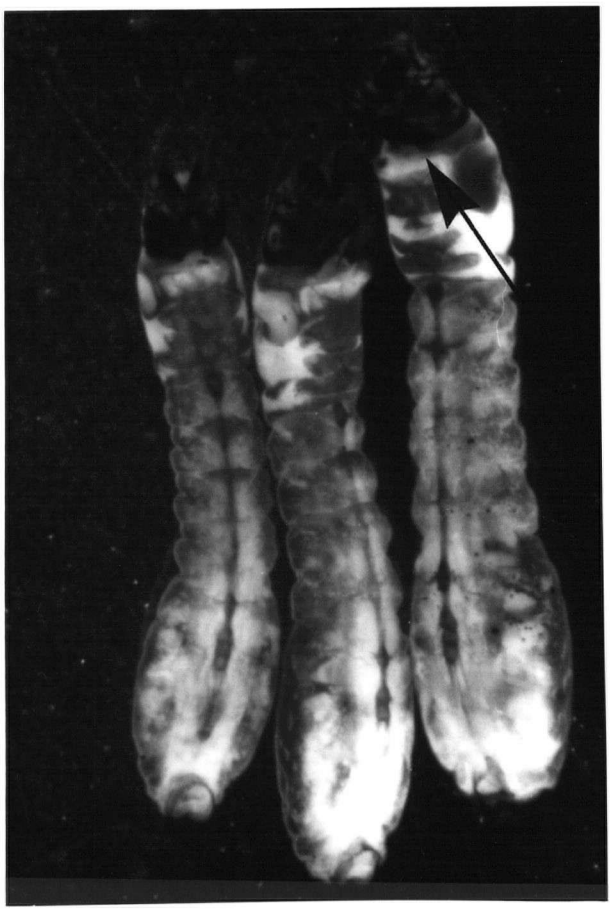


FIGURE 9: Simulium vittatum larvae. X20
Arrow marks point at which head is pulled away from the body.

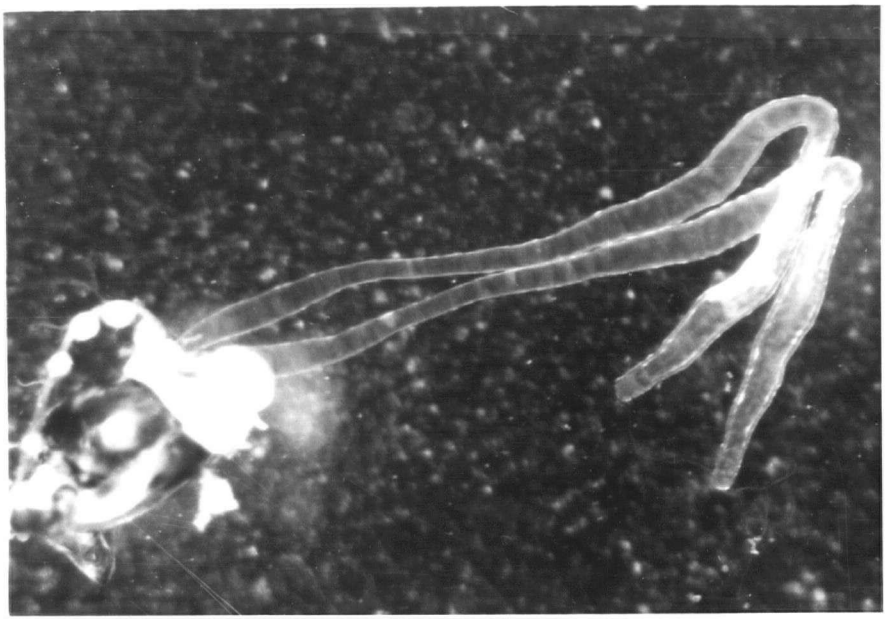


FIGURE 10: Salivary glands of S. vittatum larva before detachment from the head. X30

The fly was secured by the anterior part of the abdomen with forceps held in the left hand. A pair of Dummont number-5 Biologie forceps (Fine Science Tools Ltd., Vancouver, B.C.), held in the right hand, was used to grasp the head and pull it clear of the thorax at the neck membrane (Figure 7). In this way, the salivary glands were drawn out of the thorax and remained attached to the head, still held by forceps in the right hand. The remaining carcass was discarded, freeing the left hand to make the final break between the glands and the head (Figure 8).

The positioning of the female salivary glands in the anterior of the prothoracic "hump" made the process of glandular extraction extremely difficult. It was found to be important to pull the head from the neck membrane with the utmost caution so as not to rupture the whole glands or break the salivary ducts which unite each gland pair.

After considerable practise it was possible to perform the entire sequence of gland removal in a time period of less than 10 seconds per fly (Watts 1981).

Pupae were handled in a manner similar to adults.

Salivary glands were removed from larvae by pulling on the entire head capsule with forceps while holding onto the body of the insect by the larval pro-leg (Figures 9 and 10). Larval salivary glands are relatively large and easy to remove in comparison to those of the adult flies.

1.2.3 Codification and storage of extirpated material

A minutien pin, mounted on a toothpick (Figure 5), was used to transfer freshly extracted glands into a one microlitre drop of Ringer's

solution contained within a 10 µL glass micro-capillary pipette (Brand, Wertheim, W. Germany). One end of the capillary had previously been heat-sealed; the other end was sealed with Parafilm (American Can Co., Greenwich, Conn.) directly after loading.

Each pair of extracted glands was stored individually and labelled for date, species, origin and age before being deep frozen at -25°C.

1.3 PROTEIN ASSAYS OF SALIVARY GLAND MATERIAL

Generally, protein assays require destruction of the test sample. In this study the material available for assay was strictly limited by the short flight period of the adult flies and by the time element involved in the gland extraction procedures.

1.3.1 Maceration of glandular material

Partial tissue breakdown occurred owing to the freezing and thawing action on stored glands. As glands were required for assay, the semi-disintegrated glandular material was transferred with a fine hand-drawn capillary pipette into glass micro-centrifuge tubes (Figures 11 and 12). Complete maceration was ensured by manually grinding the tissues with a teflon pestle custom made to fit snuggly into these tubes. The micro-centrifuge tubes were made from thick walled 3 mm precision-bore glass tubing to a total length of 55 mm.

Five pairs of glands were grouped for each assay of adult glandular protein, three for larval and six for each pupal assay. The macerated material was made up to 100 µL with distilled water before the tube was sealed with Parafilm.

1.3.2 Micro-centrifugation of glandular material

Centrifugation was performed in a Sorvall Superspeed Centrifuge (Sorvall Inc., Norwalk, Conn.), at 20,000 xg for 15 minutes. Prior to

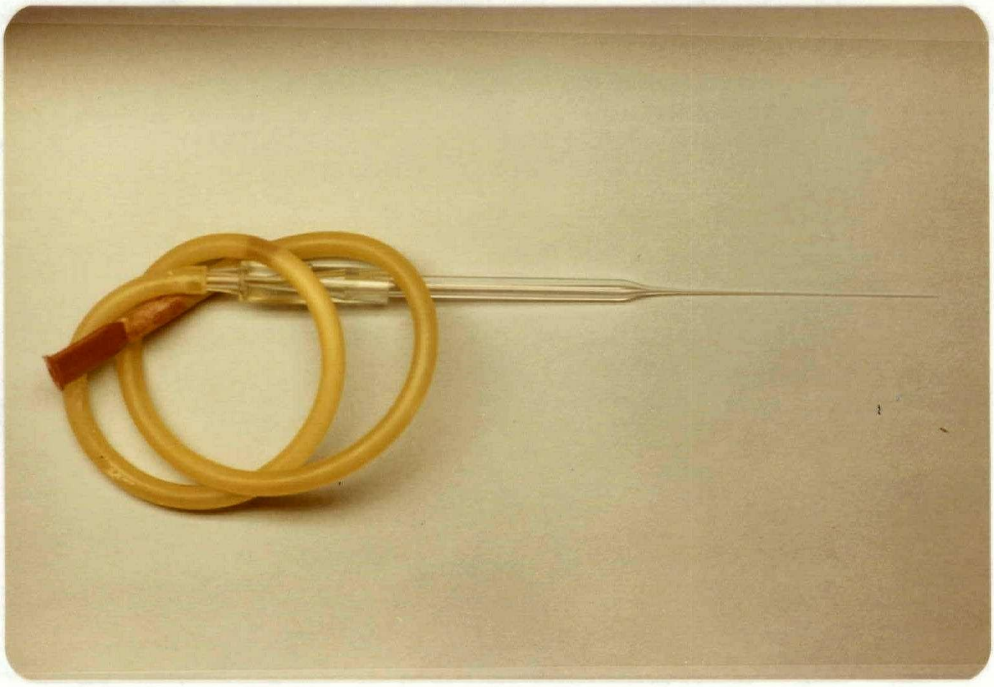


FIGURE 11: Hand-drawn microcapillary for the transfer of small volume solutions.

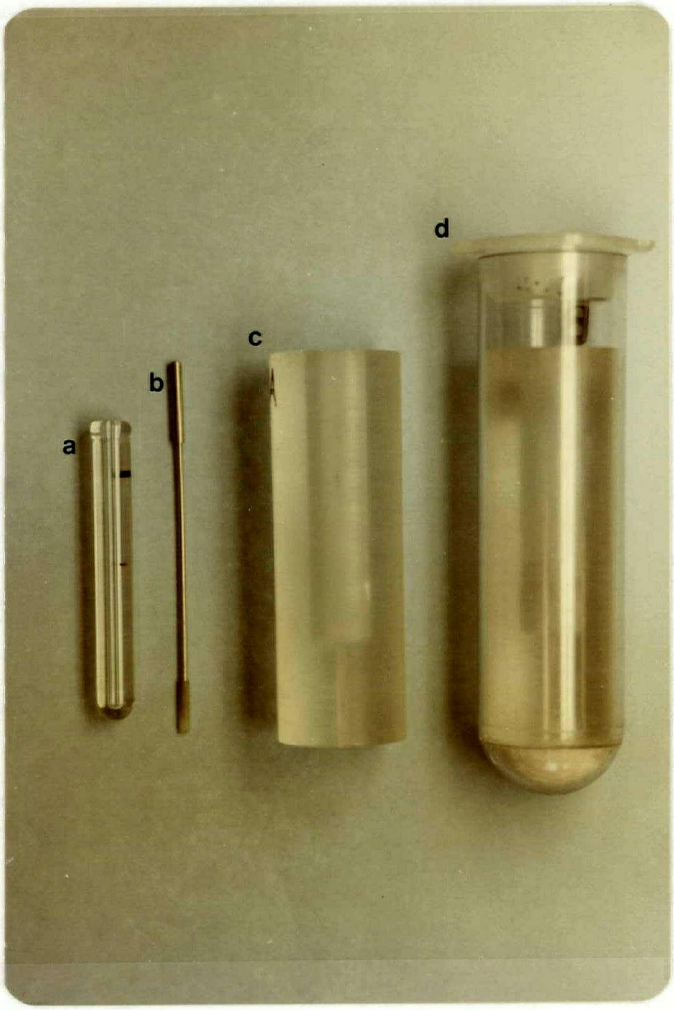


FIGURE 12: Microcentrifugation equipment: a. custom made glass micro-centrifuge tube; b. teflon pestle for a.; c. plexi-glass adaptor for holding micro-tube in standard centri-fuge tube; d. stan-dard centrifuge tube fitted with adaptor for micro-tube.

centrifugation, the centrifuge rotor was cooled under cold tap water for 10 minutes and all samples were refrigerated for 30 minutes. The centrifuge was run at room temperature.

Special plexiglass adaptors (Figure 12) were made to hold the glass micro-centrifuge tubes inside standard 28.6 mm x 103.6 mm Autoclear (Reg. Trade Mark) round-bottom centrifuge tubes.

1.3.3 Micro-protein assay procedure

An adaptation of the Lowry method (Brewer et al. 1974) was used for protein estimation. This combined biuret and phenol method of protein assay measured for peptide bonds and tyrosine residues respectively.

Adult and pupal salivary glands were found to be suited to a micro-scale assay capable of detecting protein in the range of 0-10 µg/ 500µL. Larval salivary gland proteins however, were measured by use of a higher range assay capable of detecting protein in the range of 10-300 µg/ 500 µL. The Appendix contains all formulations and procedural details.

Bovine serum albumin (Polysciences Inc., Warrington, Pa.), subsequently referred to as BSA, was run as a series of sample standards for every assay in order to establish a standard curve. Stocks of BSA were prepared at concentrations of 500 µg/mL. Each lower concentration was prepared by independent dilution from the concentrated stock solution. The seven concentrations of BSA used for the 0-10 µg assay were selected in order to establish regular intervals along the scale. Similarly, 11 concentrations were used to cover the span of the 10-300 µg assay. Duplicate assays were carried out on each standard sample and the results were averaged. Linearity of the standard curve was checked by estimation

of the correlation coefficient. Samples for salivary gland protein estimation were run in quadruplicate.

All optical density recordings were made with a Pye Unicam SP800 U.V. Visible Spectrophotometer (Canlab, Vancouver, B.C.), at a wavelength of 700 nm. A X10 total expansion factor, in conjunction with an external strip chart recorder, was used for recording the spectral absorbance of each sample. The temperature of the cell holder was maintained at 20°C by a constant temperature water bath.

Matched micro-cuvettes containing distilled water were used to zero the spectrophotometer at the beginning of each series. Each sample to be assayed was read against a phenol blank.

1.4 ELECTROPHORETIC METHODS

For research purposes polyacrylamide gel electrophoresis, designated by the acronym PAGE, is considered to offer several advantages over starch or agar gel techniques, which it has partially replaced (Smith 1968). These advantages include superior optical clarity of the gel, physical strength, chemical purity and reproducibility. The gel can be made to provide a desired effective pore radius by adjustment of the total acrylamide concentration. Increases in gel concentration produce proportionate decreases in pore size, with a consequent exclusion from the gel of molecules of larger molecular dimensions. This enables the gel to be used as a versatile sieving device in which the pore size can be selected for optimal resolution between any two chemical species.

1.4.1 Development of micro-electrophoretic techniques

Conventionally, disc-polyacrylamide electrophoresis is carried out in glass tubes with an inner diameter of 5-7 mm. The first use of the technique on a smaller scale was made by Pun and Lombrozo in 1964, in their attempt to fractionate brain proteins. In 1965, Grossbach developed a technique that utilized 1-5 µL Drummond micro-caps (internal diameter 0.2-0.45 mm) for separation and subsequent quantitative determination of proteins in the nanogram range.

Further refinements to these micro-electrophoretic techniques were prompted by the needs of neurochemists in their studies on different anatomical parts of the brain (Hyden et al. 1966; Neuhoff et al. 1967;

Neuhoff 1968; Ansorg et al. 1971). The micro-techniques that resulted have a number of advantages over conventional macro-techniques:

1. The separation obtainable on the micro-scale is comparable with conventional column techniques, but diminution of column dimensions increases the detection sensitivity by reduction of the cross sectional area of the gel (Grossbach 1965).

2. Heat dissipation problems, inherent in column electrophoretic techniques, are virtually non-existent with the micro-scale which involves columns of very small cross-sectional dimensions.

3. Staining of micro-gels takes from five to fifteen minutes, with no de-staining necessary. Diffusion problems are reduced by the more rapid fixation and staining procedures.

4. Perhaps the most important advantage of micro-disc electrophoresis is the shorter running time required for protein separation. Conventional techniques often require running periods of several hours. Micro-techniques can reduce the duration of electrophoresis to under one hour, depending somewhat on the proteins being fractionated.

The obvious advantages to using micro-electrophoresis when only very small quantities of material are available led to the adoption of this technique for the present study. In this study the columns consisted of 10 µL glass micro-capillary pipettes (Brand, Wertheim, W. Germany) filled with a 1-40% gradient of polyacrylamide gel. The gradient type of gel was chosen because it was desired to obtain a separation of protein according

largely independent of electrical charge (Leaback 1968; Slater 1969). It has been shown that a gradient of polyacrylamide in a micro-capillary can produce sharper separations of fractionated proteins than is possible on fixed density gels (Neuhoff 1973).

Methodologies for micro-disc gradient polyacrylamide gel electrophoresis are still somewhat inadequately described in the literature. Although the basic system established by Ruchel et al. (1973) was followed throughout this work, certain modifications were necessary and these are described. The "problem-solving" aspects of this stage of the study will be detailed.

1.4.2 Production of gradient gels

Formulations for the production of 1-40% gradient gels in 10 µL capillaries were adapted from those of Ruchel et al. (1973), with certain modifications:

Three stock solution were prepared as follows:

STOCK SOLUTIONS

A. Gel Monomer Solution

52.26 g Acrylamide-triply recrystallized	(Polysciences Inc., Warrington, Pa.)
1.066 g N,N-Methylenebisacrylamide	(as above)
H_2O to 100 mL	

It was found that gel production on the micro-scale required very high purity of reagents. In particular, for stock solution A, the acrylamide had to be re-crystallized three times before use. This can be done by the

manufacturer (in this case Polysciences Inc., Warrington, Pa.) or in the laboratory (Nuehoff 1973). The N,N-Methylenebisacrylamide required the same preparation attention in order to ensure correct polymerization of the micro-gels.

Once stock solution A had been made up, it was stored for a maximum of three months at 4°C.

B. Initiator stock Solution

35 mg Ammonium persulphate (Sigma, St. Louis, Mo.)
H_2O to 50 mL

The purity and freshness of the ammonium persulphate used in stock solution B was found to be critical. The level of manufacturer's purity had to be very high. The reagent was constantly monitored for possible degradation, indicated by a pronounced smell of ammonia, and discarded if necessary. Ammonium persulphate stock solution was made up into solution as required; it was never stored for more than the working day in which the gels were made.

C. Gel Buffer

2.8M Tris(hydroxymethyl)-aminomethane (Sigma, St. Louis, Mo.)
(Tris)
1.0 mL N,N,N,N-Tetramethylethylenediamine (Baker, Phillipsburg,
(TEMED) N.J.)
1.0 mL 1N H_2SO_4
6N H_2SO_4 to pH 8.4
H_2O to 100 mL

Problems arose with the purity of the TEMED used in the production of stock solution C. It was found to be necessary to re-distill this reagent

under vacuum prior to use. A Brinkmann EL-130 rotary flash-evaporator (Brinkmann Instruments Ltd., Ontario) was used for this purpose, and only the main fraction was collected. It should be noted that pH adjustment was made only after the addition of the TEMED. The final solution was stored at 4°C for a maximum period of one month.

Disposable 10 µL glass micro-capillary pipettes, 32 mm long and .635 mm internal diameter, were used for the production of gradient gels. The capillaries were subjected to a rigorous cleaning procedure before use (Neuhoff 1973). Any used capillaries were discarded.

Before the cleaned capillaries were filled, each was marked with a felt pen at a point 18 mm from one end.

For the production of gel gradients, 6 mL of stock solution A was added to 2 mL of solution C in a 10 mL beaker. A second beaker was filled with fresh solution B. Each capillary was first filled with B to the 18 mm mark, and then quickly transferred to the solution mixture of A+C. Although Ruchel et al. (1973) had suggested filling the capillaries only halfway with the ammonium persulphate solution, i.e. to the 16 mm mark, an excesss of this initiator was found to ensure reliable polymerization. The capillary, held by forceps, was filled by capillary attraction as it was dipped into the two solutions. A finger was held on top of the capillary to prevent the entrapment of air as it was being transferred between the two beakers (sterile laboratory gloves were worn throughout the entire procedure of gel production).

Each filled capillary was placed vertically along the side walls of a flat-bottomed 10 mL beaker, the base of which was covered with a 5 mm layer

of 50%(w/v) aqueous sucrose solution mixed 8:1 with stock solution E (Figure 13). The sucrose solution provided a seal at the end of the capillaries and prevented atmospheric oxygen from entering the polymerization reaction. Neuhoff (1973) suggested sealing the capillaries by placing them vertically into a plasticine cushion. This was found to be an inferior method as it was extremely difficult to ensure that each capillary was, infact, perfectly vertical.

The formation of a linear gradient was checked visually by adding enough bromophenol blue to solution A+C to give a weak blue colouration. The gradient was considered uniform across the whole diameter of the

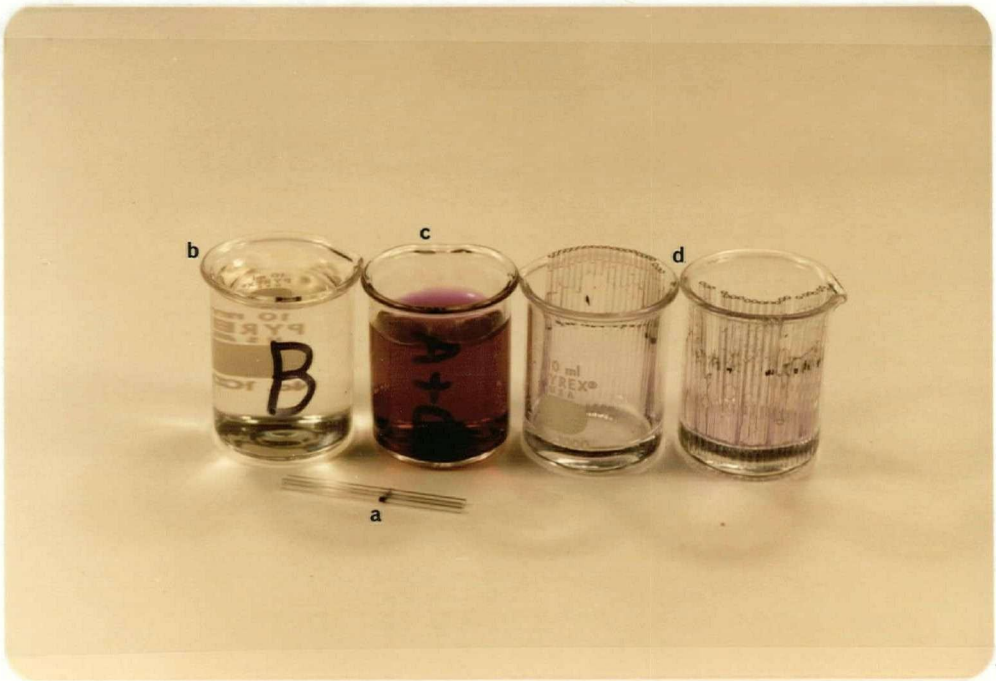


FIGURE 13: Sequence of gradient gel production: a. clean micro-capillaries; b. solution B (initiator solution); c. solution A+C (gel monomer plus buffer); d. filled capillaries aligned vertically against the side walls of a 10 mL beaker containing a 5 mm layer of sucrose.

capillary when the bromophenol blue indicated more intense colouration in a gradient from the top to the bottom of the tube.

Polymerizing and freshly prepared gels were stored for 24 hours in a moist chamber at room temperature before use. Prolonged storage of gels was possible at 4°C in a 1:8 dilution of solution E. Such gels, on retrieval, required an incubation period of one hour in a moist chamber at room temperature. Unused gels were abandoned after seven days of storage.

1.4.3 Bufer and pH selection

Electrophoresis was carried out in a continuous-pH buffer system, a uniform pH in the gel and at the electrodes. Discontinuity was provided by the different buffer compositions. The pH used by Poehling (1976) for his work with blackfly salivary glands was selected for this study.

Anode and cathode buffer solutions were prepared for the electrophoresis as described below, according to the recommendations of Poehling.

The solutions were stored for a maximum of three months at 4°C.

STOCK SOLUTIONS

D. Electrode (Cathode) Buffer Solution

3.0275 g Tris
200 mL H_2O
Glycine to pH 8.4
H_2O to 500 mL

E. Electrode (Anode) Buffer Solution

2.8M Tris
60 mL 1N H_2SO_4
6N H_2SO_4 to pH 8.4
H_2O to 100 mL

1.4.4 Sample preparation and application to the gel

Protein samples to be fractionated were prepared for electrophoresis as described in section 1.3.1.

The 9-12 mm of aqueous solution remaining above the soft gel tip after polymerization was completely removed prior to sample application. Hand drawn micro-capillary pipettes, tailored to fit into the 10 µL capillaries, were designed for this purpose, and for the following procedures. The entire empty capillary space was immediately filled with stock solution E. The top 8 mm of this was then removed and replaced with the protein solution to be fractionated. The volume of buffer removed, and hence volume of protein added, was calculated from the capillary diameter and from the exact penetration depth of a specially designed and constructed insertion-limited micro-pipette that could be introduded no further than 8 mm into the capillary. A micro-capillary pipette with a 1 mm insertion limit was used to withdraw the top fraction of the protein solution. The space was immediately filled with a 1mm layer of 20%(w/v) sucrose solution (Figures 14 and 15). The length of the protein solution applied was therefore consistently 7 mm. The capillary radius was 0.3175 mm, hence the volume of sample applied to each gel was calculated as:

$$7 \times 3.142 \times (.3175)^2 = 2.2 \text{ mm}^3 \text{ or } 2.2 \text{ µL}$$

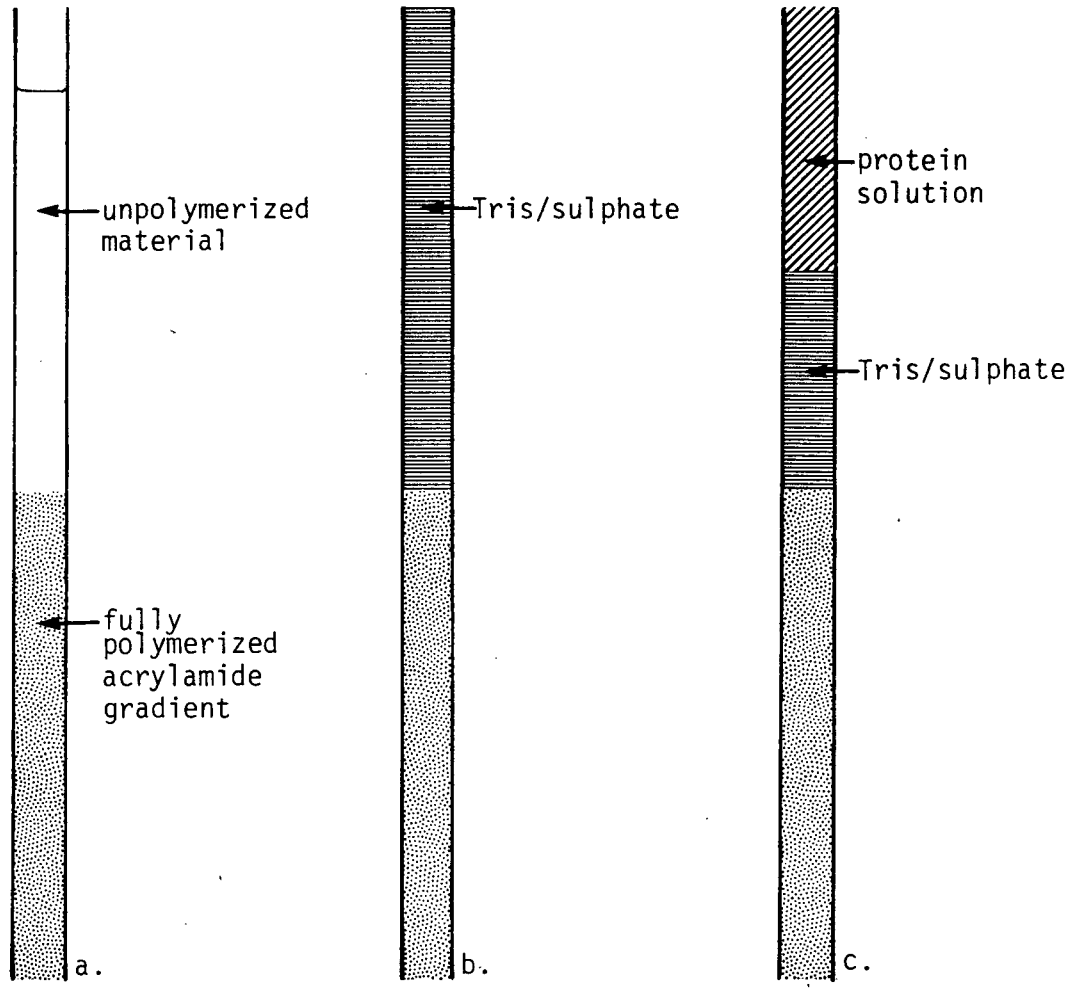


FIGURE 14: Sample loading procedure for 10 µL gels (I).

a. All unpolymerized material is removed from the top of the gel.

b. The empty space above the gel is filled with Tris/sulphate buffer (stock solution E).

c. 8 mm of Tris/sulphate is removed with an insertion-limited micro-pipette. The space above the buffer is filled with protein solution.

Scale 10:1 width; 4:1 length.

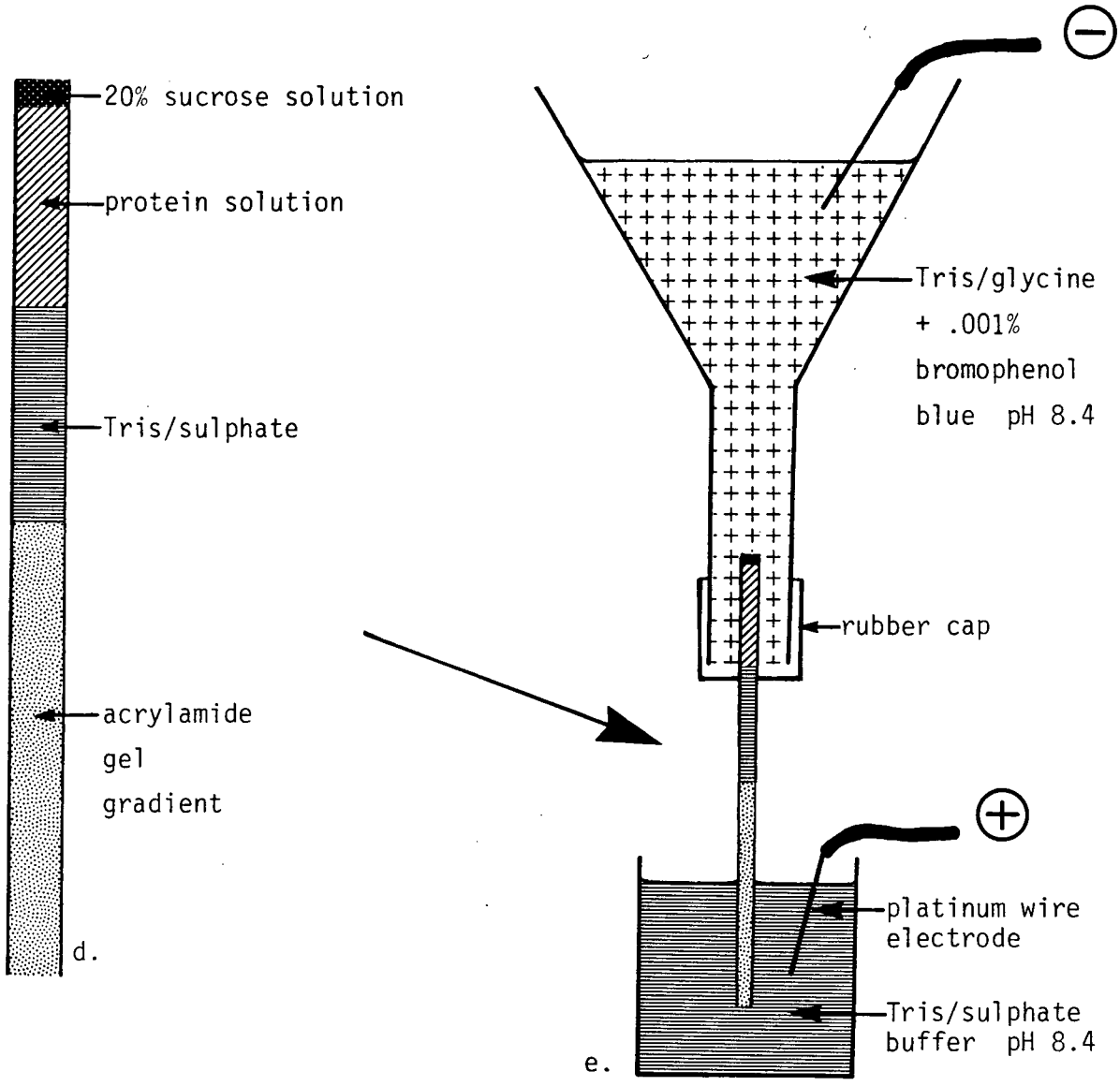


FIGURE 15: Sample loading procedure for 10 µL gels (II).

d. 1mm of protein solution is removed with an insertion-limited micro-pipette. The space above the protein solution is filled with 20% sucrose solution. Scale 10:1 width; 4:1 length.

e. The loaded capillary is inserted into a funnel containing Tris/glycine and .001% bromophenol blue. The lower end of the gel is inserted into a beaker containing Tris/sulphate buffer. Scale 2:1

Electrophoresis will not proceed through air bubbles; great care was taken not to introduce any air during the layering procedures described above.

1.4.5 Electrophoretic running conditions

The loaded capillary was mounted into the empty upper buffer funnel through a rubber funnel cap. The relatively high density of the sucrose solution effectively prevented any diffusion of the underlying protein as the funnel was filled with Tris/glycine (stock solution D). An addition of .001% bromophenol blue (Aldrich Inc., Milwaukee, Wis.) was made to the upper buffer to mark the progress of electrophoresis. Bromophenol blue binds tightly to the protein mixture at the beginning of the electrophoresis and then migrates with the buffer front.

The lower end of the capillary was inserted into a small beaker of Tris/sulphate (stock solution E). From one to ten capillaries were similarly mounted, in parallel, in the electrophoresis apparatus (Figure 16).

The platinum wire electrodes were connected with the anode in the lower buffer chamber and the cathode in the upper buffer funnel.

A direct current was supplied from a constant voltage power pack (Ernst Schütt, Göttingen, W. Germany). The unit, made specifically for this scale of electrophoresis, was capable of simultaneously monitoring both the current flowing through individual capillaries and the total current flowing through the whole system (Figure 17).

The gels were given a 10 minute concentrating period at 40 volts. Fractionation was conducted at a constant 80 volts for 60-80 minutes.

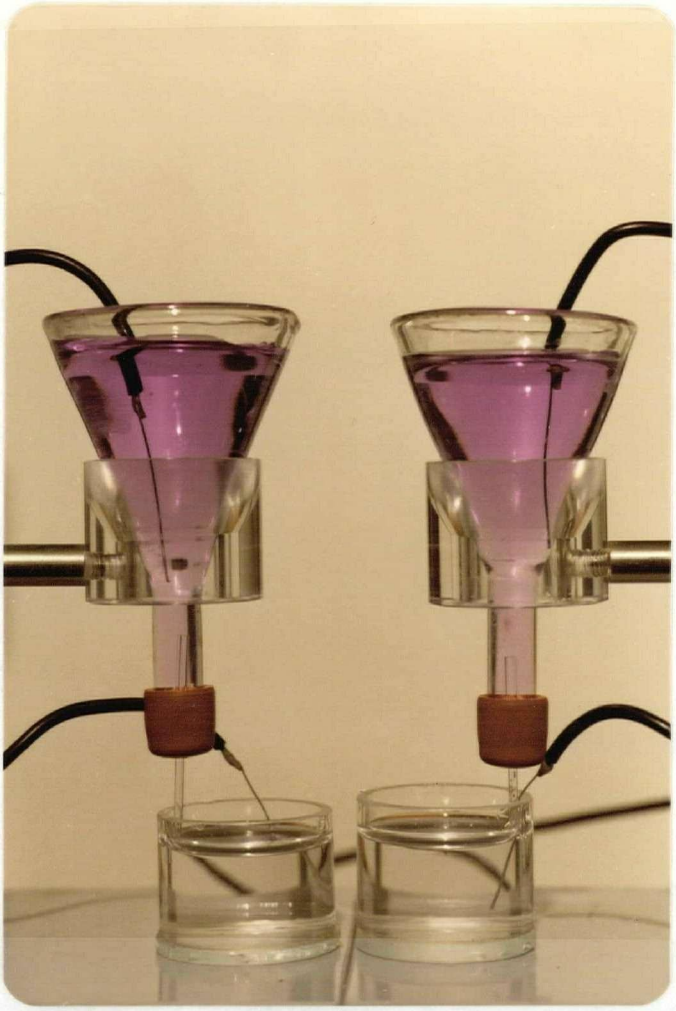


FIGURE 16: Support system for running capillary gels. Upper funnel contains Tris/ glycine + .001% bromopenol blue. Lower beaker contains Tris/ sulphate.

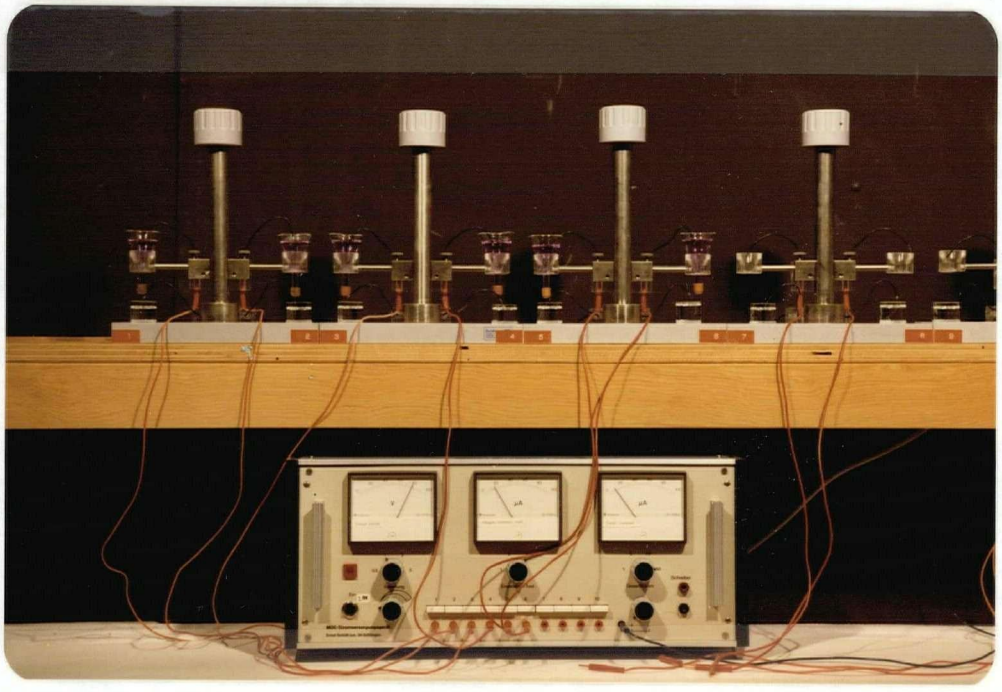


FIGURE 17: Power pack and capillary support apparatus for running 10 gels in parallel.

Electrophoresis was considered complete when the bromophenol blue front had passed through the entire gel into the lower buffer reservoir.

The total current recorded at the beginning of each run was approximately 3.4 mA, with all ten gels running. This initial current reading was carefully monitored from gel to gel. Discrepancies in individual gel current readings were considered to be an indication of possible contact failure, usually caused by air bubbles in the system. Any dubious gels were disregarded.

1.4.6 Gel staining and storage

Separate solutions were prepared for the staining and storage of gels as described below (Poehling 1976). The solutions were stored for a maximum of three months at 4°C.

STOCK SOLUTIONS

G. Gel staining solution

200 mg Coomassie brilliant blue R (ICN, Irvine, Ca.)
50 mL Methanol
10 mL Acetic acid
H_2O to 100 mL

F. Gel storage solution

7.5 mL Acetic acid
5 mL Methanol
H_2O to 100 mL

On completion of electrophoresis, the gels had to be extruded from the capillaries with an even force which was sufficient to dislodge the gel without causing any damage to the gel matrix. Several widths of steel wire

were tested for this purpose and a 25/1000 inch (0.635 mm) piano wire, cut to a length of 50 mm, was found to fit perfectly inside the tubes for this purpose. The gels were extruded directly into the staining solution G, covered, and incubated at 50°C for 20 minutes.

A Pasteur pipette was used to transfer individual gels from the staining solution to 15 mL plastic vials containing solution F. Sufficient stain was added to each vial to give a weak blue colouration.

Gels were stored in this solution at 4°C in the dark.

1.4.7 SDS electrophoresis

The anionic detergent sodium dodecyl sulphate (SDS), when used as an additive to polyacrylamide gel techniques, binds to the surface of the proteins during fractionation and cancels their charge. Hence, separation in an SDS system is based primarily on particle size.

SDS was incorporated into both the protein sample and into the cathode buffer reservoir. It was not added to the gel, as the supply from the upper buffer provided sufficient stability to the SDS-protein complex (Neuhoff 1973; Ruchel et al. 1974).

The samples to be fractionated in an SDS system were incubated with 1%(w/v) SDS and 1%(v/v) mercaptoethanol (Polysciences Inc., Warrington, Pa.) at 100°C for two minutes, immediately prior to use. SDS loaded protein was applied to the gel as described in section 1.4.4.

All buffer solutions were as previously described for regular runs with the exception of stock solution D (the cathode buffer), which was loaded with .1% SDS and .1% mecaptoethanol. Bromophenol blue was incorporated as a .001%(w/v) solution to mark the moving buffer front.

Electrophoresis was conducted at a constant 50 volts.

1.5 RECORDING OF GELS

Evaluation of the gels was performed densitometrically after an initial visual charting.

1.5.1 Visual recordings

A dissecting microscope, equipped with fluorescent illumination, was used to view and chart the gel before densitometric evaluation. The gel was transferred with a Pasteur pipette from the storage vial into a petri-dish containing 7.5%(v/v) acetic acid. The gel was aligned against a mm scale and charted (using a 5:1 scale) for total length and relative migration distance of each visibly stained protein band. The width and relative intensity of each band was also recorded.

1.5.2 Micro-densitometric scanning

A Joyce MK 111C automatic recording microdensitometer was used to scan the gel and plot density versus position in the form of a graphical trace on an integral chart recorder (Joyce-Loebl Ltd., Gateshead, U.K.). The instrument operated on a double-beam system with a calibrated grey wedge as an internal measuring standard.

The specimen table and chart recorder table were mechanically linked in a gearing ratio of 1:20, which expanded the tracing scale by a factor of 20. Two runs of the recording table were made for each complete specimen scan.

The standard Joyce microdensitometer required certain adaptations before it could be used to record the optical density of micro-gels.

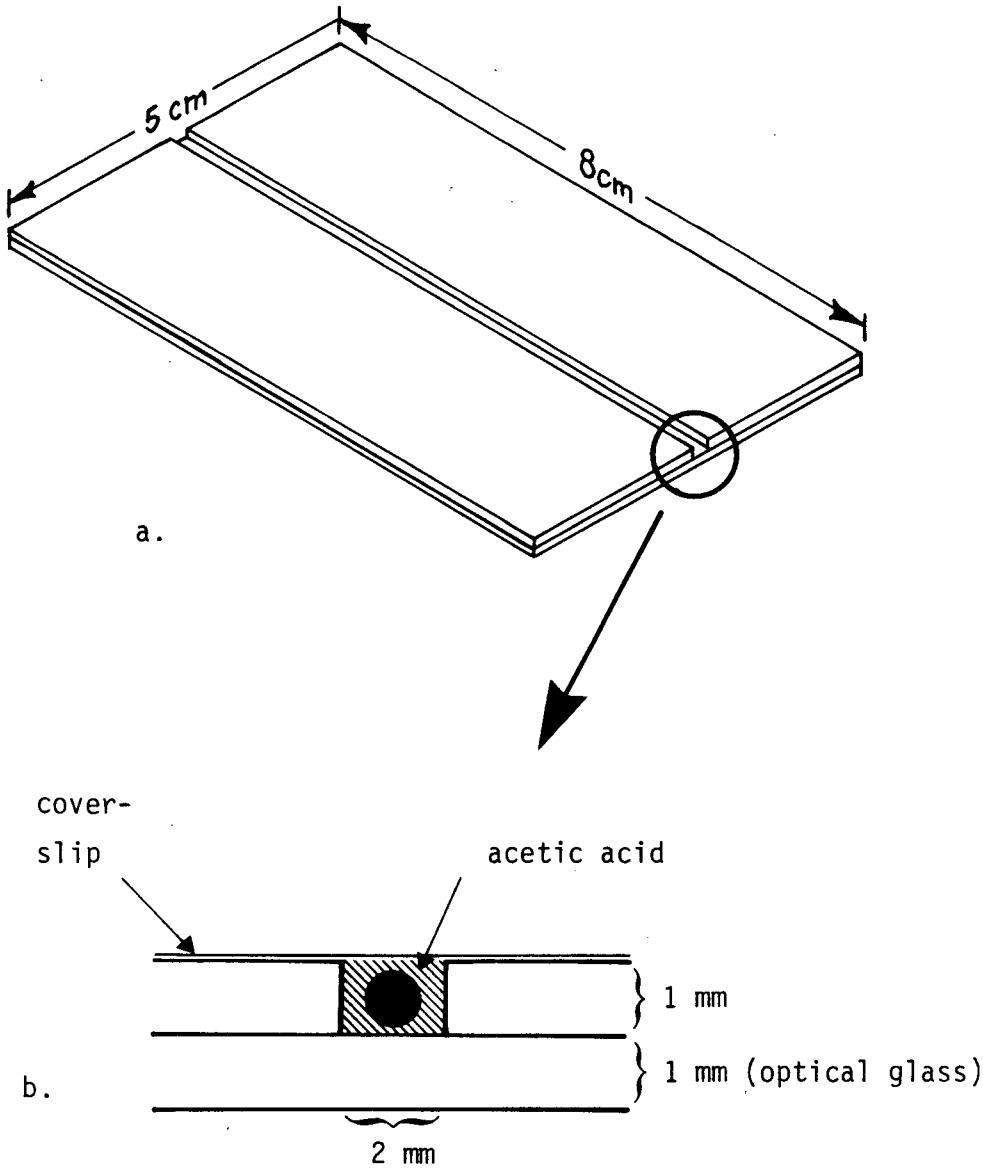


FIGURE 18: Specimen holder for Joyce-Loebl microdensitometer.

a. Isometric line drawing of custom-made gel holder. Scale 1:1.

b. End view of trough and gel submerged in 7.5% acetic acid. The trough ends are sealed with epoxy-resin and tape before the gel is placed in position. Scale 10:1.

A wedge of realtively low optical density, 0-0.5 OD, was selected as a suitable internal measuring standard. It was necessary to specially design and construct an optical-glass specimen support plate to hold a capillary gel on the flat-bed specimen table of the instrument. This plate was assembled from sections of optical glass glued together to create a 2 mm wide and 1 mm deep glass-floored trough (Figure 18). The ends of the trough were sealed with epoxy-resin. The edges of the entire holder were glued and subsequently wrapped in acid-proof tape.

The gel was lifted into the trough with a Pasteur pipette and immediately covered with 7.5% acetic acid to prevent dehydration. A 22 mm x 40 mm glass cover-slip was positioned over the gel. By careful design and construction, the narrowness of the trough prevented any curvature of the gel during the tracing procedure.

A X20 optical magnification of the gel image on the slit was used to monitor the alignment of the gel during the trace. An effective slit width (physical aperture ÷ optical magnification) of approximately one micron was used for all tracings.

1.6 MOLECULAR WEIGHT ESTIMATION METHODS

The gradation of pore sizes in the acrylamide matrix of each gradient gel allowed migration of proteins according to their molecular weights. Standard proteins of known molecular weight were fractionated on 10 µL gradient gels for comparison with unknown sample proteins. The following marker proteins of known molecular weight (MW) were used:

Bovine plasma albumin .....MW 66,000
Ovalbumin ..... MW 45,000
Pepsin ..... MW 34,700
Beta-Lactoglobulin (sub-unit) .....MW 18,400
Lysozyme ..... MW 14,300
(Marker proteins were obtained from Sigma, St. Louis, Mo.)

Each marker protein was freshly prepared with distilled water at a concentration of 0.2 µg/µL. The total protein load applied to each gel was 0.44 µg, based on a sample volume of 2.2 µL. The electrophoretic running conditions were identical with those already described in section 1.4.5.

Log MW was plotted against electrophoretic mobility (migration distance) for each protein listed above. The relationship obtained was used as a calibration curve for the estimation of unknown protein molecular weights (Andersson et al. 1972; Kopperschlager et al. 1969; Lorentz 1976; Neuhoff 1973; Ruchel et al. 1973). This method of molecular weight estimation compares native proteins according to their Stokes Radii, which are responsible for the migration properties of all but elongate protein molecules (Felgenhauer 1974). The advantage of this method, compared with

MW determination in an SDS system on homogenous gels, is that the proteins are not broken down into individual sub-units. Also, with SDS systems, variations can exist in SDS/protein binding properties that may create erroneous molecular weight estimations (Andersson et al. 1972).

1.6.1 Digital planimeter measurements of MW groupings

A Talos 600 Series Cybergraph Unit (Talos Systems Inc., Scottsdale, Ariz.), capable of converting the physical position of a cursor on an activated digitizer surface into digital output, was used to measure the relative areas of 12 molecular weight groups on each densitometric tracing. The digitizing system was programmed with a Hewlett-Packard HP9845 desktop computer (Hewlett-Packard, Mississuaga, Ontario) to calculate the perimeter and area of any complete densitometric tracing. The migration limits for the selected MW groupings were inscribed onto an acetate template which was superimposed onto the densitometric tracing for the gel. The individual area measurements, subsequently taken from the limits imposed by the calculated MW groupings, were automatically presented as percentages of the total area already measured. In this way a measurement of relative area was made for each of the 12 MW groups on every gel. The Talos digitizing system provided extremely high accuracy and stability with resolution to more than 1000 points per inch (394/cm).

1.7 SKIN REACTION TESTS WITH SALIVARY GLAND MATERIAL IN GUINEA-PIGS

Thirteen female, albino guinea-pigs, with individual weights between 300-400 g, were used in the study. The test animals were maintained indoors in a fly-free controlled-environment rearing room.

1.7.1 Sensitization of guinea-pigs with whole fly extract

Approximately 50 female adult S. vittatum were ground in one mL of physiological saline (0.85% NaCl). The suspension was centrifuged in a Fisher Model 59 bench-top centrifuge (Fisher, Vancouver, B.C.), for 10 minutes at 4,000 xg. A 0.5 mL volume of the supernatant was added to an equal volume of 50% Freund's Complete Adjuvant (BDH, Vancouver, B.C.). The mixture was vortexed immediately prior to use.

Four guinea-pigs were left unsensitized as future controls. The remaining animals were each injected, intramuscularly, with 1.5 µL of the whole fly preparation.

1.7.2 Skin tests with specific salivary gland material

A 30-day period was left between sensitization and skin testing. The animals were prepared 12 hours beforehand. Hair was removed from the back and flanks with small animal clippers. The remaining stubble was cleared with Nair depilatory cream (Trade Mark of Carter-Wallace, New York, NY.).

Fifteen pairs of salivary glands from each post-emergence age group of flies were macerated in physiological saline, as described in section 1.3.1, and made up to 2.6 mL with saline.

Whole-fly extracts were prepared, as described for sensitization but without the Freund's adjuvant, in a 1:10 dilution with saline.

The sensitized and control guinea-pigs were injected intradermally with 0.2 mL of each test material. Eight different skin tests were given to each animal in the sequence illustrated in Figure 19.

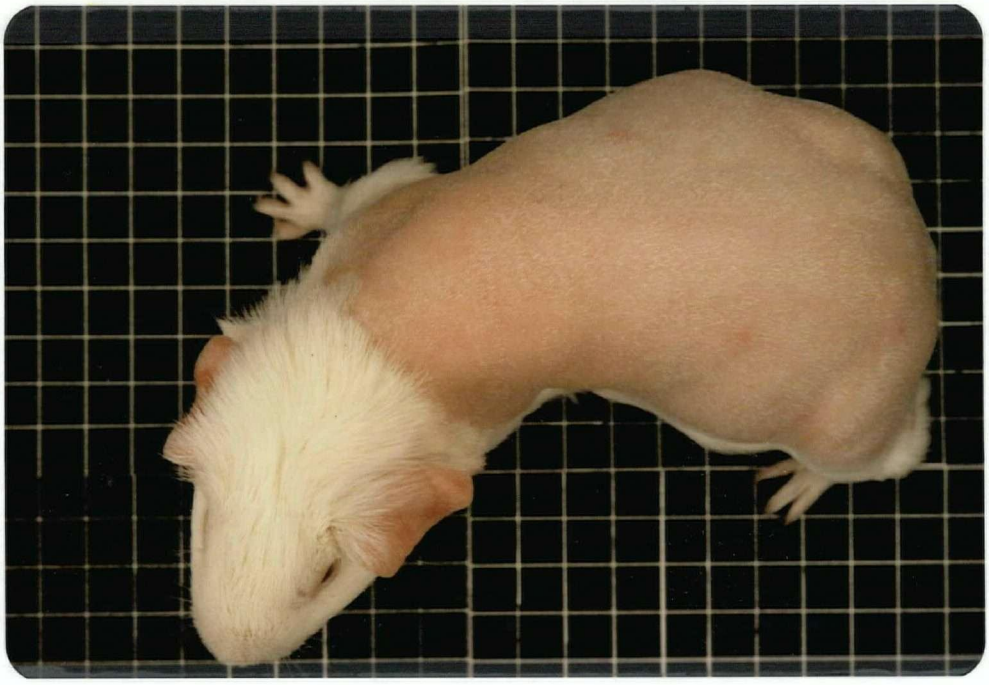
1.7.3 Measurement of skin reactions

The guinea-pig skin responses to the macerated salivary glands were assessed in several ways.

Firstly, measurements of erythema diameter were taken at regular intervals from both the control and sensitized animal groups. These measurements were made as diameters of the total area of redness after 30 minutes, and later at 1, 2, 4, 10, 24, and 48 hours. A flexible plastic ruler was used for measurements, and two such estimates were averaged for each skin test recorded at each time interval. All measurements were made "blind" (the skin-test reader was unaware of which response was being measured).

In addition to these quantitative measures, each skin test was also monitored for certain non-quantitative manifestations, such as hard raised lesions, inflammation, and intensity of induration.

The 30 minute skin-test readings were intended to define the presence or absence of an immediate reaction to the injected glandular materials; the 10 hour readings were used as a measure of the Arthus response and the 24 hour readings were intended to define the presence or absence of a delayed reaction to each of the injected glandular preparations.



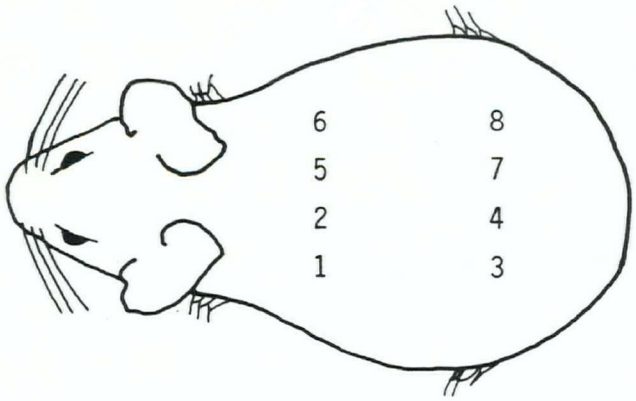


FIGURE 19: Skin-testing sites and materials.

Left flank:	1.	Salivary	glands	from	day	0	flies.
	2.	"	"	"	"	1	"
	3.	"	"	"	"	2	"
	4.	"	"	"	"	3	"
Right flank:	5.	"	"	"	"	4	"
	6.	"	"	"	"	5	"
	7. Physiological saline.						
	8. Whole fly extract X1/10.						

2. RESULTS

2.1 PROTEIN ASSAYS

The protein estimations derived from Brewer's modified Lowry assay method are presented in Tables I and II. As indicated in section 1.3.3 the total protein is represented by the sum of two components measured by the biuret and phenol tests respectively.

2.1.1 Total protein content of salivary glands

Correlation coefficients for each assay, as related to the prepared curves for bovine serum albumin, are presented in Tables I and II. It is to be noted that the correlation coefficients for all adult assays are in reference to a common standard. The number of points used to fit the standard curve was dependent on the level of assay used. The larval salivary glands were proved to have a sufficiently high protein content to validate the use of the high range assay sensitive to protein in the range of 20-600 µg/mL. Pupal and adult salivary glands were found to have a protein content within the sensitivity of the low range assay (1-20 µg/mL).

The average protein content per gland, as presented in Tables I and II, represents the pooled results of 12 (four lots of three) glands in the larval assays; 24 (four lots of six) in the pupal assays; and 20 (four lots of five) in the adult assays.

TABLE I: Total protein content of *S. decorum* salivary glands.

Assay material	n for fitting standard curve	Correlation coefficient for standard curve	Av. protein content per gland (µg), and standard error of the mean (for 4 assays)
Larva	11	.9925	24.0 ± 0.84
Pupa	7	.9912	1.5 ± 0.01
Adult:			
Day 0	7	.9813	2.5 ± 0.03
Day 1	7	.9813	2.8 ± 0.01
Day 2	7	.9813	2.9 ± 0.02
Day 3	7	.9813	3.0 ± 0.04
Day 4	7	.9813	4.5 ± 0.04

TABLE II: Total protein content of *S. vittatum* salivary glands.

Assay material	n for fitting standard curve	Correlation coefficient for standard curve	Av. protein content per gland (µg), and standard error of the mean (for 4 assays)
Larva	11	.9959	20.0 ± 0.77
Pupa	7	.9912	0.9 ± 0.05
Adult:			
Day 0	7	.9908	1.3 ± 0.07
Day 1	7	.9908	1.6 ± 0.04
Day 2	7	.9908	1.7 ± 0.01
Day 3	7	.9908	2.5 ± 0.07
Day 4	7	.9908	2.6 ± 0.01
Day 5	7	.9908	1.7 ± 0.06

Footnote: All correlation coefficients are significant at 0.01 probability level.

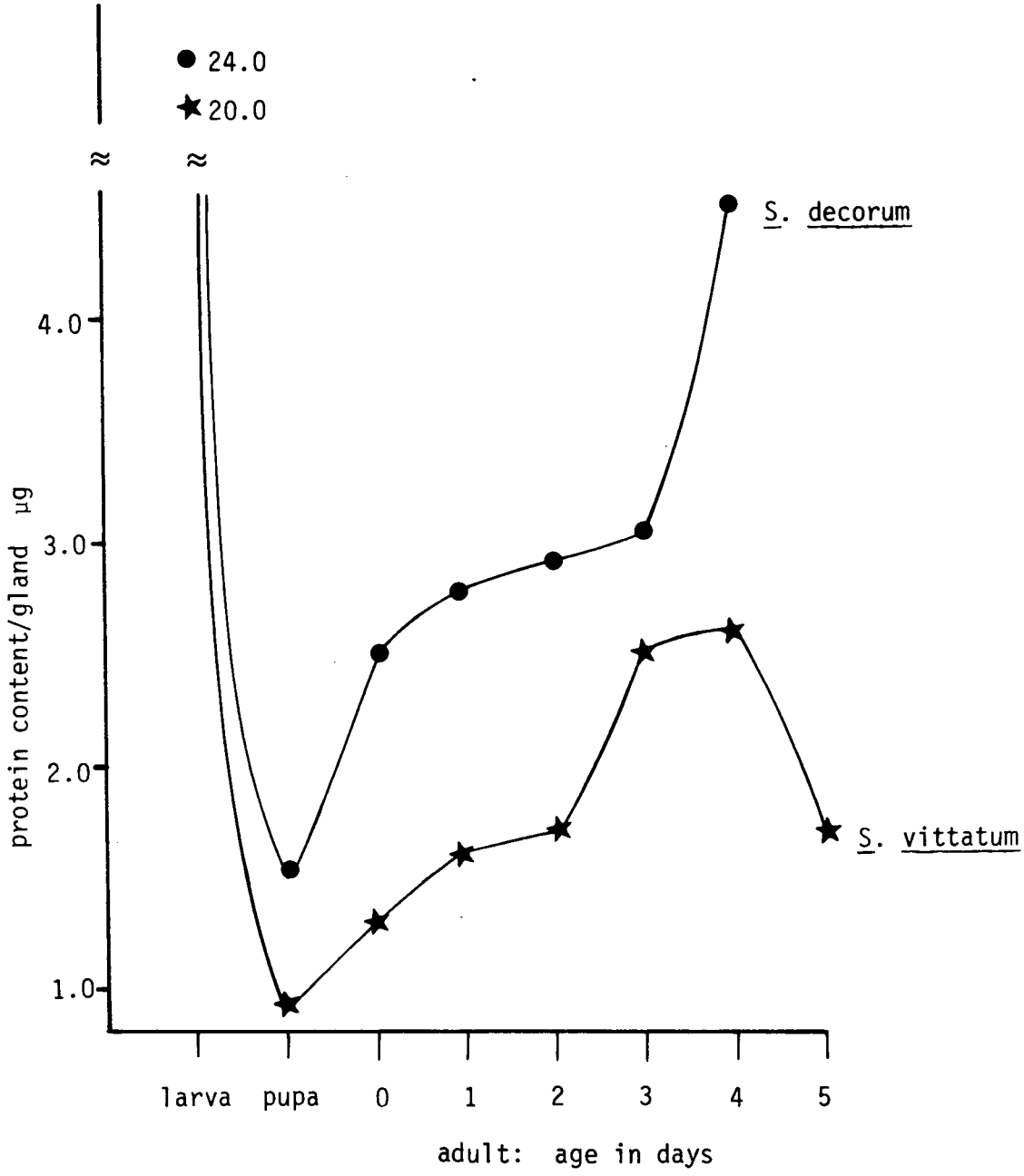


FIGURE 20: Average total protein content of S. decorum and S. vittatum salivary glands in relation to metamorphic stage and age.

2.1.2 Water-soluble protein content of adult salivary glands

The water-soluble and total protein contents for each age-group are presented for comparison in Tables III and IV. The calculation of average water-soluble protein content per gland represents the pooled results of 20 glands (four lots of five) within each age-group listed in the tables below.

TABLE III: Water-soluble protein of S. decorum salivary glands compared with total protein content.

Assay material	Average total protein per gland (Table I) µg	Average water-soluble protein content per gland (µg), and standard error of the mean (for 4 assays)	% water-soluble protein of total protein
Day 0	2.5	1.5 ± 0.06	62
Day 1	2.8	1.8 ± 0.06	65
Day 2	2.9	1.8 ± 0.05	62
Day 3	3.0	2.0 ± 0.08	66
Day 4	4.5	3.0 ± 0.09	66
		Average %	64.4

TABLE IV: Water-soluble protein of S. vittatum salivary glands compared with total protein content.

Assay material	Average total protein per gland (Table II) µg	Average water-soluble protein content per gland (µg), and standard error of the mean (for 4 assays)	% water-soluble protein of total protein
Day 0	1.3	0.9 ± 0.11	69
Day 1	1.6	1.2 ± 0.06	75
Day 2	1.7	1.3 ± 0.04	76
Day 3	2.5	1.9 ± 0.03	76
Day 4	2.6	2.1 ± 0.33	80
Day 5	1.7	1.3 ± 0.04	76
		Average %	76.3

2.2 VISUAL AND DENSITOMETRIC RECORDINGS OF SALIVARY GLAND PROTEIN SEPARATIONS

Throughout this section a scale of 10:1 has been used for the presentation of both the visual charting and the corresponding densitometric scans. The scans and visual chartings were originally recorded at scales of 20:1 and 5:1 respectively. It should be noted that all information regarding protein separations was read from the original scans and not from the reduced reproductions included in this text.

In a confirmatory test of the non-dependence of separations on the inherent charges of the component proteins, the electrophoretic patterns were not altered by the addition of sodium dodecyl sulphate. The results presented here were obtained without the addition of SDS.

2.2.1 Electrophoretic separation of S. decorum salivary gland proteins

Table V presents the total protein weight applied to each gel as calculated from the results of the corresponding protein assay. The volume of protein applied was always 2.2 µL (see section 1.4.4).

TABLE V: Sample concentration and load of S. decorum salivary gland material for electrophoresis.

Sample	No. of macerated glands; made up to 100 µL with H_2O	Conc. of sample µg/µL	Total protein per 2.2 µL application µg
Larva	2	0.48	1.06
Pupa	15	0.23	0.51
Adult:			
Day 0	10	0.25	0.55
Day 1	10	0.28	0.62
Day 2	10	0.29	0.64
Day 3	10	0.30	0.66
Day 4	10	0.45	0.99

Protein patterns obtained from the electrophoretic separation of S. decorum larvae (Figure 21), pupae (Figure 22), and adults (Figures 23-27) are presented in this section. The visual charting is included beneath the revelant densitometric scan.

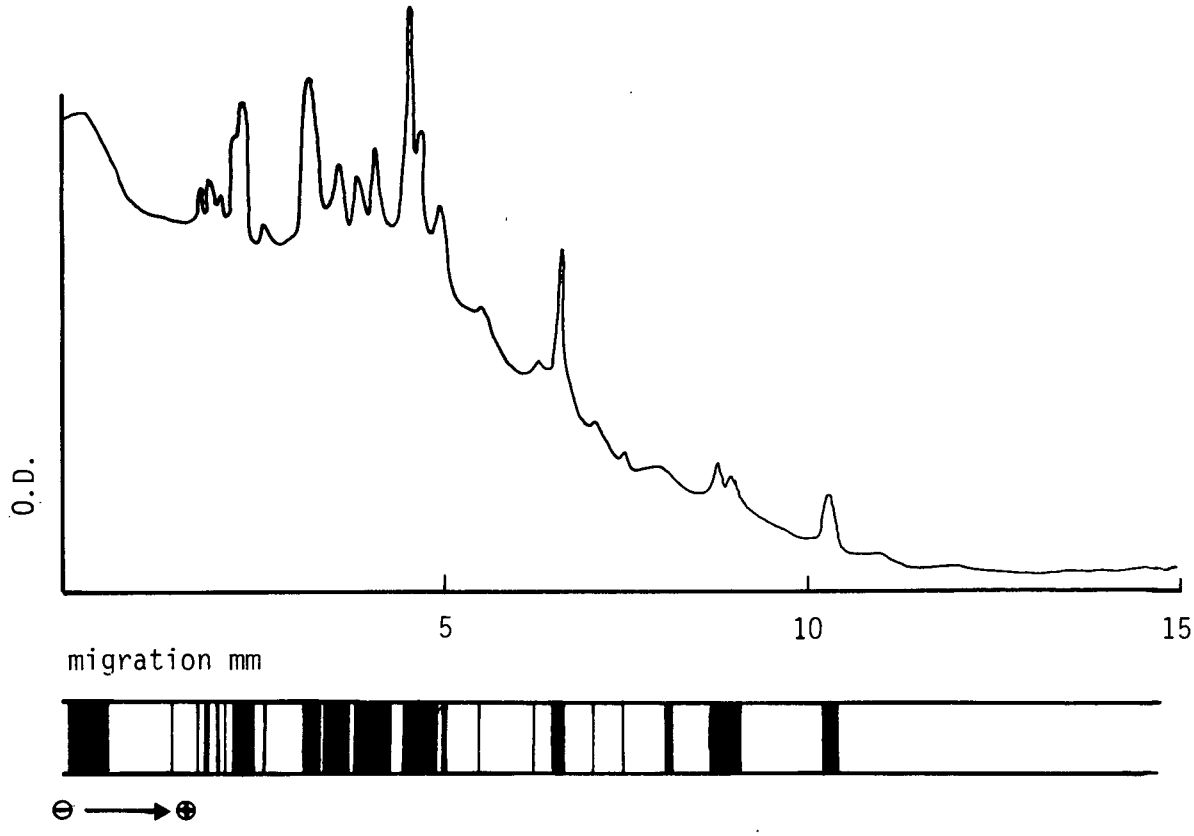


FIGURE 21: Electrophoretic separation of larval S. decorum salivary gland proteins.

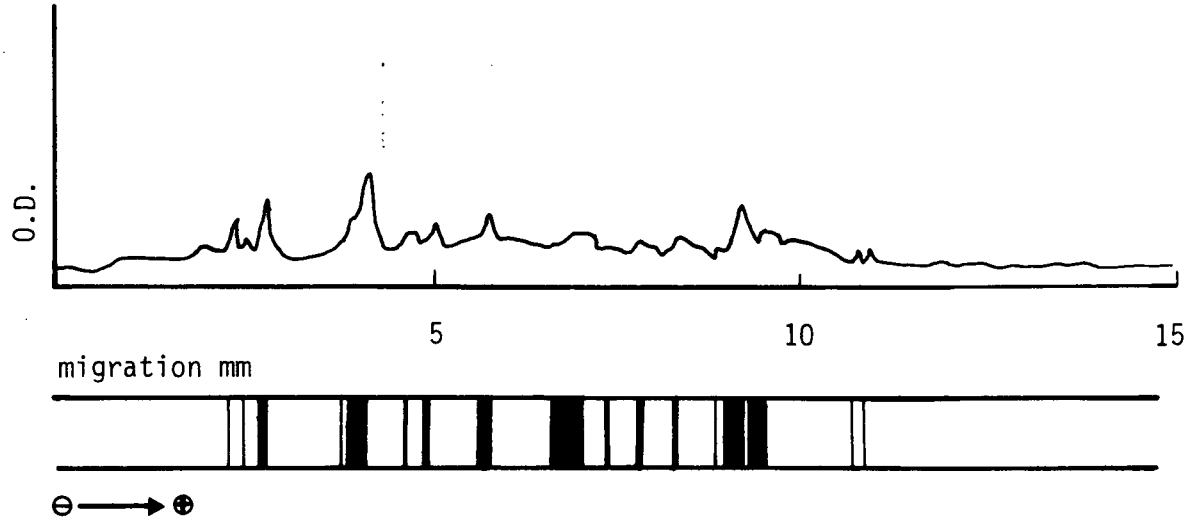


FIGURE 22: Electrophoretic separation of pupal S. decorum salivary gland proteins.

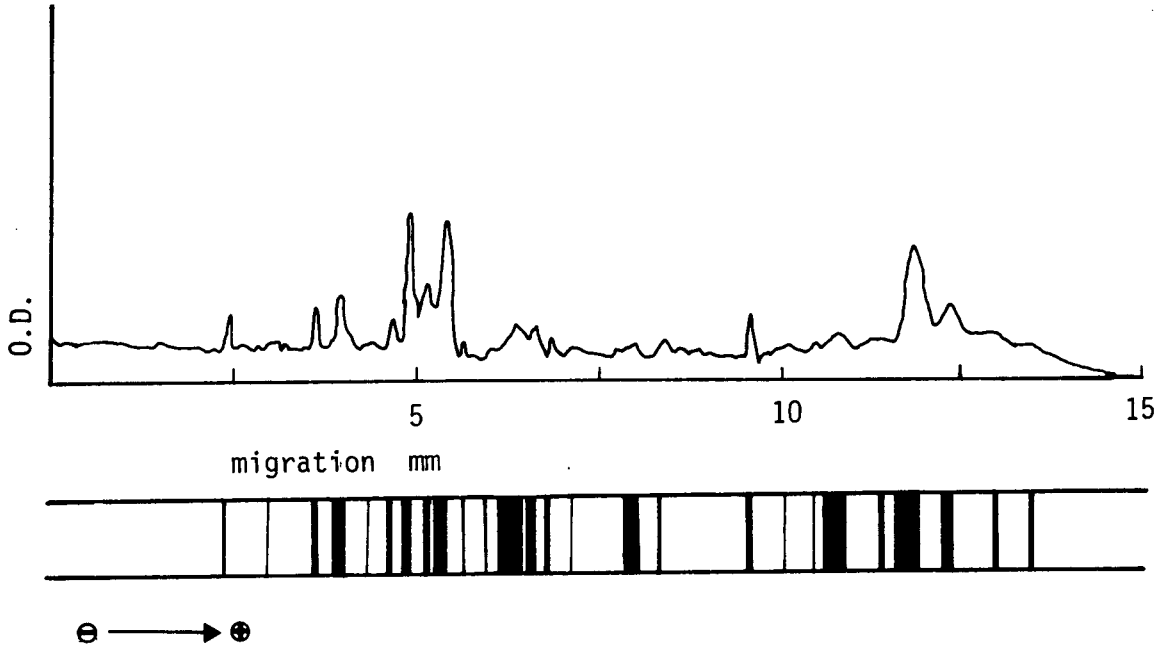


FIGURE 23: Electrophoretic separation of day 0 S. decorum salivary gland proteins.

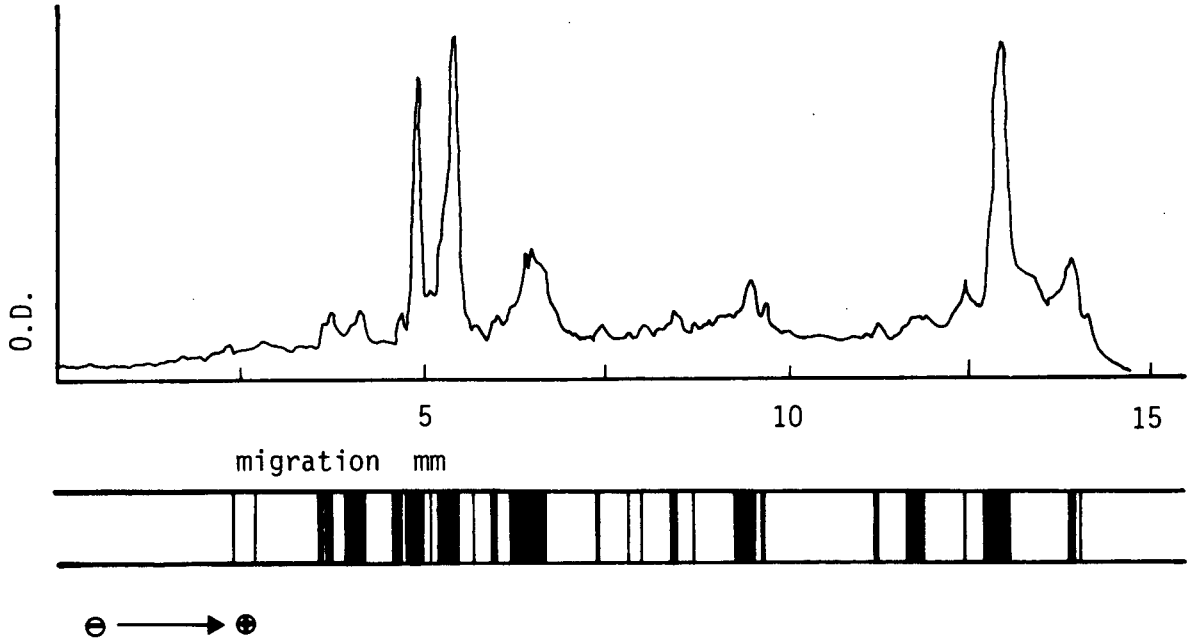


FIGURE 24: Electrophoretic separation of day 1 S. decorum salivary gland proteins.

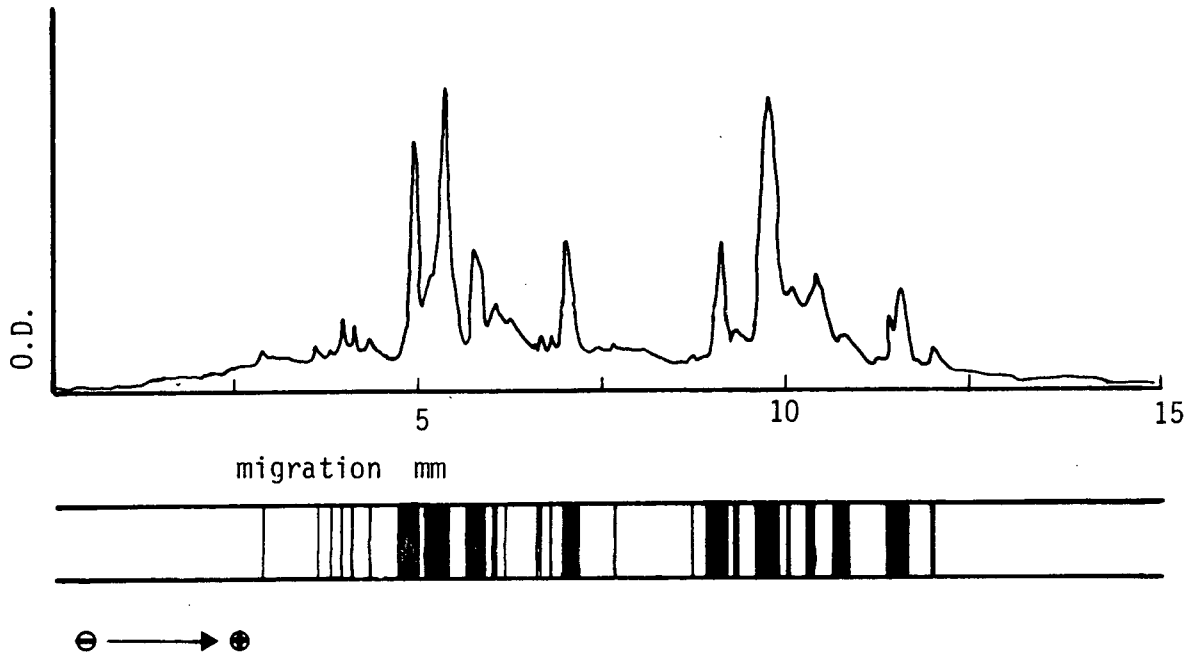


FIGURE 25: Electrophoretic separation of day 2 S. decorum salivary gland proteins.

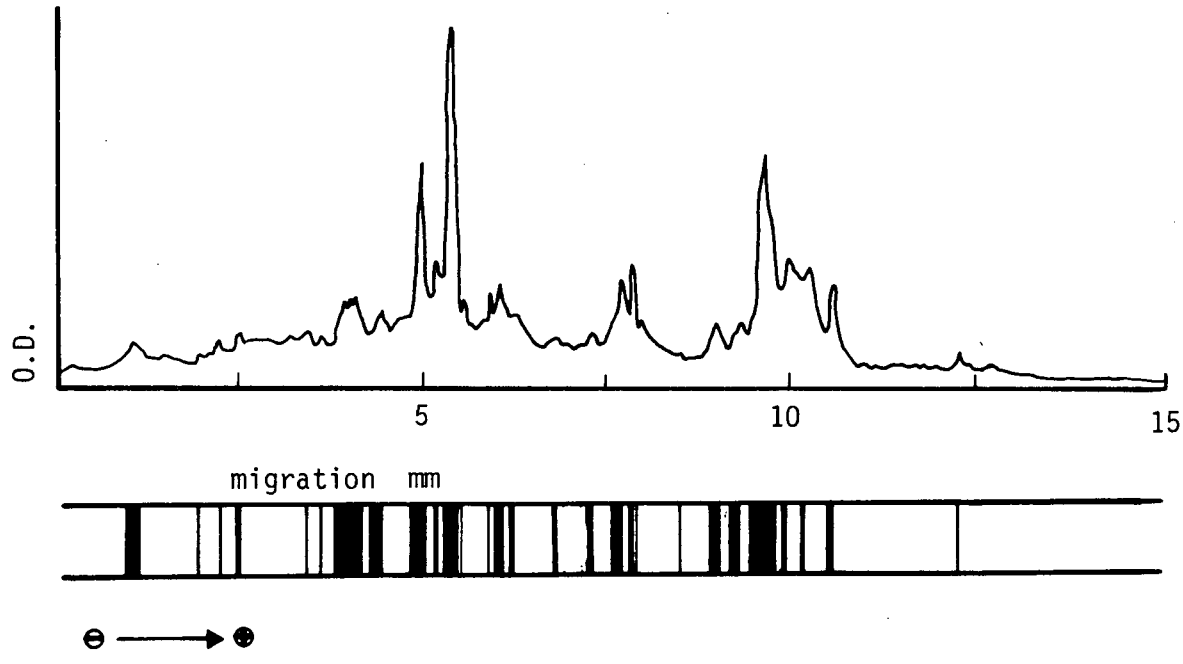


FIGURE 26: Electrophoretic separation of day 3 S. decorum salivary gland proteins.

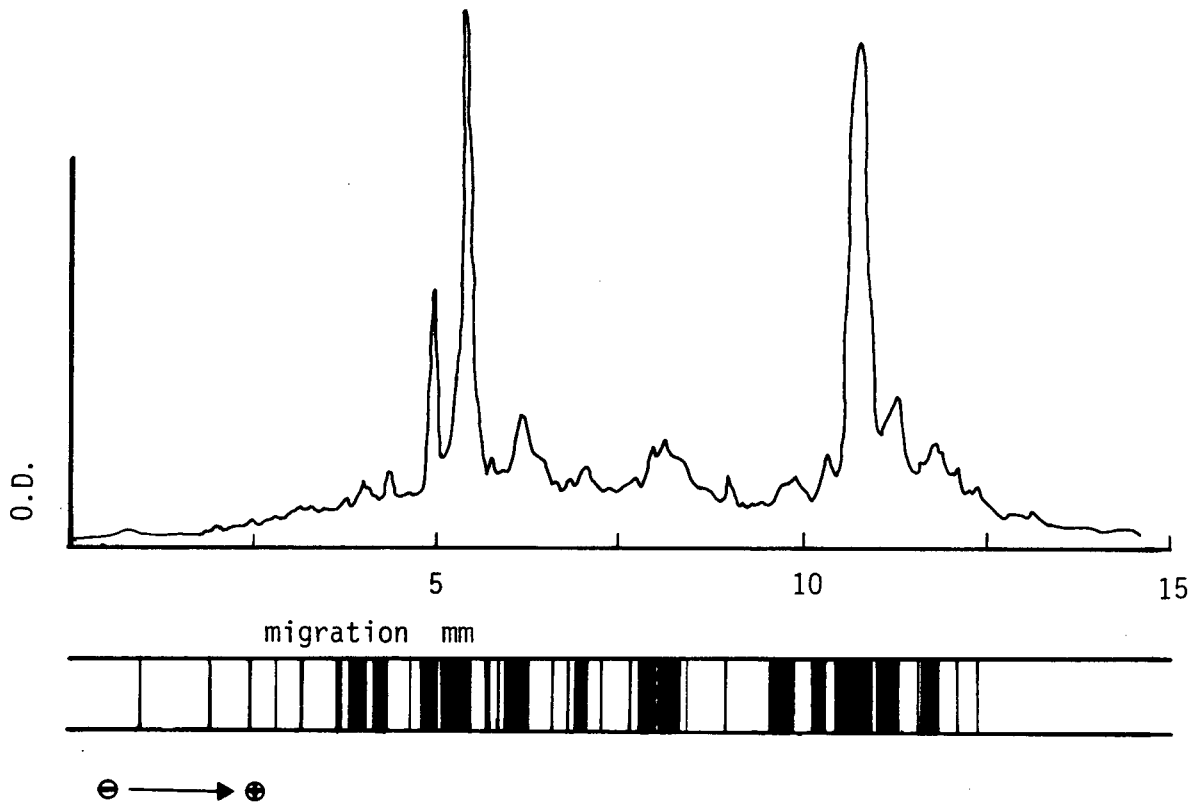


FIGURE 27: Electrophoretic separation of day 4 S. decorum salivary gland proteins.

2.2.2 Electrophoretic separation of S. vittatum salivary gland proteins

Table VI presents the total protein weight applied to each gel as calculated from the results of the corresponding protein assay.

TABLE VI: Sample concentration and load of S. vittatum salivary gland material for electrophoresis.

Sample	No. of macerated glands; made up to 100 µL with H_2O	Conc. of sample µg/µL	Total protein per 2.2 µL application µg
Larva	2	0.40	0.88
Pupa	15	0.14	0.20
Adult:			
Day 0	10	0.13	0.29
Day 1	10	0.16	0.35
Day 2	10	0.17	0.37
Day 3	10	0.25	0.55
Day 4	10	0.26	0.57
Day 5	10	0.17	0.37

Protein patterns obtained from the electrophoretic separation of S. vittatum larvae (Figure 28), pupae (Figure 29) and adults (Figures 30-35) are presented in this section. The visual charting is included beneath the relevant densitometric scan.

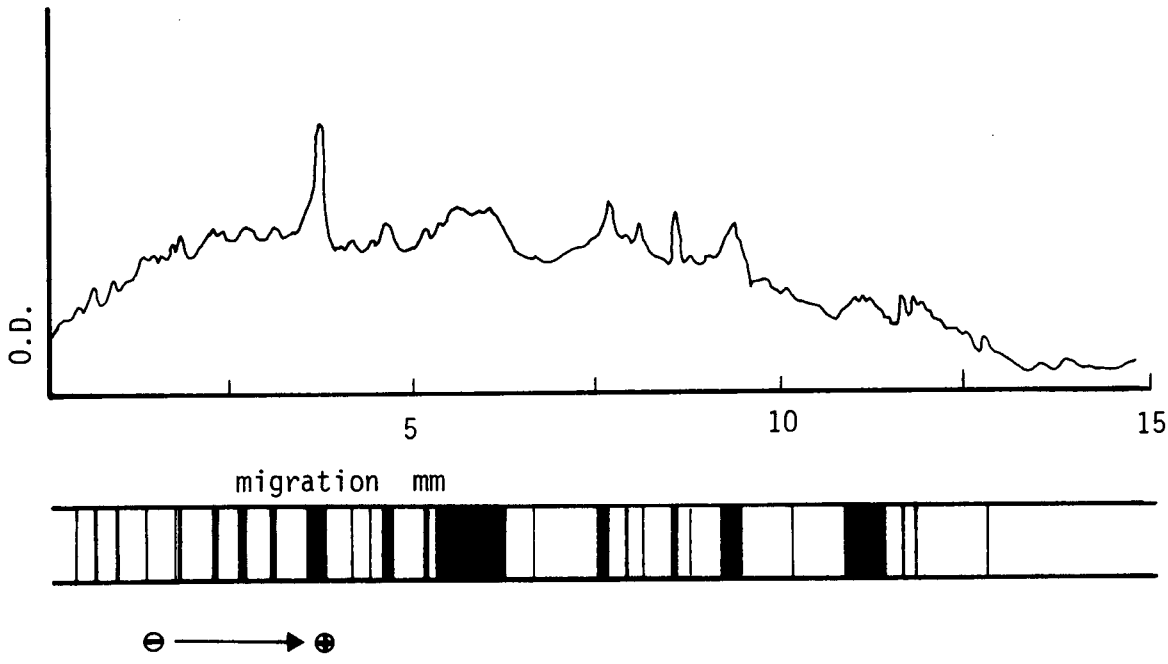


FIGURE 28: Electrophoretic separation of larval S. vittatum salivary gland proteins.

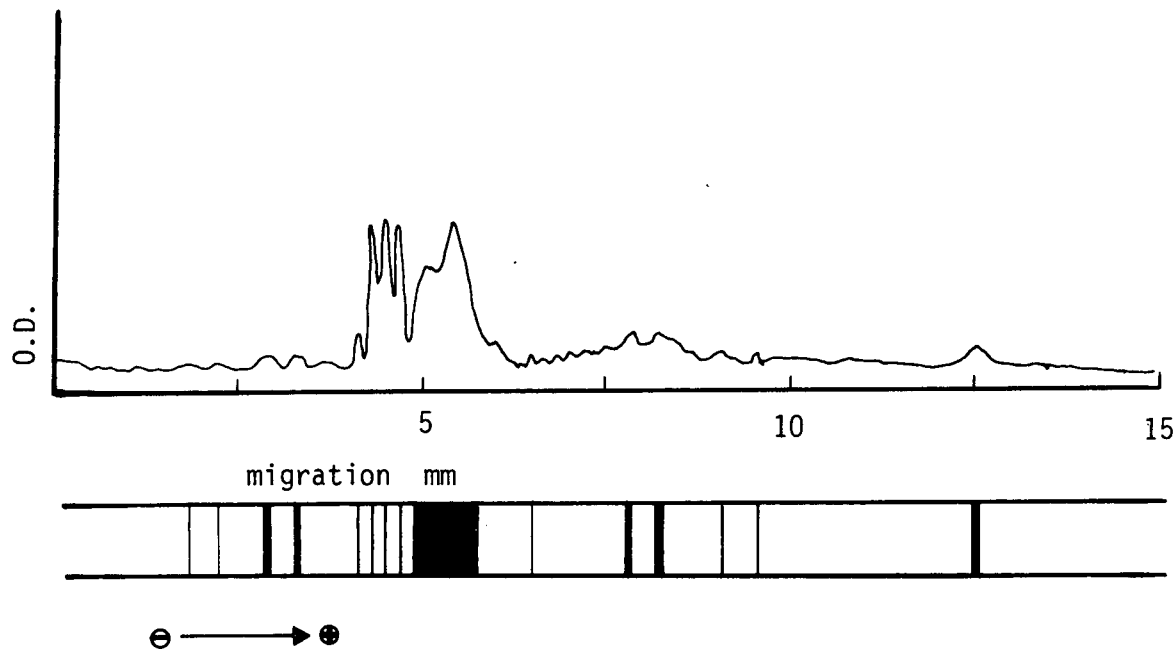


FIGURE 29: Electrophoretic separation of pupal S. vittatum salivary gland proteins.

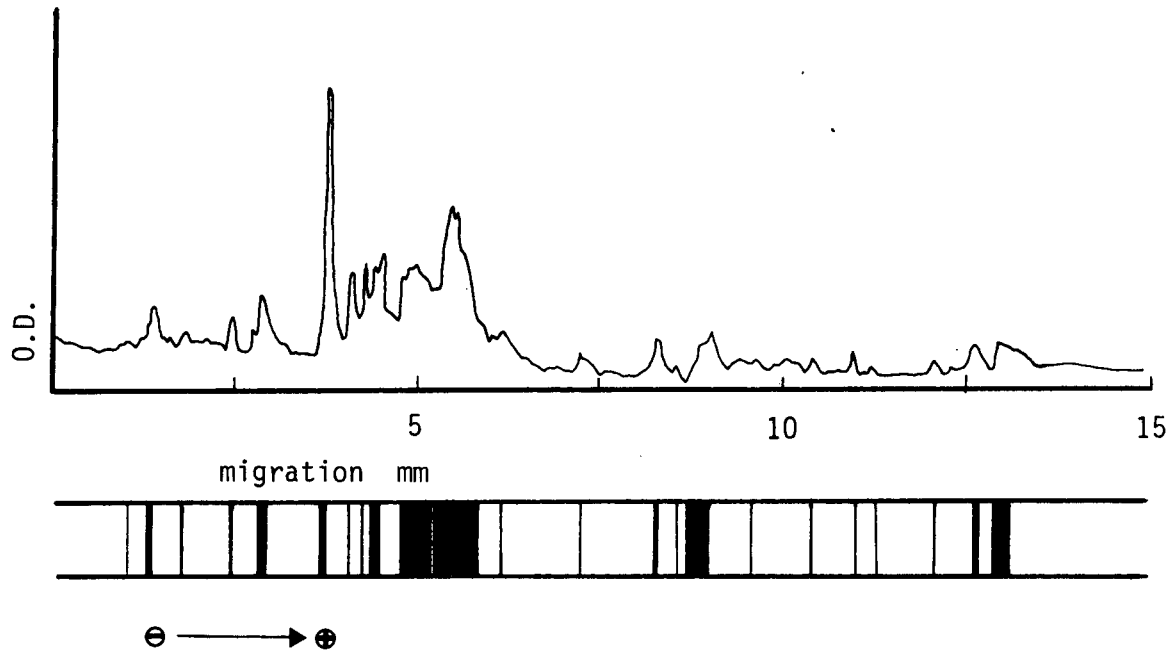


FIGURE 30: Electrophoretic separation of day 0 S. vittatum salivary gland proteins.

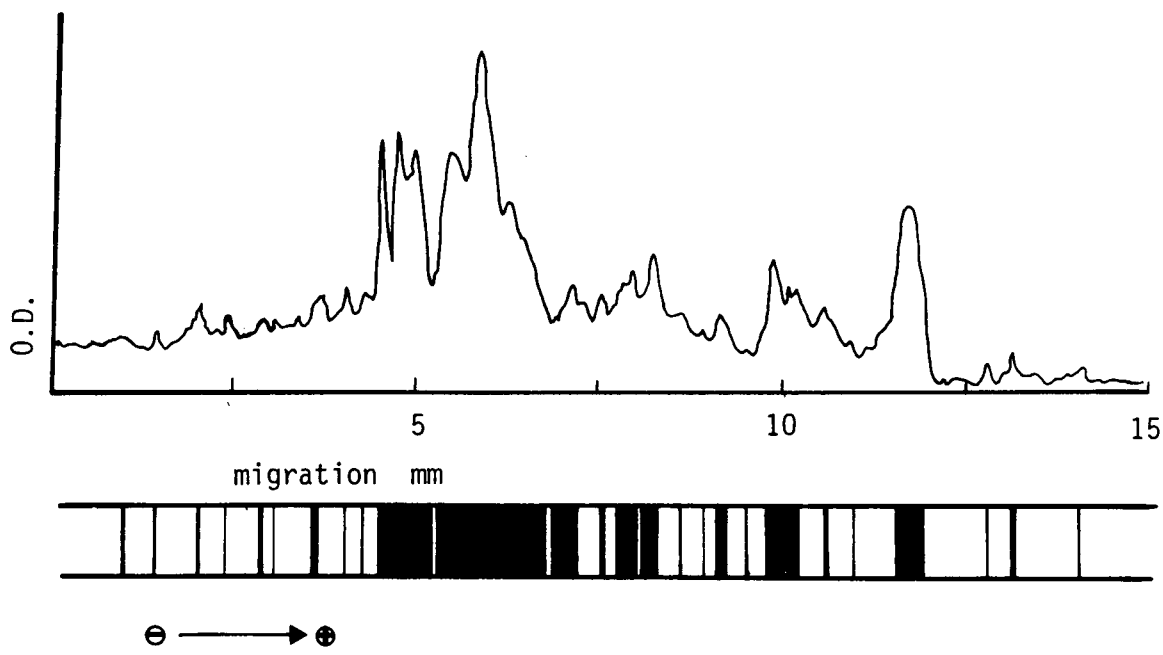


FIGURE 31: Electrophoretic separation of day 1 S. vittatum salivary gland proteins.

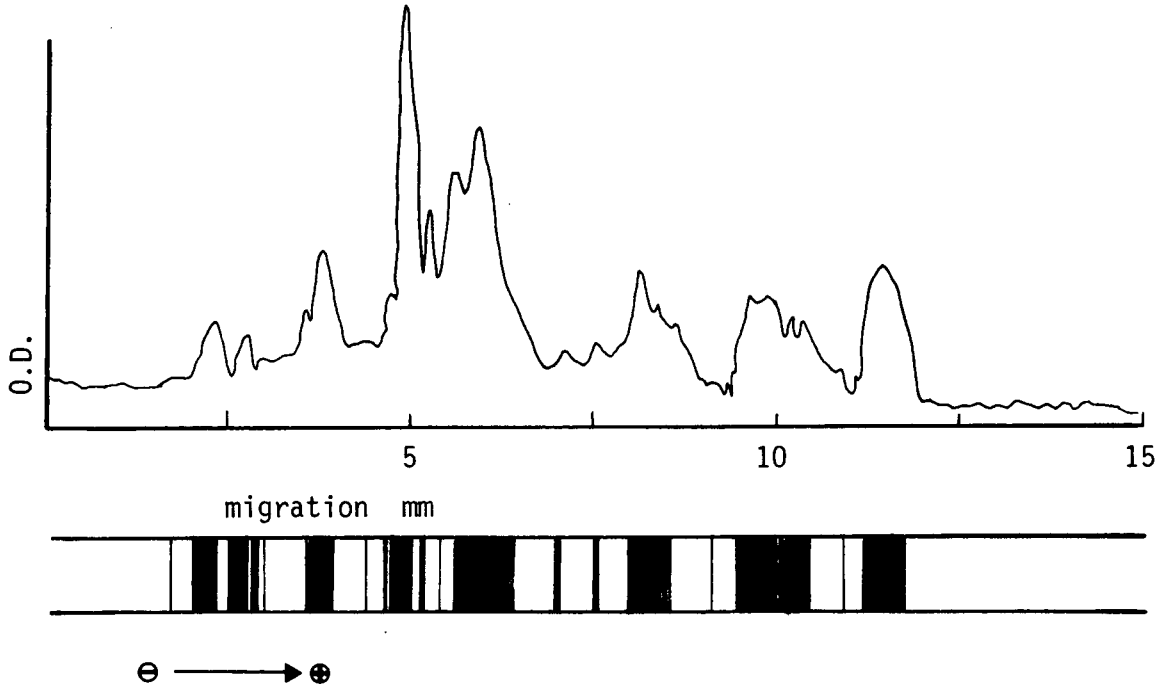


FIGURE 32: Electrophoretic separation of day 2 S. vittatum salivary gland proteins.

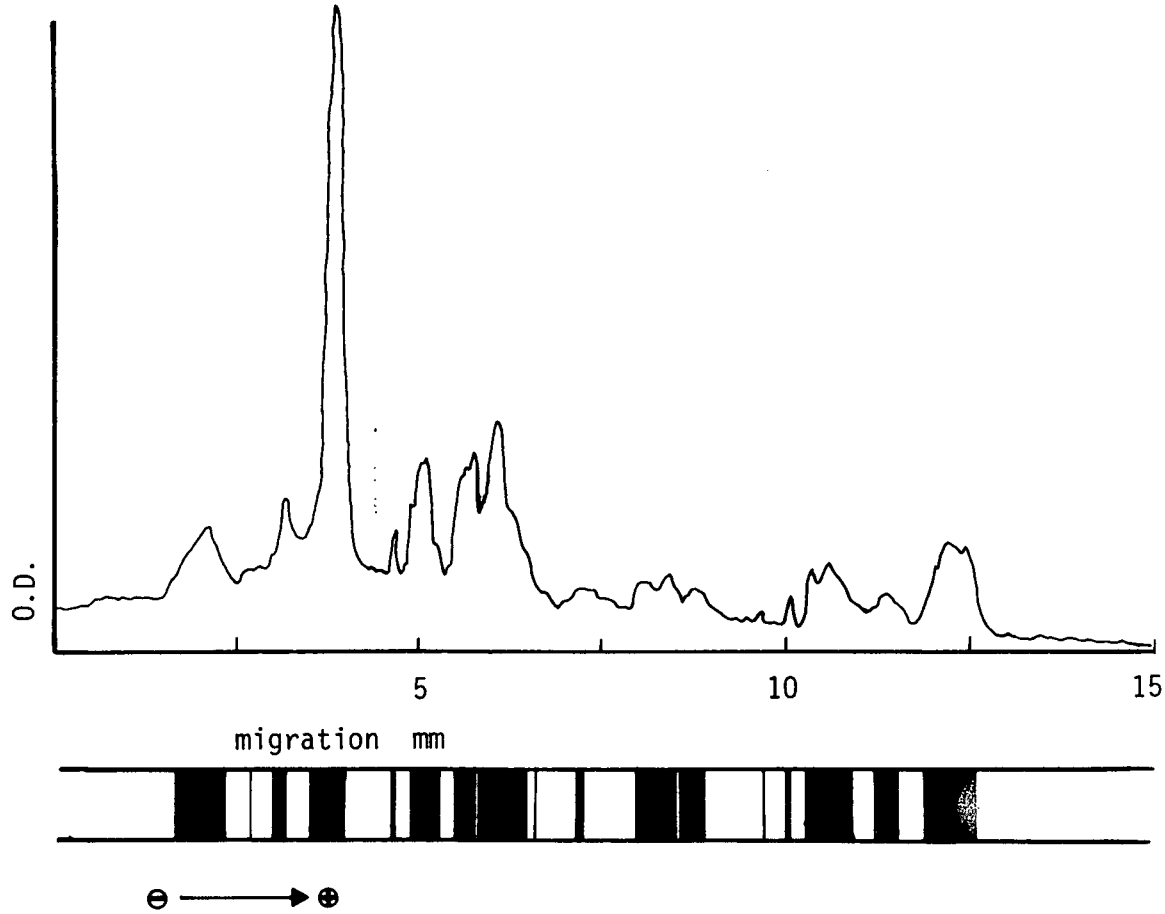


FIGURE 33: Electrophoretic separation of day 3 S. vittatum salivary gland proteins.

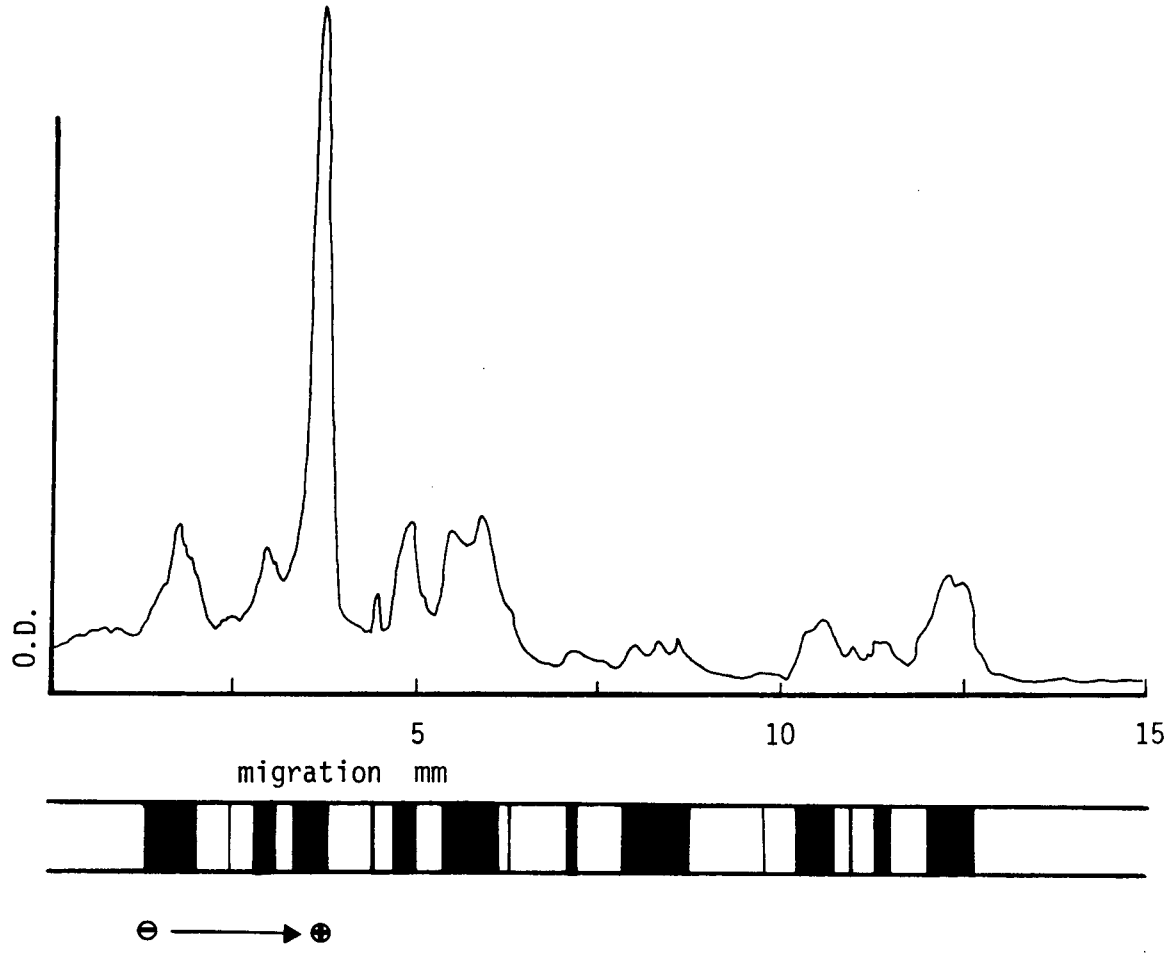


FIGURE 34: Electrophoretic separation of day 4 S. vittatum salivary gland proteins.

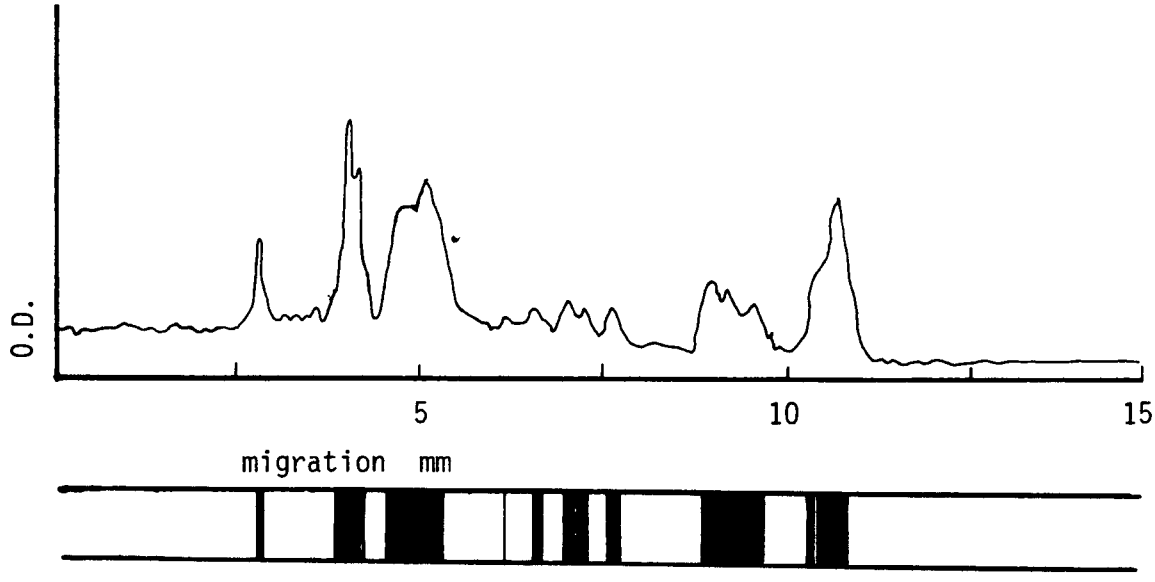


FIGURE 35: Electrophoretic separation of day 5 S. vittatum salivary gland proteins.

2.3 MOLECULAR WEIGHT ESTIMATIONS

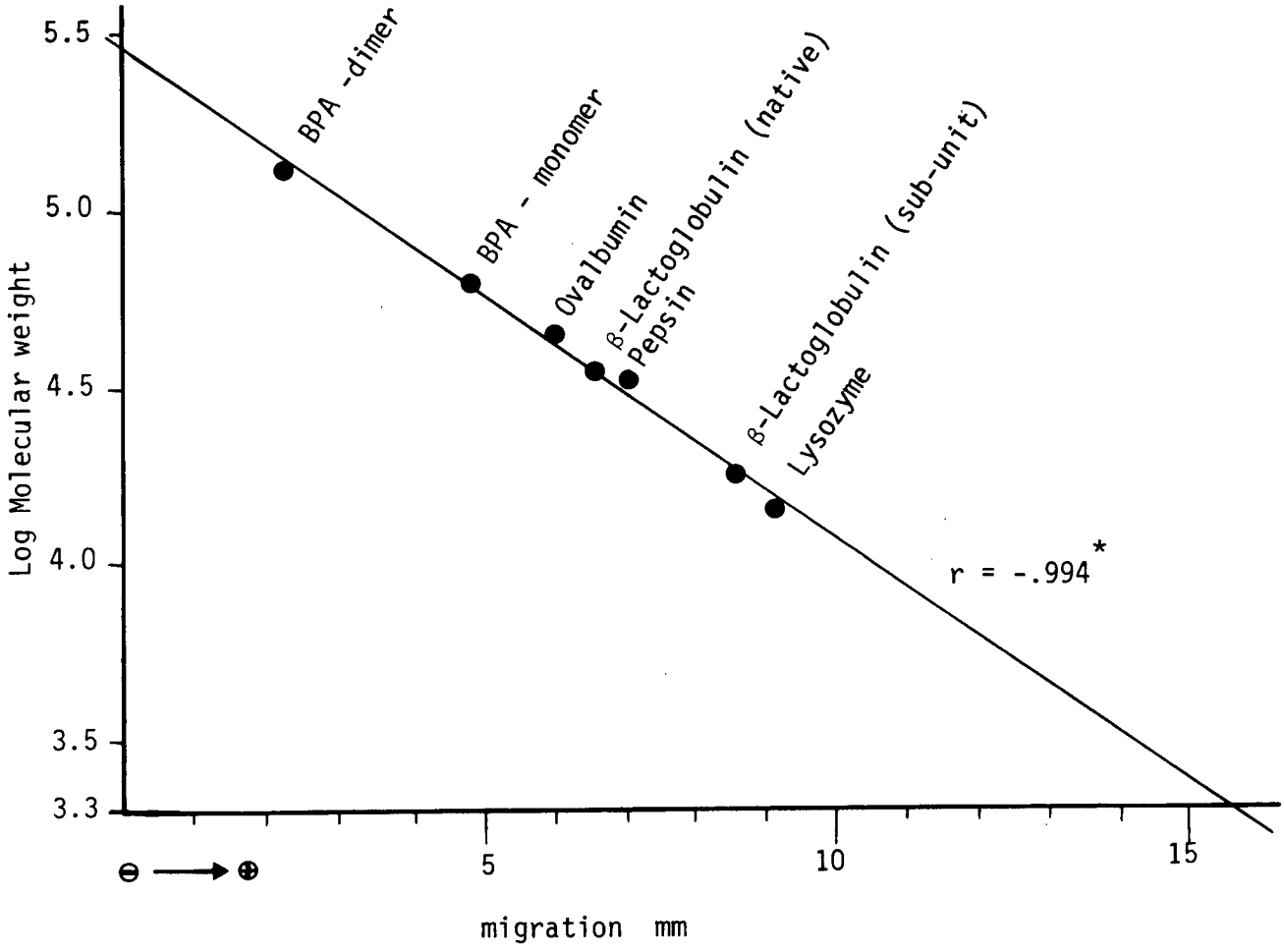
2.3.1 Migration of known protein markers

The electrophoretically-induced migration distances of known protein markers, in relation to their molecular weights and log molecular weights, are shown in Table VII.

TABLE VII: Electrophoretic mobility of marker proteins on 10 µL gradient gels.

Marker protein	MW	Log MW	Migration distance (mm)
Bovine plasma albumin (dimer)	132,000	5.1206	2.20
" (monomer)	66,000	4.8195	4.75
Ovalbumin	45,000	4.6532	6.00
Beta-Lactoglobulin (native)	36,800	4.5658	6.50
Pepsin	34,700	4.5403	7.00
Beta-Lactoglobulin (sub-unit)	18,400	4.2648	8.50
Lysozyme	14,300	4.1553	9.00

A straight line relationship, with a correlation coefficient of minus 0.994, was demonstrated between electrophoretic mobility (migration distance) and the logarithm of molecular weight for the marker proteins



* significant at the 0.01 probability level

FIGURE 36: Calibration curve for molecular weight determination. Molecular weight in relation to electrophoretic mobility for seven marker proteins on 10 µLgradient gels.

described. This linear relationship, referred to as the calibration curve, is presented graphically in Figure 36.

The separation capability of the 10 µL gradient gels was demonstrated to be in the molecular weight range of 2,000 to 294,000.

2.3.2 Estimation of unknown protein MW from known protein markers

TABLE VIII: MW groupings from the calibration curve used for estimation of the relative amounts of other proteins.

MW group	Migration distance on 10 µL gel; mm	Group label
294,035 - 250,000	0 - 0.504	1
250,000 - 200,000	0.504 - 1.195	2
200,000 - 150,000	1.195 - 2.082	3
150,000 - 100,000	2.082 - 3.342	4
100,000 - 80,000	3.342 - 4.034	5
80,000 - 50,000	4.034 - 5.490	6
50,000 - 30,000	5.490 - 7.075	7
30,000 - 20,000	7.075 - 8.330	8
20,000 - 15,000	8.330 - 9.220	9
15,000 - 10,000	9.220 - 10.480	10
10,000 - 5,000	10.480 - 12.625	11
5,000 - 2,000	12.625 - 15.491	12

Demonstration of a linear relationship between migration distance and log MW for the marker proteins provided a means of estimating the MW of any other protein by comparison of electrophoretic mobility. Although it was

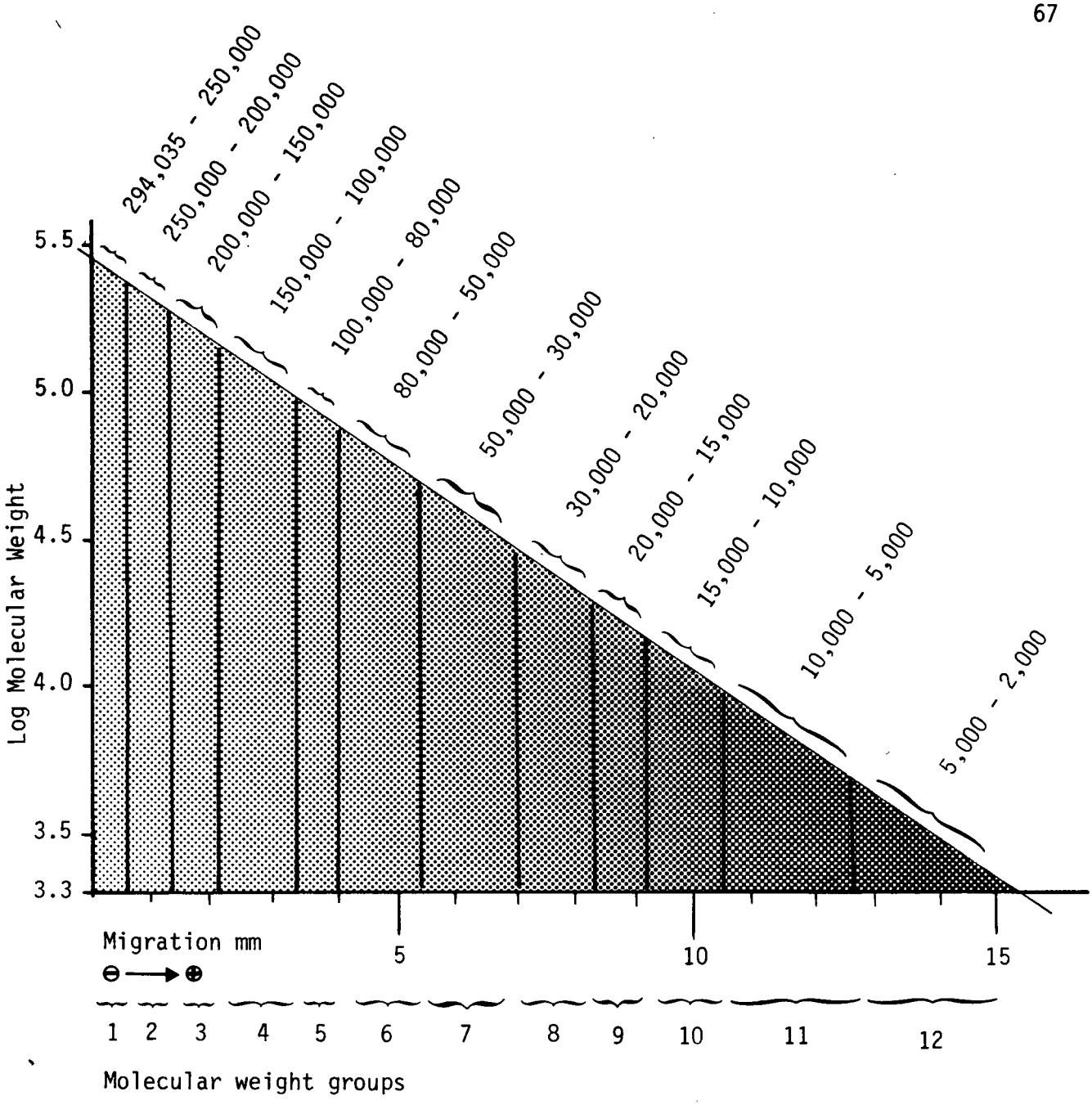


FIGURE 37: Molecular weight groupings on the marker protein calibration curve.

Footnote: Estimations of molecular weight outside of the range of data points used for construction of the calibration curve, i.e. above 132,000 MW and below 14,300 MW, are based on extrapolations from the equation:

Log MW = 5.4684 - 0.140 X migration distance (mm)

possible to estimate the MW and relative amount of any individual protein it was considered practical to estimate molecular weights in groups.

The migration limits for the 12 selected molecular weight groupings presented in Table VIII conform with the established calibration curve. The individual area measurements (taken from the migration limits imposed by the established MW groupings) for the protein groups identified on each gel are presented in Tables IX and X as percentages of the total protein measured (see section 1.6.1).

2.3.3 Comparison of electrophoretic separation patterns

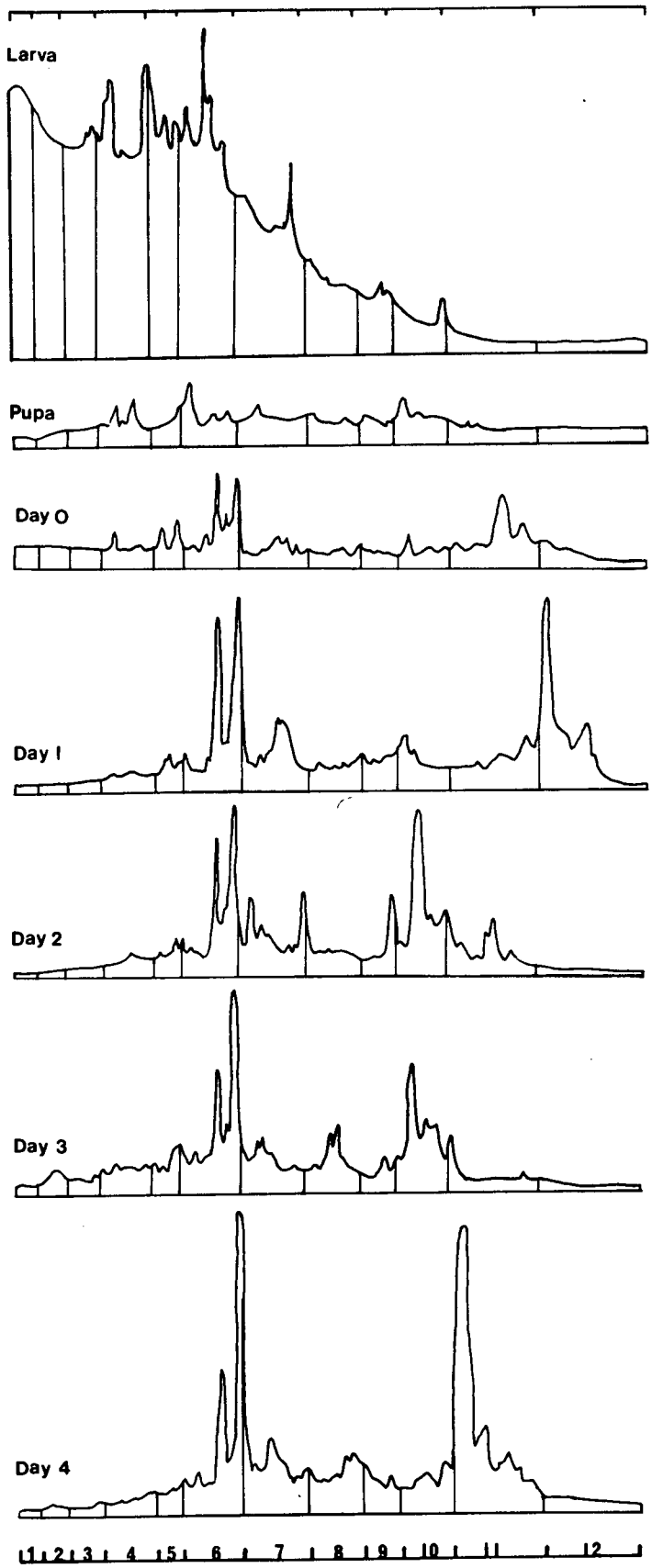
The number of visibly staining protein bands within each MW group is included beneath the relevant column in Tables IX and X. The total number of protein bands recorded for each separation is presented below in a sequence according to the biological stages of development.

	S. decorum	S. vittatum:
Larvae	24	32
Pupae	16	17
Day 0	26	28
Day 1	26	32
Day 2	26	24
Day 3	29	20
Day 4	30	20
Day 5	--	16

Figures 38 and 39 illustrate the comparative distribution of protein groups for the separated proteins of the various stages of S. decorum and S. vittatum respectively.

TABLE IX: Percent composition of S. decorum salivary gland proteins by molecular weight group.

	larva	pupa	day 0	day 1	day 2	day 3	day 4
	% composition by MW group (Number of bands visible within each MW group)						
MW group							
1	8.0 (1)	1.2 (0)	3.4 (0)	0.8 (0)	0.2 (0)	1.3 (0)	0.2 (0)
2	8.9 (0)	2.9 (0)	4.8 (0)	1.1 (0)	0.5 (0)	3.2 (1)	0.8 (1)
3	11.9 (3)	5.4 (0)	5.6 (0)	1.9 (0)	1.9 (0)	3.6 (1)	1.7 (1)
4	16.0 (5)	9.4 (3)	7.5 (2)	4.4 (2)	5.0 (1)	8.4 (3)	4.2 (3)
5	9.1 (2)	4.8 (1)	5.8 (2)	3.3 (3)	4.4 (3)	5.4 (3)	3.5 (2)
6	18.9 (4)	14.5 (3)	17.2 (5)	18.5 (4)	19.9 (4)	21.5 (4)	19.2 (4)
7	12.5 (3)	13.7 (2)	8.8 (4)	12.9 (4)	16.4 (6)	12.7 (5)	13.8 (5)
8	5.7 (3)	10.2 (2)	6.3 (3)	6.3 (3)	8.5 (2)	10.0 (4)	9.7 (4)
9	3.0 (2)	6.1 (1)	4.3 (0)	5.5 (2)	6.1 (2)	4.3 (2)	4.7 (2)
10	2.8 (1)	12.5 (2)	6.8 (3)	8.3 (2)	21.0 (4)	18.2 (4)	7.7 (2)
11	2.2 (0)	10.6 (2)	20.4 (4)	13.5 (3)	12.9 (4)	8.0 (2)	29.3 (6)
12	1.5 (0)	8.7 (0)	9.1 (2)	23.3 (3)	3.2 (0)	3.2 (0)	5.2 (0)
Totals %:	100	100	100	100	100	100	100
Bands:	(24)	(16)	(26)	(26)	(26)	(29)	(30)

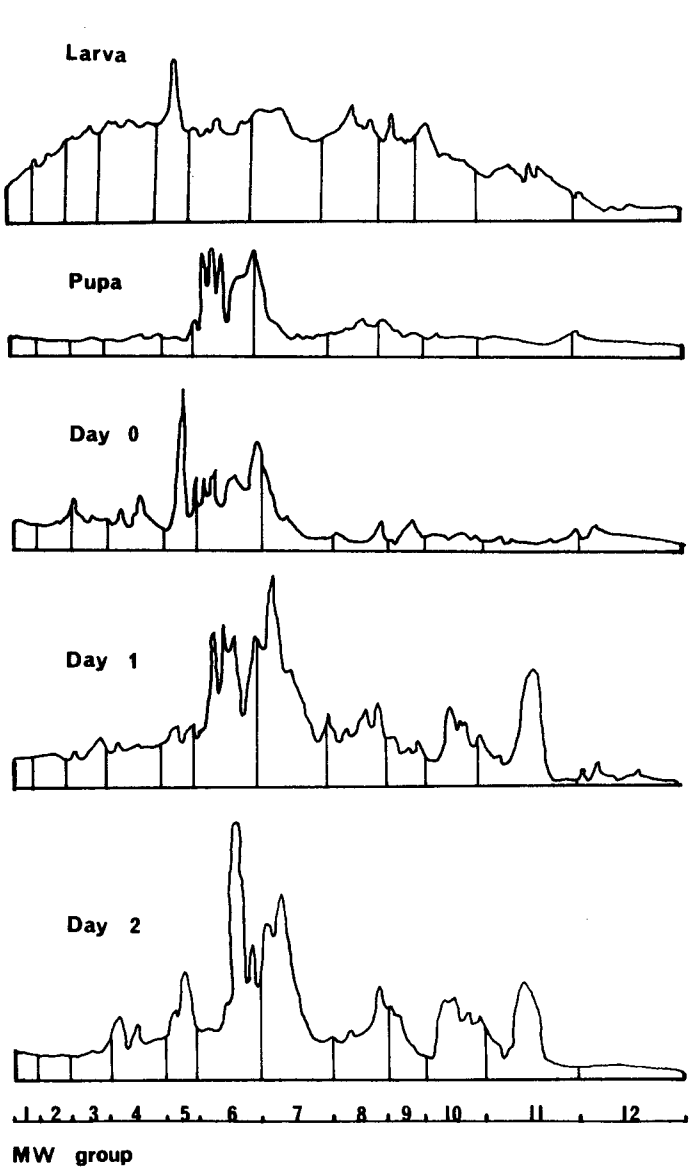


scale: 6:1 from original tracings

FIGURE 38: Distribution of S. decorum salivary gland proteins by MW group. Electrophoretic tracings and super-imposed MW group migration limits. See Table IX for relative area values.

TABLE X: Percent composition of S. vittatum salivary gland proteins by molecular weight group.

	larva	pupa	day 0	day 1	day 2	day 3	day 4	day 5
	% composition by MW group (Number of bands visible within each MW group)							
MW group								
1	2.1 (1)	2.6 (0)	3.8 (0)	2.0 (0)	1.9 (0)	1.9 (0)	2.3 (0)	2.7 (0)
2	4.1 (2)	3.3 (0)	4.8 (1)	2.8 (1)	2.1 (0)	2.9 (0)	3.6 (0)	4.4 (0)
3	7.6 (3)	3.8 (1)	7.5 (2)	4.2 (2)	3.7 (1)	5.9 (1)	8.6 (1)	5.2 (0)
4	11.7 (3)	6.4 (3)	10.7 (3)	6.4 (3)	7.4 (3)	10.5 (2)	11.3 (2)	9.2 (1)
5	13.1 (1)	3.4 (1)	9.9 (2)	5.0 (2)	7.0 (2)	18.5 (1)	21.1 (1)	8.1 (1)
6	14.5 (4)	27.9 (5)	25.7 (5)	20.2 (5)	19.3 (3)	13.5 (3)	12.9 (3)	23.3 (3)
7	11.7 (3)	12.5 (2)	12.0 (2)	22.3 (3)	20.6 (3)	16.6 (3)	12.7 (3)	10.6 (3)
8	6.9 (3)	10.7 (2)	4.0 (2)	10.3 (3)	9.1 (3)	6.3 (2)	4.5 (2)	6.9 (2)
9	8.3 (3)	5.5 (1)	4.2 (3)	4.8 (3)	4.4 (2)	4.0 (1)	3.0 (1)	5.8 (2)
10	9.0 (3)	5.8 (1)	4.7 (3)	8.1 (4)	9.6 (4)	4.6 (3)	3.5 (3)	9.1 (3)
11	3.4 (3)	10.2 (1)	6.5 (4)	10.8 (3)	10.9 (3)	11.8 (4)	12.7 (4)	9.6 (1)
12	14.5 (3)	8.0 (0)	6.1 (1)	3.1 (3)	4.0 (0)	3.4 (0)	3.7 (0)	5.0 (0)
Totals %:	100	100	100	100	100	100	100	100
Bands:	(32)	(17)	(28)	(32)	(24)	(20)	(20)	(16)



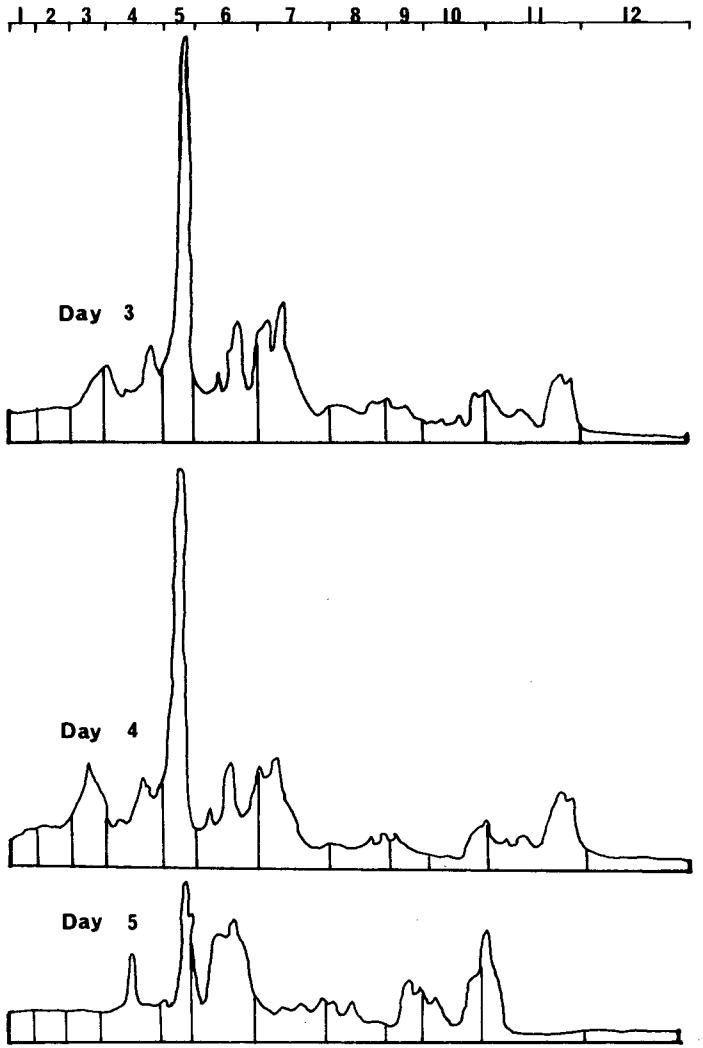


FIGURE 39: Distribution of S. vittatum salivary gland proteins by MW group. Electrophoretic tracings and superimposed MW group migration limits. See Table X for relative area values.
Scale 6:1 from original tracings.

Larvae:

At least 24 bands of protein are defined on the separations of S. decorum larval salivary glands (Figure 21). Most of these bands occur in the high MW region. S. vittatum larval salivary glands generate at least 32 bands of protein, again mostly of high molecular weight (Figure 28). Unlike S. decorum larval protein patterns, there are also a few rapidly migrating proteins of relatively low molecular weight.

Pupae:

S. decorum pupal salivary glands reveal 16 protein bands with a maximum size of 150,000 MW and a minimum of 8,000 MW (Figure 22). S. vittatum pupal proteins range between 5,000 MW and 175,000 MW with a pronounced concentration in the 50,000-80,000 MW range (Figure 29).

Post-emergence:

The salivary gland proteins of post-emergence stages of S. decorum display a trend of increase in total number from 26 at emergence to 30 at day 4 (Figure 38). Some bands, particularly those in the 30,000-80,000 MW range, are obviously present, to some extent, at every stage of development. The day 1 separation contains bands of low molecular weight (2,000-5,000 MW) which are not present at any later stage. The day 4 salivary glands contain a relatively large amount of a concentrated protein band, approximately 10,000 MW, which reaches its highest level at this stage.

The salivary gland proteins of post-emergence S. vittatum display an increasing trend in total number of bands from 28 at emergence to 32 at day 1, and then a decline to only 16 bands at day 5 (Figure 39). The day 1 salivary glands are the only separation to reveal three rapidly migrating

bands of low molecular weight. A group of protein bands in the 30,000-100,000 MW range are apparently present, to some extent, at every stage of post-emergence development. A single concentrated band, of approximately 90,000 MW, is evident in the day 3 and day 4 salivary glands.

2.4 GUINEA-PIG SKIN REACTION TESTS

The cutaneous response to skin tests demonstrated the existence of immune and/or toxic reactions to the intradermal injection of salivary material taken from blackflies of various post-emergence ages.

2.4.1 Measurement of cutaneous reactions

The average erythema (redness) diameter measurements recorded for the skin test reactions of the test animals are presented in this section. Allowance was made for the amount of reaction due to the injection of saline alone (Table XI). The averages were calculated from nine

TABLE XI: Average diameter of redness (mm) to injected *S. vittatum* salivary gland preparations (saline reaction subtracted).

0.2 mL skin test	30 MINUTE		10 HOUR		24 HOUR	
	Control	Sensitized	Control	Sensitized	Control	Sensitized
Salivary glands:						
Day 0	6.1	5.1	4.6	2.6	0.5	3.0
Day 1	6.8	8.2	7.4	5.9	3.6	5.4
Day 2	7.1	3.9	3.9	3.8	0.8	2.1
Day 3	6.5	5.5	2.0	2.8	0.4	2.7
Day 4	7.1	6.4	3.3	2.9	1.3	3.3
Day 5	7.5	5.3	3.8	1.8	0.5	1.4
Whole-fly:	2.4	2.9	9.4	10.8	8.6	13.8
(Saline:	8.0	8.5	4.4	7.1	4.2	5.5)

sensitized and four control guinea-pigs.

Standard error of the mean estimations ($S_{\bar{x}}$) for the erythema diameter measurements are presented in Figures 40-42.

2.4.2 Immediate and delayed reactions.

The cutaneous reactions to skin tests involving the original whole-fly sensitizing antigen (diluted with saline and without Freund's Adjuvant), confirmed the effectiveness of the sensitizing procedure.

Although the erythema measurements taken after 30 minutes were relatively large, skin-tests 1-5 did not indicate an immediate reaction as the sensitized animals showed no greater response than the controls (Figure 40).

The 10 hour readings did not indicate any Arthus-type responses (Figure 41).

The 24 hour readings (Figure 42) indicated a delayed reaction response to each of the glandular preparations in that the sensitized animals showed considerably greater overall skin responses than the controls. The day 4 salivary glands (skin test 5; Figures 45 and 46) produced the greatest delayed reaction response in the sensitized animals, as assessed from the erythema measurements and also from the non-quantitative manifestations outlined in section 1.7.3.

The day 1 salivary glands produced a strong reaction of similar intensity in both sensitized and control animals (skin test 2; Figures 43 and 44), indicating the involvement of toxic factors in the injected material.

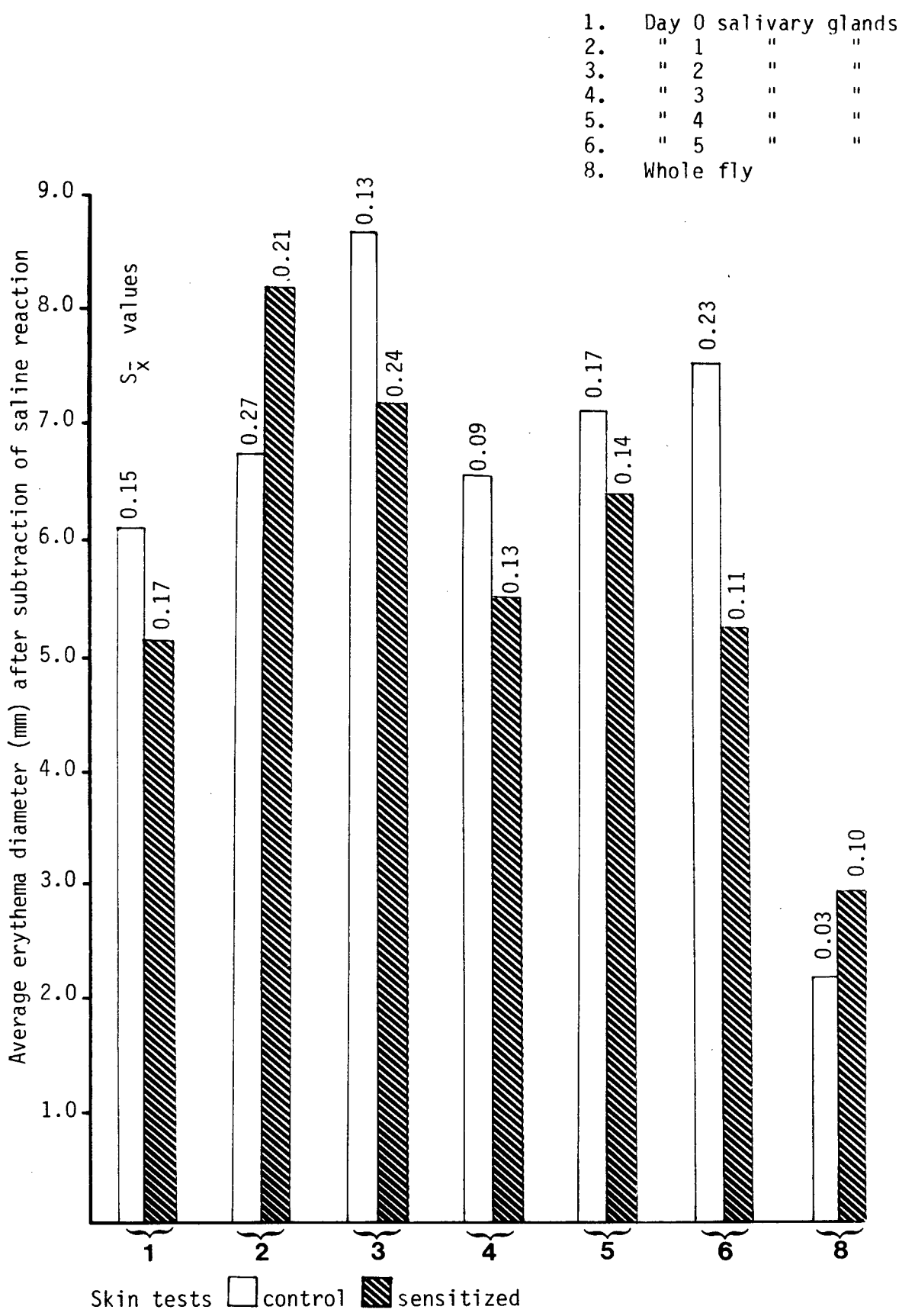


FIGURE 40: Cutaneous reactions to S. vittatum salivary gland preparations after 30 minutes (saline reaction subtracted). Averages from 9 sensitized and 4 control guinea-pigs.

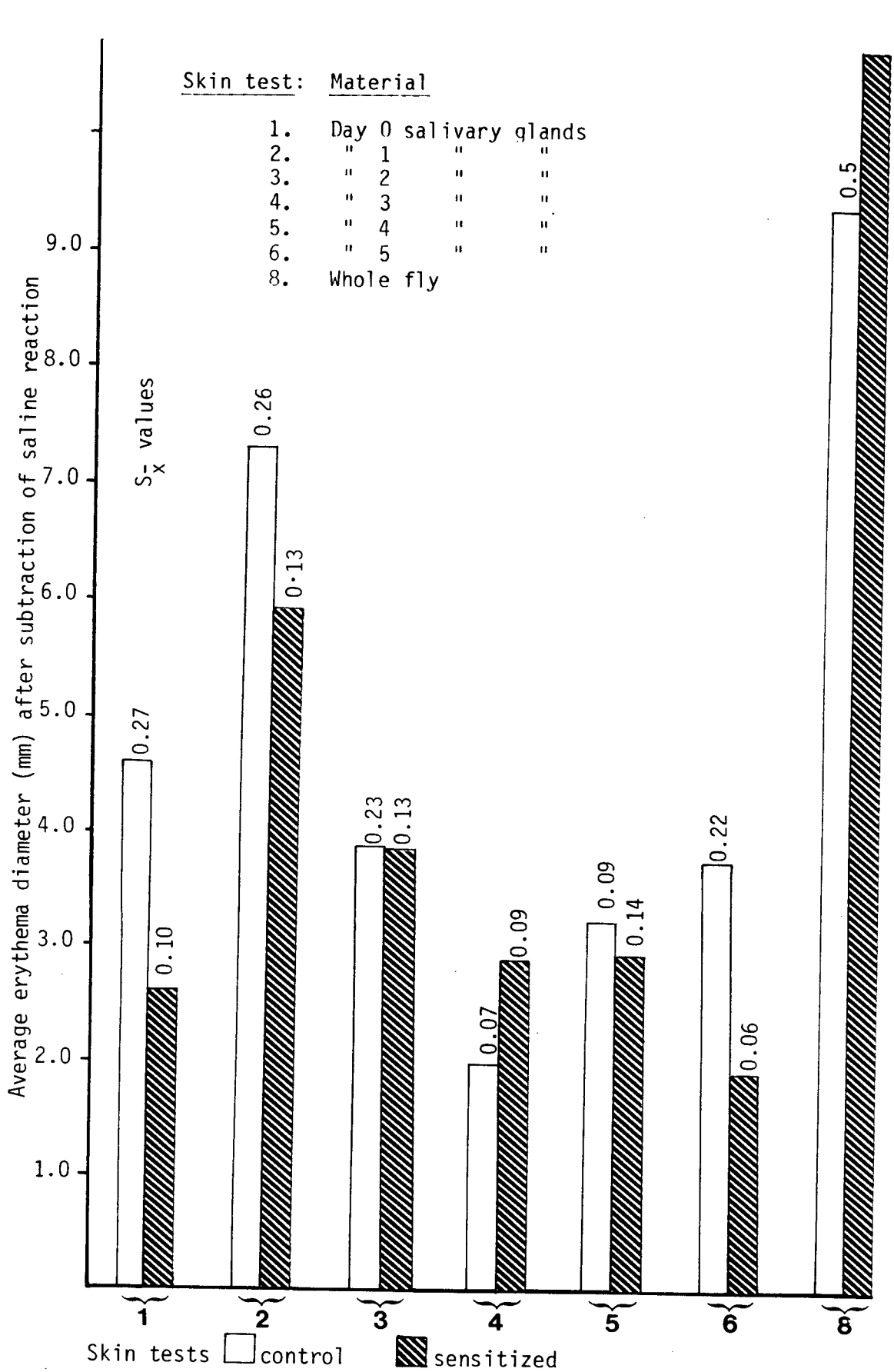


FIGURE 41: Cutaneous reactions to S. vittatum salivary gland preparations after 10 hours (saline reaction subtracted). Averages from 9 sensitized and 4 control guinea-pigs.

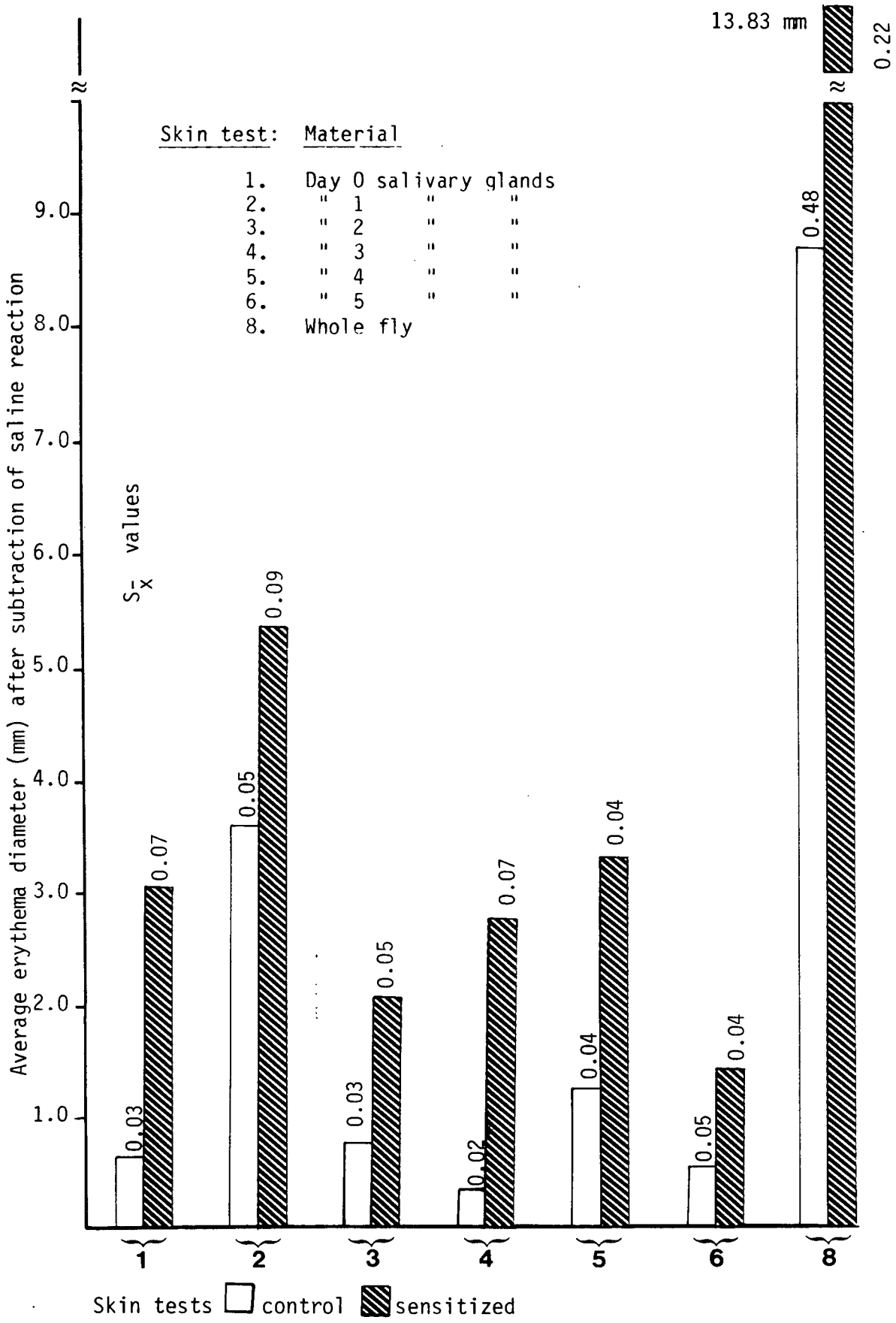


FIGURE 42: Cutaneous reactions to S. vittatum salivary gland preparations after 24 hours (saline reaction subtracted). Averages from 9 sensitized and 4 control guinea-pigs.

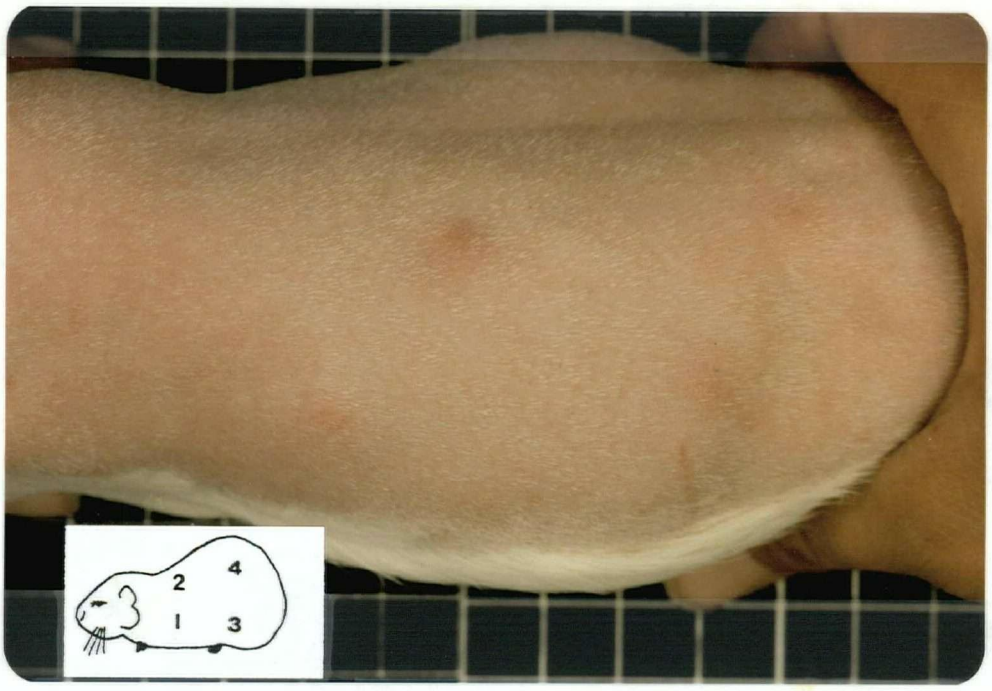


FIGURE 43: Cutaneous reactions (as per Fig. 42) in a sensitized guinea-pig after 24 hours. Left flank.

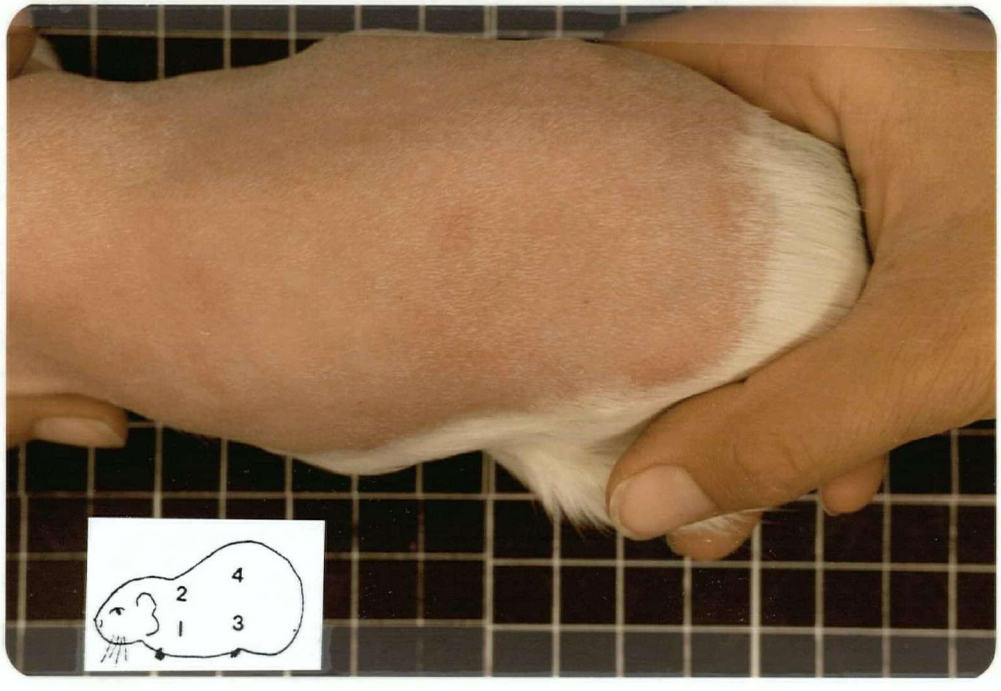


FIGURE 44: Cutaneous reactions (as per Fig. 42) in a control guinea-pig after 24 hours. Left flank.

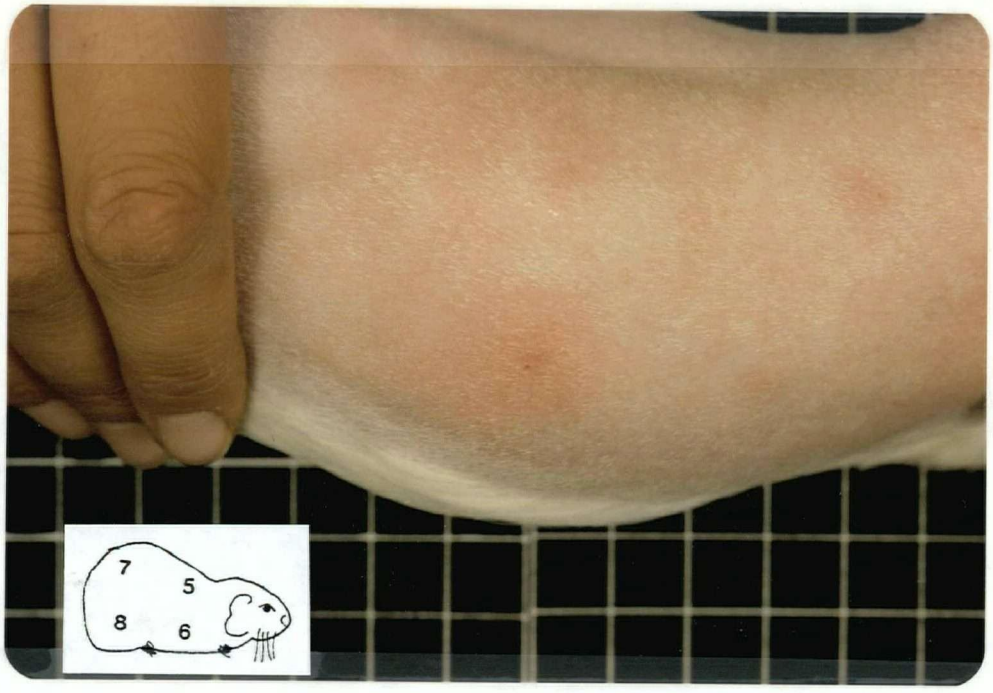


FIGURE 45: Cutaneous reactions (as per Fig. 42) in a sensitized guinea-pig after 24 hours. Right flank.

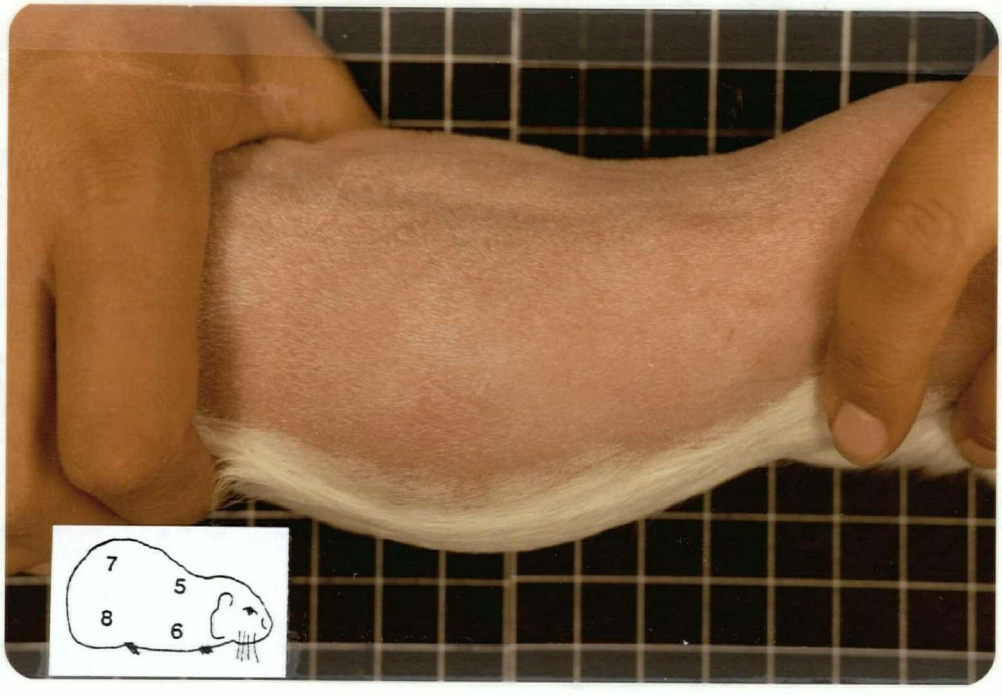


FIGURE 46: Cutaneous reactions (as per Fig. 42) in a control guinea-pig after 24 hours. Right flank.

3. DISCUSSION

Very little is known of the components of blackfly saliva. Although Hutcheon and Chivers-Wilson (1953) found histamine to be associated with several anatomical sections of the blackflies Simulium vittatum, S. venustum, and S. decorum, they considered the histamine levels too low to be of any consequence. The presence of an anaesthetic factor in the saliva has been indicated but not, as yet, identified (Frazier 1969). Unidentified agglutination and anti-coagulant factors were found in the salivary glands of adult S. venustum by Yang and Davies (1974). The anti-coagulant factors are thought to be involved in the role of maintaining the blood-meal in a fluid state during the feeding process. The exact purpose of the agglutinins is uncertain, but Yang and Davies suggested an involvement in the clumping of the blood-meal to form a semi-solid mass in the posterior mid-gut of the fly. Both these factors were absent in newly emerged female blackflies and did not develop until at least 12-24 hours after emergence.

On the basis of certain histological characteristics of the bite reactions, workers have suggested that the saliva of blackflies contains a toxin, or toxins, (Stokes 1914; Rempel and Arnason 1947; Hutcheon and Chivers-Wilson 1953; Dem'yanchenko 1960; Fallis 1964; Frazier 1969), but the exact nature of this postulated toxin has not been elucidated.

The investigations presented here have focussed on the protein component of blackfly salivary glands. While it is understood that the factors responsible for the host reaction are not necessarily of proteinaceous origin, such substances are generally believed to be the most biologically active components of venoms, and a detailed accounting of the possible involvement of various proteins is a logical primary step.

Although these studies concern the total protein content of extracted blackfly salivary glands, and the reactions elicited by test animals to these gland preparations, it is recognized that the source of certain of the noxious substances involved in a blackfly bite may, in fact, not be generated by the glands themselves. It is possible that certain of the noxious substances are taken up from the fly's haemolymph at some point immediately before biting. In fact it is known that certain proteins found in dipteran salivary glands can also be recognized in the individual fly's haemolymph (Doyle and Laufer 1969; Poehling 1976; Poehling et al. 1976; Schin and Laufer 1974).

Benjamini and Kartman (1963) proposed that the allergic reaction to a flea bite was the result of the combination of haptenic components in the saliva with certain components in the skin of the host to form sensitizing antigens. Blackfly bite reactions may involve a similar complexity of interactions which under certain circumstances of attack may result in immunity, but under other circumstances where a sensitizing antigen is not formed, may result in an intensified injury.

Notwithstanding these uncertainties, it is clearly worthwhile to consider the salivary glands as a primary source of the salivary secretions

and as a source of the noxious salivary substance capable of inducing debilitating host reactions.

The basic micro-electrophoretic techniques applied in this investigation were derived, with modifications, from those reported by Neuhoff in 1973. Poehling (1976; 1977; 1978; 1979) utilized micro-electrophoresis for studies on dipteran salivary proteins, but his purposes differed from those of this study. Poehling has been concerned primarily with the evolving glandular protein components and with features of different glandular regions. The present investigation is concerned rather with the salivary gland proteins as the source of noxious substances in the salivary secretion. Also, Poehling's work on blackflies (1976; Poehling et al. 1976) dealt exclusively with cattle feeding Boophthora, Wilhelmia and Odagmia species, representing sub-genera of Simulium confined to the Palaearctic regions of the world (Stone 1963). The two species of blackfly dealt with in this study are both of Holarctic distribution (Smart 1945; Stone and Jamnback 1955).

The results obtained in this investigation demonstrate that the techniques as selected, modified and applied are capable and appropriate for handling the extremely minute quantities of protein contained in the salivary glands of such small insects as the blood-sucking blackflies.

The application of polyacrylamide gels in a linear gradient in microcapillaries, showed good capability for handling volumes of macerated glandular material as small as 2.2 microlitres.

The application of the modified biuret-phenol test which measured total protein by the summation of two components, namely, tyrosine and

peptide bonds, showed that the micro-electrophoresis was handling quantities of protein as small as 0.5 µg. The electropherograms demonstrated the high resolving power of the process in separation of such minute quantities of protein into their numerous molecular components. The visually recorded stained protein bands in the gel corresponded to the spikes in the photoelectrically scribed scans.

Inasmuch as the gel was made to form as a gradient of acrylamide concentration (1-40%) in the microcapillaries, it constituted a molecular sieve consisting of a gradient of progressively decreasing pore sizes (Margolis and Kenrick 1967b). Consequently, the larger molecules were held back as the smaller ones moved along, then the larger molecules of that group encountered a limiting pore size, while still smaller ones travelled on. Because the gradient gel technique does not depend on electrical charge of the moving particles for their separation, we must infer that the different bands of protein recorded represented a graded series, essentially as a sequence of molecular size. A close estimate of the exact size (molecular weight) of the fractionated proteins was possible by reference to the migration patterns of known protein markers.

Glandular protein content:

Protein assay results indicate higher glandular protein levels for S. decorum than S. vittatum (Figure 20). This difference can be attributed partly to the size difference between the two flies.

Although longevity studies indicate the survival time of wild adult blackflies to be up to six weeks (Dalmat 1952), laboratory survival rates are considerably less. In this study it was possible to maintain

S. vittatum adults for only five days, while S. decorum expired after four (maintenance at 13°C and 85% RH). Extension of laboratory life of the blackflies was precluded for several reasons. It might be supposed that lack of their normal access to blood and/or nectar could have been a limiting factor in survival time. Researchers have only recently been able to demonstrate reproducible techniques for colony maintenance of blackflies through all life stages (Simmons and Edman 1981). In the present study dissections were made to include all post-emergence stages up to the age when they would presumably be ready for blood feeding.

Although blackfly larvae and pupae are not involved in blood-feeding, their extracted salivary glands were included in this study for the purpose of completing the overall picture of salivary protein development through the post-hatching stages. The differences in protein levels and electrophoretic patterns recorded for blackfly larvae and pupae are a fundamental accompaniment to the ontogenetic changes in the glandular tissues. The larval salivary glands function as secretory organs producing the fine silk threads required for locomotion (Crosskey 1973). All larval dissections were performed on mature individuals that were almost ready to pupate, a process requiring the production of vast quantities of silk from the salivary glands. The high levels of protein recorded for the larvae (18X that of the pupae for S. decorum and 22X the pupae for S. vittatum) might be accounted for, to some extent, by the immediate silk production requirements placed on the glands. Generally, the salivary glands cells of Dipterous larvae are believed to remain constant in number throughout larval life, growth occurring solely through increase in cell size and

reaching a maximum size shortly before pupation (Jenson and Jones 1957). However, it has been reported for the fly Rhynchosciara americana that the larval salivary glands store only 10% of the protein needed to make a cocoon, 90% of the secretion proteins being synthesized after the beginning of spinning (De Bianchi and Terra 1975). Such a sequence in blackfly larvae has not been investigated.

The pupal salivary glands were taken from fully metamorphosed individuals close to eclosion. Structurally these glands resembled those of the adult rather than the larva, but they were consistently smaller than those of newly emerged adults; S. decorum pupal glands averaged 60% of the protein content of newly emerged adults, S. vittatum pupal glands averaged 69% of young adults. This corresponds with Poehling's observations in 1977 that the salivary glands of Wilhelmia lineata (Simuliidae), although fully differentiated at the time of pupal-adult ecdysis, exhibited a lower overall protein content in the pupa than the adult.

During adult post-emergence development the gland lumen is filled with salivary secretions, as is reflected in the results generated from protein assays in this study (Tables I and II). However, results obtained here for S. vittatum indicate a sudden drop in protein content at day 5. S. decorum could not be maintained in sufficient numbers for more than 4 days, so it is not known whether such a limitation would occur for them in nature. It is possible that four days is the maximum time that laboratory raised individuals of S. vittatum can endure without a blood meal, or some other kind of nutrient. It is likely that day 5 S. vittatum do not represent

flies in a vigorous blood-thirsty condition primed for feeding, but rather individuals declining beyond the ability to blood-feed.

The amount of water-soluble protein in S. decorum salivary glands appeared to be remarkably constant at between 62 and 66% of the total protein (Table III). Results from S. vittatum reflected a slightly larger range of 69-80% water-soluble protein (Table IV). It appears that the glands are accumulating salivary secretions progressively from day 0 to day 4, at the same rate as they are increasing in protein content through cell growth. Indeed, such is the case, according to Barrow et al. (1975), with the salivary glands of Culex pipiens which continue to develop and accumulate salivary gland secretions for the first five days of adult life. However, day 5 for Simulium vittatum marked a drop in overall protein content and a reduced proportion of water-soluble protein. At this stage the glands may have re-absorbed the secretions or even released them externally. If the glands secrete continuously, regardless of nutrients, as was suggested for Anopheles by Jenson and Jones (1957), it may be that some factor, yet to be investigated, causes the secretory mechanism to switch off after the fourth day. The duration of biting activity in the wild for either S. decorum or S. vittatum is not documented, nor is it known how long these flies can endure without a blood meal.

Electrophoretic interpretations:

The electrophoretic separation results allowed ready comparison of the component proteins from larvae through to day 5 adults (day 4 in S. decorum). The banding sequences showed definite patterns, whereby some proteins could be recognized, by their molecular weights, as occurring in

all developmental stages; others appeared and disappeared quite suddenly at particular stages of glandular development (Figures 38 and 39).

The overall protein load for electrophoresis was marginally different for each age group due to the disparities in gland sizes (see sections 2.2.1 and 2.2.2). Consequently, direct comparisons of individually separated proteins from one age group to another were precluded. Comparisons between age groups were made by collation of the relative amounts of separated protein groups as described in section 2.3.2.

S. decorum protein patterns:

As might be anticipated from the obvious functional differences, glands of S. decorum larvae displayed very little resemblance in protein fractionation to the later stages of development. Most protein, (approximately 85%), occurred in the high molecular weight range, 30,000 to 290,000 MW (MW groups 1-7). A maximum of 50% occurred in this range at any later stages. Three protein bands, between 50,000 and 80,000 MW (MW group 6), appeared in larval glands and also followed through, to some extent, in every subsequent stage. Similarly, with one or two bands in MW group 5 in the range of 80,000 to 100,000 MW (Figure 38 and Table IX).

The most intensely staining protein bands occurred as a distinct group of four in the MW range of 50,000 to 80,000 (MW group 6), at every adult stage. One additional band was recorded in this group at day 0. Also of interest is the low molecular weight protein, MW 2,000 to 5,000 (MW group 12), that appeared as 23% of the day 1 separation; the preceding stage revealed only 9% in this weight range while no later stages show any such rapidly migrating protein bands.

Inasmuch as the S. decorum protein assays indicated a progressive increase in protein content from the pupa through to day 4 adults, it might be postulated that the protein developments observed from electrophoretic results reflect the preparation of the glands for the blood-feeding process. The more developed the gland becomes the more likely it might be to contain the substance, or substances, which will become of consequence to the host. The day 4 separations featured one very concentrated band in MW group 11 of approximately 10,000 MW protein, which is maximal at this stage.

S. vittatum protein patterns:

For S. vittatum, the only high MW protein revealed by electrophoretic techniques was one in the range of 200,000 to 250,000 MW (MW group 2), which appeared at both day 0 and day 1 (Figure 39 and Table X). These separations also displayed two bands between 150,000 and 200,000 MW (MW group 3). Later separations (day 2-day 4) contained only one visible band in this range.

The most intensely staining protein bands occurred initially as a group of nine (day 0), and later only seven bands (days 3, 4 and 5), in the 30,000 to 100,000 MW range (MW groups 5, 6 and 7). Day 1 separations revealed a total of ten bands in the same MW range. Another feature of the day 1 separations was the occurrence of three distinct and rapidy migrating bands in the range of 2,000 to 5,000 MW (MW group 12). The only other occurrence of protein in this low MW range was at day 0, where one band was evident. A concentrated band of protein, with an approximate MW of 90,000,

occurred at day 3 and day 4. It was also evident as a minor band prior to this, and had dropped again by day 5.

The protein assay results from S. vittatum salivary glands indicated a progressive increase in overall protein content from day 0 to day 4. The time lapse in the trend of increase in protein appears to conform to the time for the onset of feeding activity after emergence. It might, therefore suggest that the day 4 should also contain the maximal amount of the sought-after protein, or proteins, involved in the debilitating host response to a blackfly bite.

Guinea-pig skin reactions:

The skin tests of the different salivary gland preparations from S. vittatum were intended to be exploratory only. They sought to examine the proposition that the substances obtained in the manner described are capable of inducing reactions paralelling those which accompany the bites of the flies. Therefore, it is possible to assume that the study of the salivary gland proteins is one of the valid approaches to understanding of the biochemical mechanisms in bite reactions. Additional testing was considered to represent a phase of research beyond the limits set for the present study.

The whole fly preparations administered to nine guinea-pigs sucessfully sensitized the animals to subsequent injections of salivary glands extracted from S. vittatum of various ages. Although no immediate reactions to the salivary gland preparations were evident, the delayed reactions (Figures 42-46) indicated that the sensitizing dose of whole flies had contained at least some of the antigens present in each of the

glandular age groups injected. A strong response, possibly of a toxic nature as it was also recorded in the controls, was observed for the injection of day 1 salivary glands. Apart from this apparent toxic reaction, the day 4 glands provoked the greatest degree of delayed skin response (assessed from the erythema diameter measurements and other non-quantitative manifestations) in the test animals. Indeed, it was suggested earlier that day 4 might be the most likely stage to contain the noxious salivary element. Electrophoretic separations of day 4 salivary glands exhibited a large amount, relative to younger and older flies, of a very strongly staining and well defined protein band in the 90,000 MW range (Figure 39). Further studies are warranted for a closer analysis of each protein recorded from the day 4 salivary glands. As it is possible to isolate individual protein fractions after separation on a micro-gel (Neuhoff 1973; Neuhoff and Schill 1968), the suspect band at 90,000 MW might profitably be examined more closely in future research.

If the "toxic" factor, observed as a reaction to day 1 salivary glands, is assumed to be a protein, the electrophoretic results would imply a possible involvement of the three low MW bands between 3,000 and 5,000 MW, which do not occur at any other stage. Poehling and co-workers (1976) drew attention to several low molecular weight proteins in the salivary glands of three species of blackfly, which occur only as a secretion from a specific region of the female glands. These proteins are not found in the male salivary glands or in either the male or female haemolymph. They suggest that these low MW proteins are a highly specific secretion somehow implicated in the female's blood-feeding role. Although

the salivary glands of S. vittatum appear to be growing and filling continuously for the first four days of post-emergence activity, the number of different proteins recorded by electrophoretic separation is maximal at day 1 (Table X). It is possible that some of the discomfort caused by blackfly bites is attributable to the feeding activity of young flies harbouring apparently toxic substances in their glands.

A definite sequence of skin reactivity in response to arthropod bites has been established for mosquitoes by Mellanby (1946) and McKiel (1959), and for fleas by Benjamini et al. (1961). Benjamini and Feingold (1970) have suggested that existence of this sequence existing, whereby, after repeated exposure delayed reactions give way to immediate plus delayed reactions, later immediate reactions alone, and finally no reaction, suggests that the oral secretions of fleas and mosquitoes are antigenic in nature. A toxic reaction would be expected to occur with equal severity at any point in time. However, no such sequence of skin reactivity has ever been documented for blackflies. Indeed, the bite reaction experienced by many people is consistently more severe than could be attributed to either a mosquito or flea bite. A toxic component in the saliva, as is suggested by the guinea-pig skin response to day 1 salivary glands, could perhaps help explain the unflagging reaction phenomena experienced by the host to a blackfly bite. In fact, the combination of a toxic factor and substances capable of inducing specific sensitization has been suggested as a partial explanation for the reaction induced by mosquito bites (Rockwell and Johnson 1952; McKiel and West 1961; Feingold et al. 1968).

Quite apart from the implications of the salivary gland constituents and products for the host, questions arise about the relationship of the chemical sequences in the developing glands as they pertain to the strategy of survival of the flies. There are already plausible explanations for the functions of the anti-coagulant and agglutination factors in the blood uptake and subsequent digestion. On the other hand, there remains the question as to whether the substances which cause reactions in the host comprise the presumptive anti-coagulant and agglutinating factors, or whether there are additional substances. In any event, it is conceivable that the irritating substance might produce an increased blood flow at the feeding site by causing a histaminic reaction in the host. Such an effect might serve in reinforcing the weak action of the meagre amount of histamine in the saliva. An antibody response in the host, naturally occurring or induced by appropriate techniques, might then be disadvantageous to the flies in subsequent attacks. In the event that the desired immunity cannot be developed in the host, alternative countermeasures against the bite reaction might be sought by treatments such as, e.g. antihistamines or cortisone to reduce the secondary effects.

4. SUMMARY AND CONCLUSIONS

New methods devised here for the rapid handling and dissecting of live blackflies made possible the accumulation of large numbers of extirpated salivary glands within the limited activity period of the adult flies.

The short activity period of the adult flies under study imposed certain limitations on the amount of glandular material that could be collected. Future research might profitably investigate improvements to the techniques of laboratory rearing and maintenance, and perhaps the possibly of deep-freezing of the adult flies for subsequent gland removal and analysis.

A sensitive micro-scale protein assay proved effective for the determination of both total and water-soluble protein contents of the glands at various stages of development. The following conclusions can be drawn:

1. The salivary glands of Simulium decorum are growing and filling continuously for the first four days of adult life. The water-soluble protein content of the glands increases at a rate similar to that of the total protein content, of which it constitutes approximately 64%.
2. S. vittatum salivary glands increase in size and water-soluble protein content for the first four days of post-emergence activity, and then show a marked reduction by day 5. The

water-soluble protein content averages 76% of the total protein content.

Refined techniques of micro-electrophoresis employing 10 µL gradient polyacrylamide gels to separate the protein fractions by molecular weight, independently of electrical charge, demonstrate that the procedure is both capable and appropriate for handling the extremely small quantities of proteins contained in the salivary glands of blood-sucking blackflies. The high resolving power of the process has revealed as many as 32 individual protein bands in one separation (day 1 S. vittatum glands).

The following conclusions are based on comparisons of the separated proteins from the different metamorphic stages and post-emergence age groups of the salivary glands under study:

3. The protein constituents of larval salivary glands differ qualitatively and quantitatively from those of pupae or adults.
4. Pupal salivary glands are smaller than those of post-emergence stages but structurally resemble those of adults, and display a similar sequence of proteins in electrophoretic separations.

Progressive changes in the quantity and properties of adult salivary gland proteins were observed from electrophoretic results.

5. Three protein bands from S. decorum, in the MW range of 50,000-80,000, show a progressive increase in relative predominance from day 0 through day 4, the stage presumed to be the most prepared for blood feeding. There is also a concentration of more rapidly migrating protein (approximately 10,000 MW) in the day 4 salivary glands. These proteins merit

further investigation as to their possible role in the noxious properties of blackfly saliva.

6. Most of the protein in S. vittatum post-emergence salivary glands occurs in the range of 50,000-100,000 MW. The glands contain a progressively increasing concentration of 90,000 MW protein which culminates as one very distinct band in the day 4 glands. Day 1 salivary glands are the only age group to reveal three bands of rapidly migrating low MW protein in the 2,000-5,000 MW range.

Skin tests of S. vittatum salivary gland preparations in guinea-pigs previously sensitized by whole fly tissue gave presumptive evidence of a combination of antigenic and toxic factors:

7. An antigenic factor in the salivary glands was implied by the delayed reaction in the skin tests after prior injection of macerated salivary glands. Day 4 salivary glands induced the strongest delayed reaction in the sensitized animals.
8. Both control and sensitized guinea-pigs showed a strong cutaneous reaction to the injection of day 1 salivary glands, implicating a mildly toxic factor in the sequence of host bite reaction.

The establishment of both toxic and sensitizing components of the salivary glands indicates a possible mechanism by which the salivary products of these glands might act to cause an initial short-lasting erythema followed by a delayed and potentially more severe and sustained or recurrent reaction.

The conclusions drawn here are based on whole blackfly salivary glands, and the reactions they elicit in test animals. The need exists for further studies to investigate the relationships between the whole glands and the salivary products known to be injected into the host during the feeding process.

The demonstrated capability of the micro-electrophoretic procedure in resolving as many as 32 component proteins in minute samples of salivary glands, now makes it possible in future research to isolate any particular noxious proteins which may occur in the saliva itself. Further skin tests with the isolates, individually or severally, could throw light on the proteins of concern in any postulated aspect of the bite reaction. Then it should be possible to select particular proteins as antigens for developing active antibody responses, or for creating immune sera for passive protection.

From the exploratory skin tests described above, in which both antigenic and toxic factors are implied, the indicatons are that special attention be given to particular bands in day 1 and day 4 salivary glands. The recognition of two different categories of noxious substances, namely toxins and sensitizers, paves the way for improved prognosis, prophylaxis involving antigens and therapy by anti-inflammatory agents.

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APPENDIX

MICRO-PROTEIN ASSAY PROCEDURAL DETAILS (section 1.3.3)

Reagents:

4% Na_2CO_3 (w/v) (MCB, Norwood, Ohio)
2% $CuSO_4 \cdot 5H_2O$ (w/v) " " "
4% $Na_2C_4H_4O_6 \cdot 2H_2O$ (w/v) " " "
The above were all stored for up to one month at 5°C.

Folin-Ciocalteau Phenol Reagent(2N) (Fisher, Fair Lawn, N.J.)
diluted 1:2 with distilled water
(made up daily as required)

Stock solution BSA 500 µg/mL (Polysciences Inc., Warrington, Pa.)
made up as 50 mg BSA in 50 mL
distilled water, dispersed with
ultra-sonic homogenizer and made up
to 100 mL (made up daily as required)

Protein detection range 10-150 µg/500 µL

High range reagents:

150 mL 4% Na_2CO_3
1.5 mL 2% $CuSO_4 \cdot 5H_2O$
1.5 mL 4% $Na_2C_4H_4O_6 \cdot 2H_2O$

Procedure:

All microlitre applications were made with lambda volumetric transfer pipettes.

1. Duplicate samples of 11 concentrations of BSA were made up from the BSA stock solution in clean boiling tubes. Each total sample volume was 500 µL, concentration range 0-250 µg/500 µL.
2. 5.0 mL of high range reagent was added (and immediately vortexed) to each sample. The samples were covered and left at room temperature for one hour.
3. 500 µL of freshly diluted Phenol Reagent was added (and immediately vortexed) to each sample. The samples were covered and left at room temperature for 90 minutes.
4. Each sample was transferred to a micro-cuvette and read at 700 nm.

Protein detection range 1-10 µg/500 µL

Low range reagents:

100 mL 4% Na_2CO_3
5 mL 2% $CuSO_4.5H_2O$
5 mL 4% $Na_2C_4H_4O_6.2H_2O$

4 mL of BSA stock solution (500 µg/ mL) was made up to 100 mL with distilled water, thereby giving a 20 µg/ mL solution.

Procedure:

1. Duplicate samples of seven concentrations of BSA were made up from the diluted BSA solution. Each total sample volume was 500 µL, concentration range 0-10/500 µL.
2. 5.0 mL of low range protein reagent was added (and immediately vortexed) to each sample.
3. and 4. as described in high range assay.

INSECT RINGER'S SOLUTION (Section 1.2.2)

Reference: Child 1943

6.075 g NaCl
0.283 g KCl
0.170 g $CaCl_2$
Distilled water to 1000 mL

GLOSSARY

ACTIVE IMMUNITY: The immunity elaborated by the activity of an individual's own tissues, cells or body fluids. This primed population of cells will rapidly expand on renewed contact with antigen, and adequate levels of the antibody will be established.

ALLERGENIC: Acting as an antigen to induce an antibody response.

ANAPHYLAXIS: The reaction of antigen with a specific class of antibody bound to mast cells (reaginic antibodies). This leads to degranulation of the mast cell and release of vasoactive amines. Anaphylaxis may occur after the second injection of very small amounts of protein. Symptoms include intense contraction of smooth muscle, dilation of capillaries, and release of histamine.

ARTHUS REACTION: This reaction is due to antibody excess; the injected antigen precipitates with antibody and the complex binds complement. Histamine liberation occurs. The reaction usually peaks at 8-10 hours after the injection of antigen.

DELAYED REACTION: This is a cell-mediated reaction occurring approximately 24 hours after the injection of antigen into a sensitized animal.

ERYTHEMA: Redness of the skin due to congestion of the capillaries.

IMMEDIATE REACTION: This is a humoral antibody response characterized by the synthesis and release of free antibody into the blood and other body fluids. The immediate reaction occurs within 30-60 minutes after the injection of antigen.

PASSIVE IMMUNITY: Antibody protection acquired by giving preformed antibodies from another individual. As these antibodies are utilized the protection is gradually lost.

PRURITUS: Intense itching.

URTICARIA: Smooth elevated patches on the skin, often whiter than the surrounding skin.