TEMPORAL SYNTHESIS OF CUTICLE PROTEINS DURING LARVAL DEVELOPMENT IN THE TSETSE FLY, *GLOSSINA MORSITANS MORSITANS*

BY

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DECLARATION

I, Vincent Oduol Ochieng, hereby declare that this thesis is my original work and the work described here has not been presented to any other University.

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This thesis is dedicated to my parents, brothers and sisters for their patience and moral support throughout my studies.

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ABBREVIATIONS

BSA	-	Bovine serum albumin
IEF	-	Isoelectrofocussing
JH	-	Juvenile hormone
NADA	-	N-acetyldopamine
NANE	-	N-acetylnorepinephrine
NBAD	-	N-ß-alanyldopamine
NBANE	-	N-ß-alanylnorepinephrine
NEPHGE	-	None-equilibrium pH gel electrophoresis
NP-40	-	None ionic detergent P-40
PAGE	-	Polyacrylamide gel electrophoresis
PAS	-	Periodic acid Schiff reagent
PMSF	-	Phenylmethylsulfonylfluoride
PTTH	-	Prothoracicotropic hormone
RP-HPLC	-	Reverse phase high performance liquid chromatography
SDS	-	Sodium dodecyl sulphate
Tris	-	Tris-(hydroxy methyl)-amino methane
W/v	-	Weight by volume

UNIT ABBREVIATIONS

gm	-	gram
h	-	hour
M	-	molar concentration
mA	-	milliampheres
min	-	minute
mg	-	milligram
ml	-	millilitre
mm	-	millimetre
mM	-	millimolar
M_r	-	molecular weight
nm	-	nanometre
pH	-	-Log hydrogen ion concentration
pI	-	isoelectric point
μg	-	microgram
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SUMMARY

The tsetse fly is an insect of great economic importance to man as a vector of both human and animal trypanosomiasis.

The important functions of the cuticle are support, including muscle attachment, protection, and permeability barriers. Cuticle proteins are an important component, that define much of specialized structural and functional nature of the cuticle. A thorough knowledge of the patterns of cuticle proteins during larval development in *G. m.morsitans* is important in understanding their possible roles in cuticular sclerotization. Such knowledge might be useful in management of tsetse flies.

In this study proteins extracted from larval, pupal and adult cuticles of the tsetse fly, *Glossina morsitans morsitans* were compared electrophoretically by both SDS polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis. Proteins extracted from the third instar resolved into ten major bands (M_r 10 KD, 12 KD, 14 KD, 26 KD, 28 KD, 38 KD, 45 KD, 70 KD, 93 KD, 112 KD, 200 KD). In SDS-PAGE, two major proteins (45 KD and 200 KD) were common to all stages of development. The 45 KD had a carbohydrate moiety as it stained with Periodic acid schiff reagent (PAS). Cuticles from three larval instars (first, second and third) contained six low molecular weight proteins (10 KD, 12 KD, 14 KD, 30 KD, 50 KD, 80 KD). Two proteins emerging in pupal cuticle (29 KD and 98 KD) persisted upto the adult stages.

Further analysis by two-dimensional gel electrophoresis and silver staining showed that few cuticular proteins were synthesized between the 1st and 2nd instars. By third instar (2 days before larviposition), a large number of proteins were induced (M_r < 30 KD). These proteins persisted upto the brown pupal stage and showed a rapid decline thereafter. Most of the proteins with molecular weights M_r < 30 KD were undetectable at apolysis (5 days after larviposition). By 15 days pupal stage, the number of cuticle proteins was very small. Ligation of adults at eclosion resulted in notable changes of cuticle proteins.

CHAPTER 1

INTRODUCTION

1.1. **Insect Growth and Development**

The growth of insect larvae is restricted by the cuticle which only allows limited expansion. Growth is, therefore, punctuated by a series of moults. The number of moults which can occur is variable but is generally less in more advanced insects (Chapman, 1971). The last larval instar of most insects undergo metamorphosis, a process in which changes in the internal as well as external parts of the body accompany the transition from the last larval instar to adult (Chapman, 1971; Wigglesworth, 1972). In those insects with incomplete metamorphosis (hemimetabola), the process occurs in one step with the last larval stage transforming directly into the adult. In contrast those, insects with complete metamorphosis (holometabola), the changes during metamorphosis are more extensive and a pupal stage is interposed between the last larval stage and the adult (Chapman, 1971; Wigglesworth, 1972).

The cyclic process of growth and moulting is brought about by the prothoracicotropic hormone (PTTH) and ecdysone. Secretion of PTTH stimulates the prothoracic glands to secrete ecdysone which is subsequently converted to the active moulting hormone (ß-ecdysone). The moulting process is initiated by ß-ecdysone acting on the epidermal cells. The type of moulting is determined by the amount of juvenile hormone (JH) secreted by the corpora

allata. Thus, high JH titters dictates the larval-to-larval moult whereas low titters, larval-to-pupal moults. The pupal-to-adult metamorphosis occurs in the absence of JH (Riddiford and Truman, 1978; Gilbert *et al.*, 1980).

1.2. The Life Cycle of Tsetse Fly

Among insects, the tsetse fly and other viviparous flies have a unique mode of reproduction. The larva completes its entire development within the uterus of the female and is then larviposited at the completion of feeding (Riddiford, 1990). The evolution of viviparity in tsetse flies has resulted in a complex interaction between the mother and its progeny. Each pregnancy cycle, lasting from 9 to 10 days, culminates in the production of a single fully grown third instar larva which burrows into the soil and forms puparium within a few hours of larviposition (Buxton, 1955). The *in utero* development of the larva is made possible by the provision of nourishment from a modified female accessory gland called the milk gland.

At 25 °C, ovulation in *Glossina morsitans morsitans* occur one hour after the previous larviposition, the egg hatches on day 3.8, ecdysis to second instar occurs on day 4.9, the third instar cuticle is formed on day 6.8, and parturition occurs on day 9.0 (Denlinger, 1974).

1.3. Structure and Composition of Insect Cuticle

The insect cuticle provides essential protection from the environment and defines the form of the organism. The cuticle is also a dynamic structure with

the old cuticle in part recycled and in part lost, and a new cuticle formed at each moult in order to accommodate the growth and metamorphosis of the insect.

The insect cuticle is made up of three components: protein, chitin (polymer of N-acetylglucosamine) and the exterior surface lipids (Willis, 1987). Studies by light microscopy have shown that the outermost part of cuticle is a layer distinct from the rest, and that this layer, (the epicuticle) is a tightly cross-linked network of lipids and proteins (Weis-Fogh, 1960). The sequence of deposition of the various parts of the epicuticle has been determined. The cuticulin layer is the first to be deposited when a new cuticle is formed after apolysis (Filshie, 1970; Locke, 1966; 1974). The protein epicuticle is deposited beneath the cuticulin (Andersen, 1979). Chitin has not been demonstrated in the epicuticle.

The procuticle is that region of the cuticle located between the epicuticle and epidermal cells, and the former constitutes the main part of the total cuticle. It can be subdivided into several layers with different staining properties: exocuticle, mesocuticle, endocuticle, and deposition layer. The differences in ability to bind various stains are presumably due to different degrees of secondary modification of the cuticle, such as sclerotization. Insect procuticles consist predominantly of protein and chitin, but other materials (lipids, pigments, inorganic materials, and small organic molecules) can also be present (Andersen, 1979). Most of the major properties of cuticle depend upon the

amount and organization of the individual components in the procuticle, as well as upon the interaction between the components.

Chitin is present as long microfibrils, usually about 2.8 nm thick and of indefinite length. Each microfibril may contain 18-21 chitin molecules. The microfibrils are embedded in a protein matrix, but the relative amounts of chitin and protein can vary considerably from cuticle to cuticle.

1.4. Sclerotization of Insect Cuticles

During sclerotization of insect cuticle the O-diphenols, N-acetyldopamine (NADA) and/or N-ß-alanyldopamine (NBAD), are enzymatically oxidized after which the major parts react with cuticle protein and/or chitin resulting in a chemically stabilized structure (Hopkins *et al.*, 1982; Lipke *et al.*, 1983; Andersen, 1985). Two enzymes are involved, a desaturase and phenoloxidase with the last part of the reaction occuring spontaneously (schemes I and II). A minor fraction of the oxidation products react with low molecular weight compounds (O-diphenols and water) to form soluble products. These may be inessential for hardening but they are useful for obtaining information on the chemical reactions during sclerotization. Various schemes have been proposed for the chemical reactions during sclerotization. They are mainly based on the structure of some of the reaction products formed *in vivo* or *in vitro*. The formation of dimeric products of the benzodioxin type has been used as evidence for ß-sclerotization, α, ß-dehydro-N-acetyldopamine (dehydro-NADA) being an essential intermediate (Scheme 1). In this type of sclerotization,

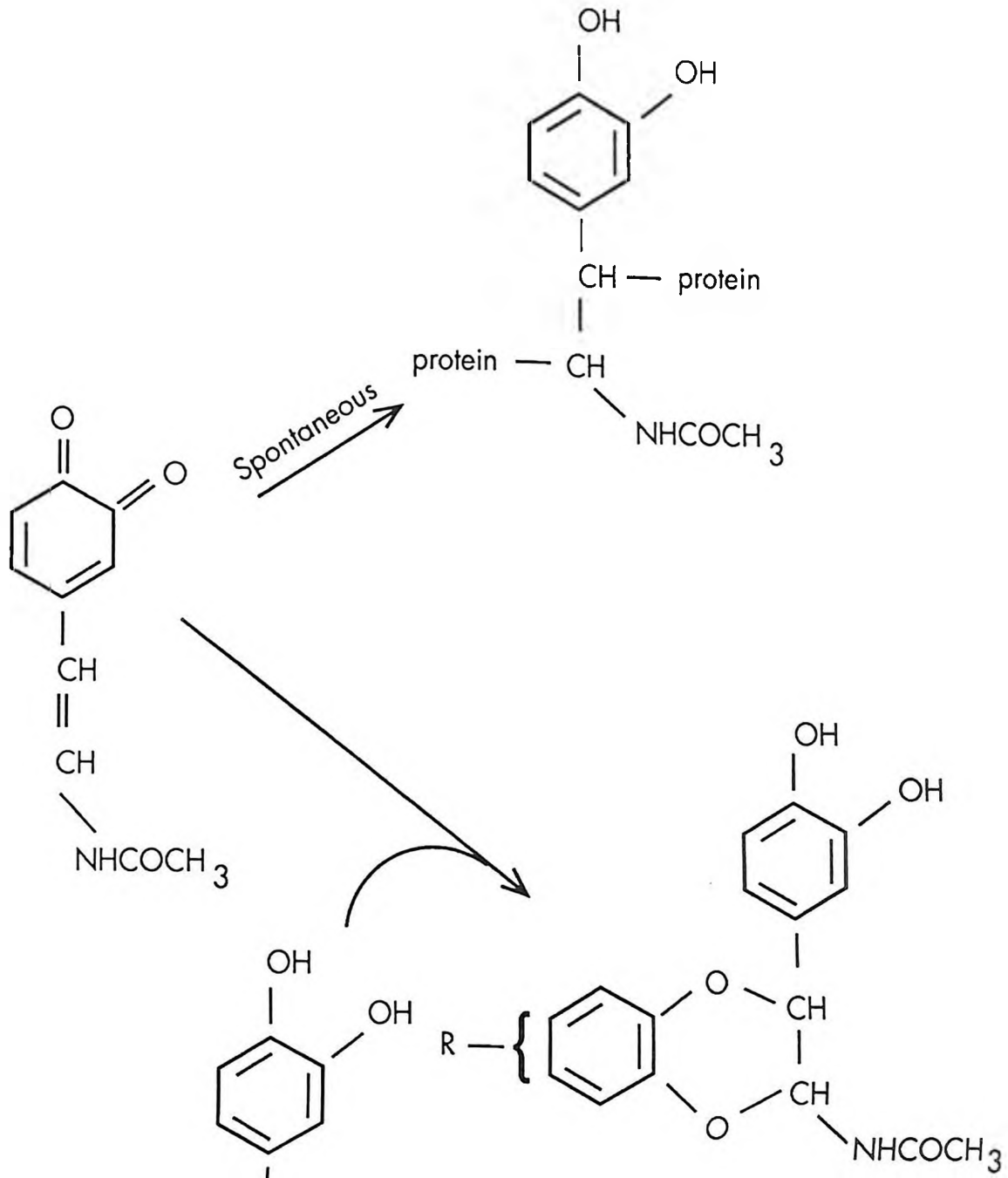
significant amounts of ketocatechols are released by acid hydrolysis of the sclerotized cuticle (Andersen and Roepstorff, 1982; Andersen, 1985). The formation of N-acetylnorepinephrine (NANE) has been used as evidence for quinone sclerotization, quinone methides being intermediates (Suguraman and Lipke, 1983; Lipke *et al.*, 1983; Suguraman, 1987). The main features of quinone methide sclerotization as well as of O-quinone tanning are indicated in Scheme II.

The products formed when various types of cuticles are incubated with N-acetyldopamine (NADA) have been studied using reverse phase high performance liquid chromatography (RP-HPLC) (Andersen, 1989 a, b). The results indicate that NADA-sclerotization occurs via both ß-sclerotization, quinone methide sclerotization and O-quinone tanning, all at the same time, and one of the pathways may dominate depending upon the relative amounts of various enzymes.

Evidence has been presented suggesting that NBAD may be treated somewhat differently from NADA during its incorporation into cuticle. It has been observed that NADA is a better precursor than NBAD for products giving ketocatechols after acid hydrolysis (Morgan *et al.*, 1987). It has been suggested that NBAD sclerotization is mainly correlated with formation of brown sclerotized cuticles, whereas NADA sclerotization would give almost colourless cuticles (Hopkins *et al.*, 1982; 1984). In cuticles using NBAD for sclerotization, the formation of the ß-hydroxylated product, N-ß-alanylnorepinephrine (NBANE), has been observed (Morgan *et al.*, 1987) to be analogous to the formation of

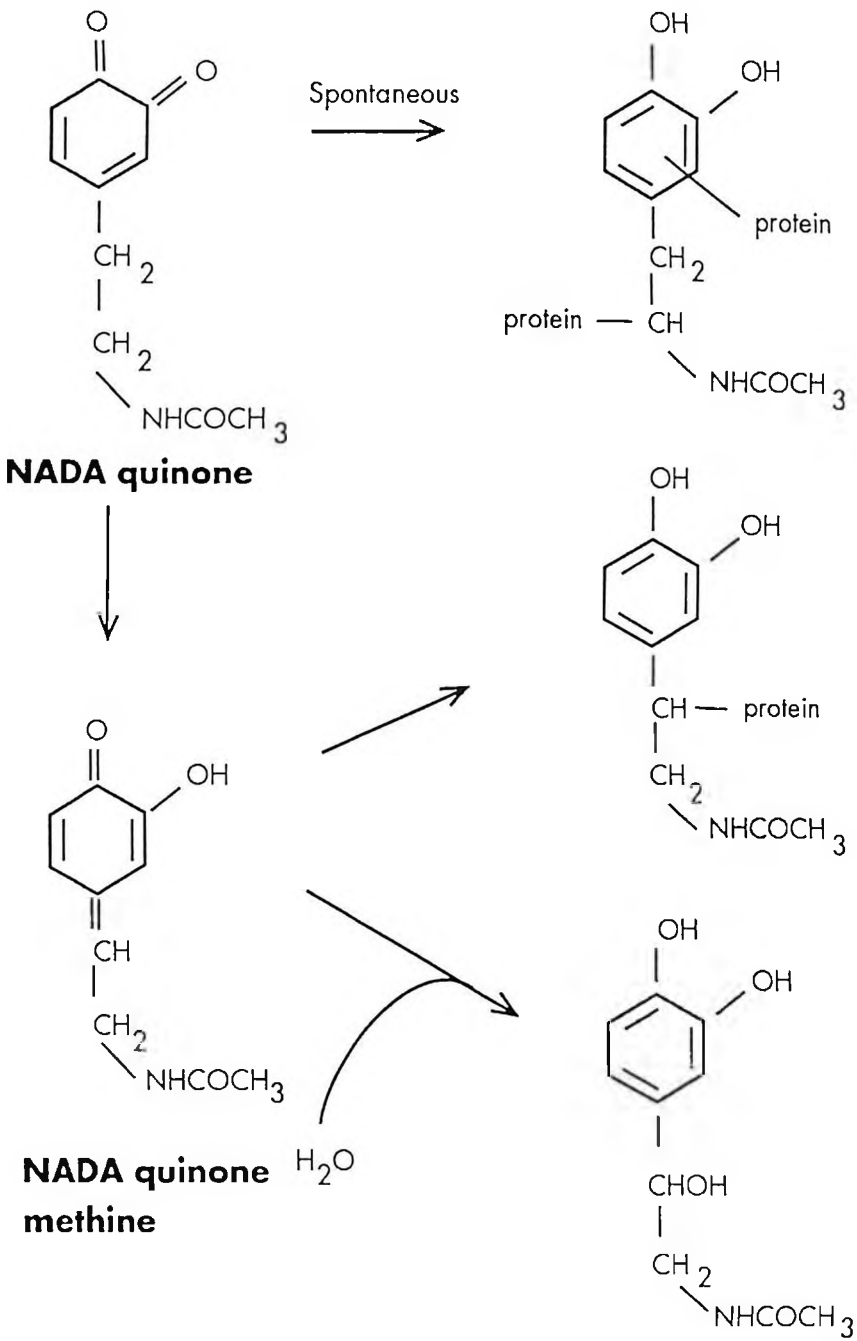
SCHEME 1: The formation of dimeric products of the benzodioxin during β-Sclerotization.

NADA **dehydro–NADA**


OH
OH
CH — protein
protein — CH
NHCOCH 3
Spontaneous
O
O
CH
CH
NHCOCH 3
OH
OH
OH
OH
R
O
CH
CH
O
NHCOCH 3

SCHEME 2: The formation of N-acetylnorepinephrine (NANE) during quinone methide and O-quinone sclerotization.

NADA


Spontaneous
protein
protein
NADA quinone
NADA quinone methine
H_2O

N-acetylnorepinephrine (NANE) from NADA in cuticle. The formation of NBAD-dimers or of α, ß-dehydro-N-ß alanyldopamine (dehydro-NBAD) has not been noticed in any cuticle, although the corresponding NADA-derivatives are formed in several types of cuticles (Andersen, 1989 b).

1.5. Insect Cuticle Proteins

Proteins are a major component of the cuticle constituting approximately 50% of the dry weight (Richards, 1978). Chitin makes up most of the rest of the bulk while exterior surface lipids complete the composition (Willis, 1987). Insect cuticle is an extracellular composite structure that consists of chitin filaments embedded in a protein matrix. The mechanical properties of cuticle are influenced by the relative amounts of chitin and proteins, the architecture of the chitin filaments, the degree of hydration of the proteins, the degree of sclerotization, and presumably also the properties of the individual proteins (Andersen, 1979). When analyzed by electrophoresis or on the basis of solubility, cuticle protein extracts appear to be heterogeneous. The major soluble cuticle proteins from a variety of insects share certain properties (Hackman, 1974 b, Neville, 1975); they are generally of relatively low molecular weights (M_r < 30 KD) and low isoelectric points (pI 3-6). The soluble cuticle proteins are generally assumed to be derived largely from the procuticular region where they exist unbound or associated to varying degrees with microfibrils of chitin and other cuticular components.

Proteins provide a great deal of diversity to the cuticle composition. It seems likely that they define much of the specialized nature of the insect cuticle both in different anatomical regions and at different metamorphic stages (Silvert, 1985; Willis, 1987).

Several detailed biochemical analysis of the insect cuticle proteins have emerged in the last decade mainly on three insect orders, the Lepidoptera, Diptera and Orthoptera (Shawky and Vincent, 1978; Sridhara, 1983; Silvert *et al.*, 1984; Andersen *et al.*, 1986; Hojrup *et al.*, 1986; Willis, 1987; Skelly and Howells, 1987, 1988; Andersen, 1988). Coleopteran cuticle proteins have been studied in *Tenebrio* (Roberts and Willis, 1980 a-c; Lemoin and Delachambre, 1986; Lemoine *et al.*, 1989, 1990) and *Anthonomus* (Stiles and Leopold, 1990; Stiles *et al.*, 1991).

A few studies have examined the regulation of the synthesis of the cuticle proteins (Roberts and Willis, 1980 c; Kiely and Riddiford, 1985 a, b; Fristrom *et al.*, 1986; Wolfgang and Riddiford, 1986; Cassier *et al.*, 1988) or regulation of expression of cuticle protein genes by the endocrine system (Synder *et al.*, 1981; Sridhara, 1985; Riddiford, 1986; Riddiford *et al*, 1986; Kimbrell *et al.*, 1988; Apple *et al.*, 1991). Cuticle proteins specific to individual developmental stages of the insect have been identified (Roberts and Willis, 1980 b; Chihara *et al.*, 1982; Sridhara, 1983; Kiely and Riddiford, 1985 a; Lemoine and Delachambre, 1986; Andersen and Hojrup, 1987; Skelly and Howells, 1988).

1.6. Properties and Functions of Cuticle Structural Proteins

In all insects studied so far, a number of different cuticle proteins are present. Serial extraction with a number of solvents, which are taken to affect different intermolecular types of chemical bonds, are often used to separate the proteins into different fractions. The extracted protein fractions have been compared with respect to amino acid composition, separation by gel electrophoresis, electrofocusing and gel filtration. Solvents generally used for extraction are distilled water; weak solutions of neutral salts, taken to break electrostatic and Van der Waals interactions between macromolecules; strong solutions of urea, taken to break hydrogen bonds; and dilute sodium hydroxide, taken to break stronger bonds, such as Schiff's bases and susceptible peptide bonds. The sequential extraction of cuticle proteins is of direct importance in understanding protein structure within the cuticle (Hackman, 1972; 1974; 1975).

The form of the cuticle differs between different insect species and also at various times during the development of a particular species. This change in form is often accompanied by changes in the protein composition of the cuticle. For instance, the soluble cuticle proteins of different metamorphic stages (larval, pupal and adult) of *Galleria mellonella* (Lepidoptera), *Tenebrio molitor* (Coleoptera) and *Drosophila melanogaster* (Diptera) show unique electrophoretic profiles (Srivastava, 1970; Roberts and Willis, 1980; Chihara *et al.*, 1982). The soluble cuticle proteins of three larval instars of *T. molitor*, however, have the same profile (Roberts and Willis, 1980). In the case of *Drosophila melanogaster*, the electrophoretic profiles of the cuticle proteins of

first and second larval instars are largely identical but the profile changes markedly during the third instar (Chihara *et al.*, 1982).

It is now widely recognized that the cuticle contains many different proteins. Five protein fractions were identified using electrophoretic separation of proteins extracted in pH 9.2 borate buffer solution from larvae of the Scarab beetle, *Diaphonia dorsalis* (Hackman, 1953 a). The occurrence of many different cuticle proteins in the larvae of *Musca domestica* has been demonstrated (Downe, 1962). Subsequent applications of immunological methods have revealed several proteins in the cuticle of *Hyalophora cecropia* (Willis, 1970), *Periplaneta americana* (Fox *et al.*, 1972), and *Manduca sexta* (Koeppe and Gilbert, 1973). A large number of proteins have been identified by gel electrophoresis and Sephadex thin layer studies. For example, fourteen bands have been identified in *Periplaneta americana* (Fox and Mills, 1969).

1.7. The Importance of Characterizing Cuticle Proteins in Tsetse Fly

The cuticles of insects function both as exoskeleton and as barriers between the living tissues and the environment. Also to a large extent, the cuticle also determines the shape and appearance of insects. Insect growth depends on the precise timing of the shedding of the old cuticle and the synthesis of a new one. The cuticle, therefore, is an essential and a dynamic organ. Cuticle proteins are an important component, that define much of specialized structural and functional nature of the cuticle. Patterns of cuticle proteins during larval development have been established in most other insects of great economic importance to man. However, no information is available on

the tsetse fly, an insect of great economic importance to man as a vector of both human and animal trypanosomiasis. A thorough knowledge of the patterns of cuticle proteins during larval development in the tsetse fly is important in understanding their possible roles in cuticular sclerotization. Such knowledge might be useful in the management of tsetse flies as the vector of both human and animal trypanosomiasis.

The tsetse larva has a very brief free living existence. The third instar, after completing its development within the uterus of the female, is deposited and burrows into the substrate where it pupariates within 1-2 hours. This rapid sclerotization is possibly linked evolutionarily to the exposure of the larva to dangers of predation by ants, birds, spiders and lizards as well as parasites such as fungi, protozoa, bacteria and viruses. Accelerated pupariation may also be an adaptation to protect larva against environmental factors such as sunlight, heat, or low humidity. Cuticle hardening can be prevented by interfering with the proteins during development. Soft and unsclerotized cuticles would not provide protection against a variety of factors. Unfavourable environmental factors would result in larval death through desiccation.

The whole process of cuticle hardening in tsetse is a very rapid process compared to most other insects. For example *Lucilia cuprina* larvae (Dipteran) takes 66 hours to pupariate while tsetse fly larvae takes only 2 hours. During the process there is sequential expression of cuticle proteins. This provides a unique model for studying gene expression where genes are switched on and off.

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1.8. Specific objectives of the study

The aims of the present study were to:

(a) Establish the conditions for the separation of cuticle proteins by 2-dimensional gel electrophoresis.

(b) Use the technique to study the temporal synthesis of cuticle proteins during larval development in tsetse flies.

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CHAPTER 2

MATERIALS AND METHODS

2.1. Reagents

The laboratory chemicals used in the experiments were of analytical grade obtained from BRL, Particle Data Laboratories, Serva, Sigma, Pierce, Pharmacia-LKB and Bio-Rad.

2.2. Experimental Insects

Tsetse flies (*Glossina morsitans morsitans* Westwood) were obtained from Insect and Animal Breeding Unit (IABU) of the International Centre of Insect Physiology and Ecology (ICIPE). They were maintained at 25 °C and 70% relative humidity under a 12 Light: 12 Dark photoperiod and fed daily on rabbit ears. Three day old female and seven day old male flies were allowed to mate. Pregnant flies were dissected to obtain first and second instar larvae. Third instar larvae about two days to larviposition were forcibly expelled from the uterus by applying pressure on the female abdomen. Some flies were left to larviposit normally and pupae obtained at different stages. Some pupae were left to emerge into adults.

2.3. Preparation of the Cuticle

Cuticles were isolated from first instar larvae, (day one) and second instar, (day one). The polypneustic lobes of the third instar larvae (seven days after ovulation) become black about two days before parturition and are visible as dark spots through the abdominal wall of the female (Denlinger and Ma, 1974). The larvae were obtained by abortion (early third instar) and cuticles isolated. Cuticles were also isolated from third instar normally larviposited (late third instar).

Puparia of *G. m. morsitans* were separated from the underlying pupal and pharate adult structures at various times by dissection. Pupal to adult development begins with the formation of the white puparium. Pupal cuticle forms beneath and separates from the puparium within five days (at 25 °C) of white puparium formation. The outer covering case was dissected out leaving the developing pupa from which cuticle was dissected. Cuticles were also isolated from 10 and 15 days old puparia after white puparium formation. Pupal cuticles were isolated by dissection.

In the case of adults, only abdominal integument was used. Cuticles were freed of contaminating tissue under a dissecting microscope. Isolated cuticles were rinsed three times in tsetse Ringer solution and blotted dry on filter paper and stored at -80 °C.

2.4. Extraction of Cuticle Proteins

For one-dimensional gel electrophoresis, the tissue was homogenized for 2 minutes in 100 μl/insect of boiling 6.25 mM Tris pH 6.8, 2% SDS, 1 mM phenylmethylsulfonylfluoride (PMSF). Samples for two-dimensional gels were homogenized at 4 °C in 100 μl/insect of 6.25 mM Tris pH 6.8, 1% Nonidet P-40 and 1 mM PMSF.

2.5. Protein Estimation

Protein estimation was carried out by BCA protein assay method (Rockford, IL. USA). Bovine serum albumin (BSA fraction V) was used as the protein standard.

2.6. One-Dimensional Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Gradients (4-20%) were cast using a gradient marker (BRL). Samples were dissolved in an equal volume of sample buffer (130 mM Tris-HCl, 20% glycerol, 0.002% bromophenol blue, 4% SDS, 1% ß-mercaptoethanol, pH 6.8) and boiled for three minutes in a water bath, prior to application onto the gel. Electrophoresis was performed at room temperature at a constant current of 25 mA through the stacking gel, then 30 mA through the separating gel.

Gels were stained with Coomassie Brilliant Blue (Weber and Osborn, 1969) in a solution of acetic acid, methanol and distilled water (9.2:50:40.8)

overnight. The gels were then treated with several changes of destaining solution (acetic acid, methanol, distilled water, 9.2:50:40.8) for 12-15 hours at room temperature. Destained gels were stored in 7% acetic acid until photographed using Kodak, Panatomic X films.

2.7. Molecular Weight Determination

SDS-PAGE was used to determine the molecular weights of the cuticle proteins using protein standards from Bio-Rad. The protein standards used were: trypsin inhibitor (M_r ~ 21,500), bovine carbonic anhydrase (M_r ~ 31,000), ovalbumin (M_r ~ 42,699), BSA (M_r ~ 66,200), phosphorylase b (M_r ~ 97,400), lysozyme (M_r ~ 116,000) and myosin (M_r ~ 200,000). After staining the gels with Coomassie Brilliant Blue and destaining, the molecular weights were determined from plots of log molecular weight versus relative migration of the protein standard.

2.8. Two-Dimensional Polyacrylamide Gel Electrophoresis

2.8.1. Preparation of Samples

Proteins to be separated on the first dimension of 2-D PAGE were prepared in the isoelectric focusing (IEF) sample buffer which contained urea, NP-40, ß-mercaptoethanol and Ampholines, pH range 5 to 7 and 3 to 10 mixed in a ratio of 4:1 for establishing the pH gradient. Dilute samples were used but because it was necessary to use more of a dilute sample, precautions were

taken to prevent excessive lowering of the urea concentration in the sample buffer. The following proportions were used:

Aqueous sample = 10 μl

Crystalline urea = 10 mg

IEF sample buffer = 10 μl.

The above preparation ensured complete solubilization and denaturation of the proteins as well as appropriate chemical make up of the samples.

2.8.2. **Sample Storage**

Samples in IEF sample buffer were stored in tightly sealed tubes at -80 °C to prevent evaporation and samples breakdown.

2.8.3. **First Dimension Electrophoresis**

The procedure for first dimension gel electrophoresis was carried out according to O'Farrel *et al.,* (1977) with a few modifications. Briefly, isoelectricfocussing gels were cast in glass tubing (139 x 3 mm) sealed at the bottom with Parafilm. The length of the gel tube and gel affect reproducibility. Consequently, these parameters were kept constant between samples that were being compared. To prepare 10 ml of gel mixture, 5.5 g of urea was added to a 125 ml side arm flask, followed with 1.33 ml of 30% acrylamide stock, 2 ml of stock Nonidet P-40: 10% (w/v) NP-40 in H_2O, 1.97 ml of H_2O, 0.4 ml of Ampholines, pH range 5 to 7, and 0.1 ml of pH 3 to 10. In all, the gel contained 9.2 M urea, 4% acrylamide, 2% NP-40, and 2% Ampholines. The

monomer solution was mixed in a flask omitting the NP-40; TEMED and ammonium persulfate. The urea was completely dissolved by gentle warming. The solution was degassed with vacuum for about 5 min with regular stirring. The NP-40 was added and gently swirled to mix, then 10 μl of 10% ammonium persulfate was added. Immediately after addition of 7 μl of TEMED, the solution was loaded into the gel tubes care being taken not to introduce air bubbles. The tubes were filled to approximately 14 mm from the top. This could be varied to hold samples of different volumes. The gel was overlaid with 8 M urea and after 1 to 2 hours, the overlay solution was removed and replaced with 20 μl of lysis buffer (LB: 9.5 M urea, 2% (w/v) NP-40, 2% Ampholines comprised of 1.6% pH range 5 to 7 and 0.4% pH range 3 to 10 and 5% ß-mercaptoethanol) overlaid with a small amount of water. The gels were allowed to polymerize for 1 to 2 hours. The Parafilm was removed, and the gels placed in a standard tube gel electrophoresis chamber. The lysis buffer and water were removed from the surface of the gel and 20 μl of fresh lysis buffer were added. The tubes were then filled with 0.02 M NaOH. The lower reservoir was filled with 0.01 M H_3PO_4 and the upper reservoir was filled with 0.02 M NaOH which was extensively degassed to remove CO_2. The gels were then prerun according to the following schedule for ten gels: (a) 200 volts for ¼ hour; (b) 300 volts for ½ hour; (c) 400 volts for ½ hour. The power supply was turned off, the upper reservoir was emptied, lysis buffer and NaOH were removed from the surface of the gels, and the samples were loaded (119 μg). The samples were overlaid with 10 μl of sample overlay solution (to 100 μl lysis buffer was added 20 μl

deionized distilled H_2O) then 0.02 M NaOH, and the chamber was refilled. After loading the samples, the gels were run at 400 volts for 16 hours and then at 800 volts for 1 hour.

In order to remove gels from tubes, they were "rimmed" by inserting a blunt thin needle and gently running it around the edge between the gel and the glass.

2.8.4. **Equilibration**

Gels were extruded into screw capped vials half filled with equilibration buffer (10% glycerol, 2.3% SDS, 5% ß-mercaptoethanol, 62 mM Tris, pH 6.8). The gels were equilibrated for about 30 minutes at room temperature at which point they were loaded on the second dimension gel, or frozen at -80 °C for later use.

2.8.5. **Measurement of pH Gradient**

The isoelectric focusing gel was cut into 5 mm sections which were placed in individual vials with 2 ml of degassed water. These vials were capped and shaken for 5 to 10 min; then pH was measured on a pH meter.

2.8.6. **Second Dimension Electrophoresis**

The second dimension SDS polyacrylamide slab gels were carried out on gradients 6-20% or 8.5% polyacrylamide gels topped by a 3% stacking gel (Laemmli, 1970).

deionized distilled H_2O) then 0.02 M NaOH, and the chamber was refilled. After loading the samples, the gels were run at 400 volts for 16 hours and then at 800 volts for 1 hour.

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The second dimension SDS polyacrylamide slab gels were carried out on gradients 6-20% or 8.5% polyacrylamide gels topped by a 3% stacking gel (Laemmli, 1970).

The stock solutions were mixed in the following way to make volume of the running gel (8.5%): 17 ml of 30% stock acrylamide, 12.0 ml of 5 X separating buffer (1900 mM Tris-HCl pH 8.8, 2% SDS), 31 ml distilled water, 200 μl 10% ammonium persulfate and 60 μl TEMED. To make SDS separating solution (6-20%) the following proportions were mixed: 6 ml of 30% stock acrylamide, 6 ml of 5 X separating buffer, 18 ml distilled water, 200 μl 10% ammonium persulfate, 10 μl TEMED and 20 ml of 30% acrylamide, 6 ml of 5 X separating buffer, 4 ml distilled water, 150 μl 10% ammonium persulfate, 10 μl TEMED. Gradients (6-20%) were cast using a gradient marker (BRL). The gel solutions were mixed as above omitting the TEMED and ammonium persulfate. The solutions were filtered through a Millipore 0.22 μ filter. The separating gel solution was degassed for 5 min. TEMED and ammonium persulfate was then added to the separating gel solution, swirled to mix and poured between the glass plates to within about 1 cm of the notch. The gel solution was overlaid with 1-2 ml of butanol-saturated with water. The mixture was allowed to polymerize for 45 min at room temperature. Butanol overlay was removed and top of the gel rinsed with 1 X stacking buffer (500 mM Tris-HCl, pH 6.8). The stacking gel was poured (2 ml of 30% stock acrylamide, 2 ml 10 X stacking buffer (1300 mM Tris-HCl pH 6.8, 2% SDS), 16 ml distilled water, 60 μl ammonium persulfate and 24 μl TEMED) to within 2-3 mm of the top of the glass plate.

When the stacking gel had polymerized, the top was rinsed with the tank buffer (25 mM Tris, 192 mM Glycine, pH 8.3, 0.1% SDS). The sample buffer

was poured off from equilibrated IEF tube gel and thawed for 15 minutes before required if frozen. Tube gels were laid onto a piece of Parafilm. The cathode end was identified (usually uneven and slightly shrunken). The tube gel was placed on top of the slab gel orientating so that the cathode was to the right and overlaid with 0.5-1 ml agarose (1% in equilibration buffer: 10% glycerol, 2.3% SDS, 5% ß-mercaptoethanol, 62 mM Tris, pH 6.8). The gels were loaded into the tanks and the tank buffer was added to both chambers. Electrophoresis was carried out at 25 mA/gel (constant current) until sample had migrated through the stacking gel and increased to 35 mA/gel until bromophenol dye marker reached a few millimetres from the bottom of the gel.

Silver staining

Silver staining was done according to Wray *et al.*, (1981). Double distilled water was used. Freshly prepared reagents was used. The procedure was as follows:

1. **Fixation of Proteins in the gel**

		Time (minutes)
CYCLE I	1. Acetic Acid 10% + Methanol 50%	2
	2. Methanol 50%	10
	3. Methanol 50%	10
CYCLE II	1. Distilled H_2O	5
	2. Methanol 50%	10
	3. Methanol 50%	10
	4. 2.5% Glutaraldehyde	30
CYCLE III	1. Distilled H_2O	5
	2. Methanol 50%	10
	3. Methanol 50%	10

The proteins were fixed onto the gel as shown in the table.

2. **Staining**

Solution A - 0.8 g Silver nitrate was dissolved in 2.5 ml distilled water.

Solution B - 1 ml 2 M NaOH was placed in a clean flask and volume adjusted with distilled water to 20 ml. 1.6 ml NH_4OH (concentrated) was added.

Solutions A and B were mixed dropwise to avoid precipitation. The volume was adjusted to 100 ml with distilled water. After fixation, the gel was placed in the solution for 15 minutes and then rinsed in distilled water for 5 minutes.

3. Development

To 2.5 ml 1% citric acid, was added 125 μl 38% formol and adjusted to 250 ml with distilled water. The gel was placed in this solution with shaking until clear colouration was seen. The reaction was stopped using 5% acetic acid for 5 minutes.

CHAPTER 3

RESULTS

3.1. General considerations of the results

An essential prerequisite for any study of cuticle proteins is the assurance that one has eliminated cellular and haemolymph contamination. In this regard, cuticles were thoroughly cleaned and were observed to be free of contaminating tissue when examined under a dissecting microscope. Repeated attempts have previously been made to obtain cuticle proteins from different stages in the life cycle of a variety of insects and to compare them, either by amino acid or electrophoretic analysis (Srivastava, 1970; Hackman and Goldberg, 1976 and Andersen, 1979). The varied results obtained have been mainly due to the various solvents utilized to extract the proteins. The solubility of the proteins depend on the bonding of the polypeptides to other cuticular components, principally chitin as well as degree of sclerotization. Since analysis of the extracted cuticular polypeptides are usually carried out by SDS-PAGE or isoelectric focusing in urea, SDS and N-P40 were used for extraction and separation of samples on SDS and IEF, respectively.

The soluble protein fraction of isolated tsetse fly cuticle was heterogeneous as judged by electrophoresis. The proteins extracted at different developmental stages also differed as determined electrophoretically (Figure 1a).

3.2. Analysis of Cuticle Proteins by One Dimensional Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). The separating gel was a 4-20% gradient of acrylamide.

3.2.1. Larval cuticle

Figure 1a illustrates protein pattern for extracts of cuticle proteins of larval, pupal and adult. Molecular weight values were determined by comparison with Bio-Rad protein standards (Fig. 1b and table 1). The electrophoretic patterns of cuticle proteins from first and second instars larvae ($L_{1,2}$) were essentially identical, the major bands being present in both. Proteins 21 KD and 14 KD were more prominent in L_2 than in L_1 (Fig. 1 Lane L_1 and L_2). The greatest amount of protein was obtained from 3rd instar larval cuticle (larviposited) (Fig. 1a, LL_3). Eleven major proteins were consistently observed, with molecular weights ranging from 10 KD to 200 KD. Most of the larval proteins, except six, appeared to be significantly present in pupae. Thus, there appeared to be at least six larval specific cuticle proteins Fig. 1a (10 KD, 12 KD, 14 KD 30 KD, 50 KD and 80 KD).

3.2.2. Pupal Cuticle

The pupal cuticle proteins were resolved into seven major bands that were consistently observed in the three different pupal ages examined and

Fig. 1a. A 4-20% gradient SDS-PAGE of cuticle proteins extracted from various developmental stages of the tsetse fly. The proteins separated for different stages are identified by letters on top. L_1, first instar larval cuticle; L_2, second instar larval cuticle; LY_3, third instar larval cuticle (aborted); LL_3 third instar larval cuticle (larviposited); P_1, five-day old pupal cuticle; P_2, ten-day old pupal cuticle; P_3, fifteen-day old pupal cuticle; A, adult cuticle 2 hours after eclosion; MH and ML are high and low molecular weight markers, respectively.

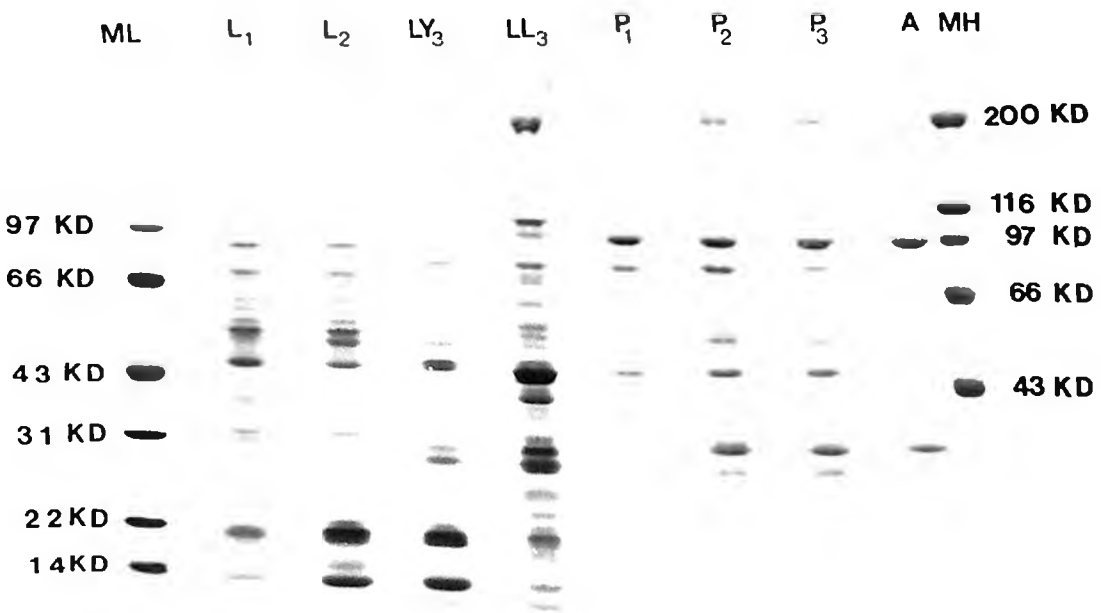

ML
L1
L2
LY3
LL3
P1
P2
P3
A
MH
97 KD
66 KD
43 KD
31 KD
22KD
14KD
200 KD
116 KD
97 KD
66 KD
43 KD

Fig. 1b. Standard curve of log molecular weight against relative mobility on a 4-20% SDS-PAGE polyacrylamide gel.

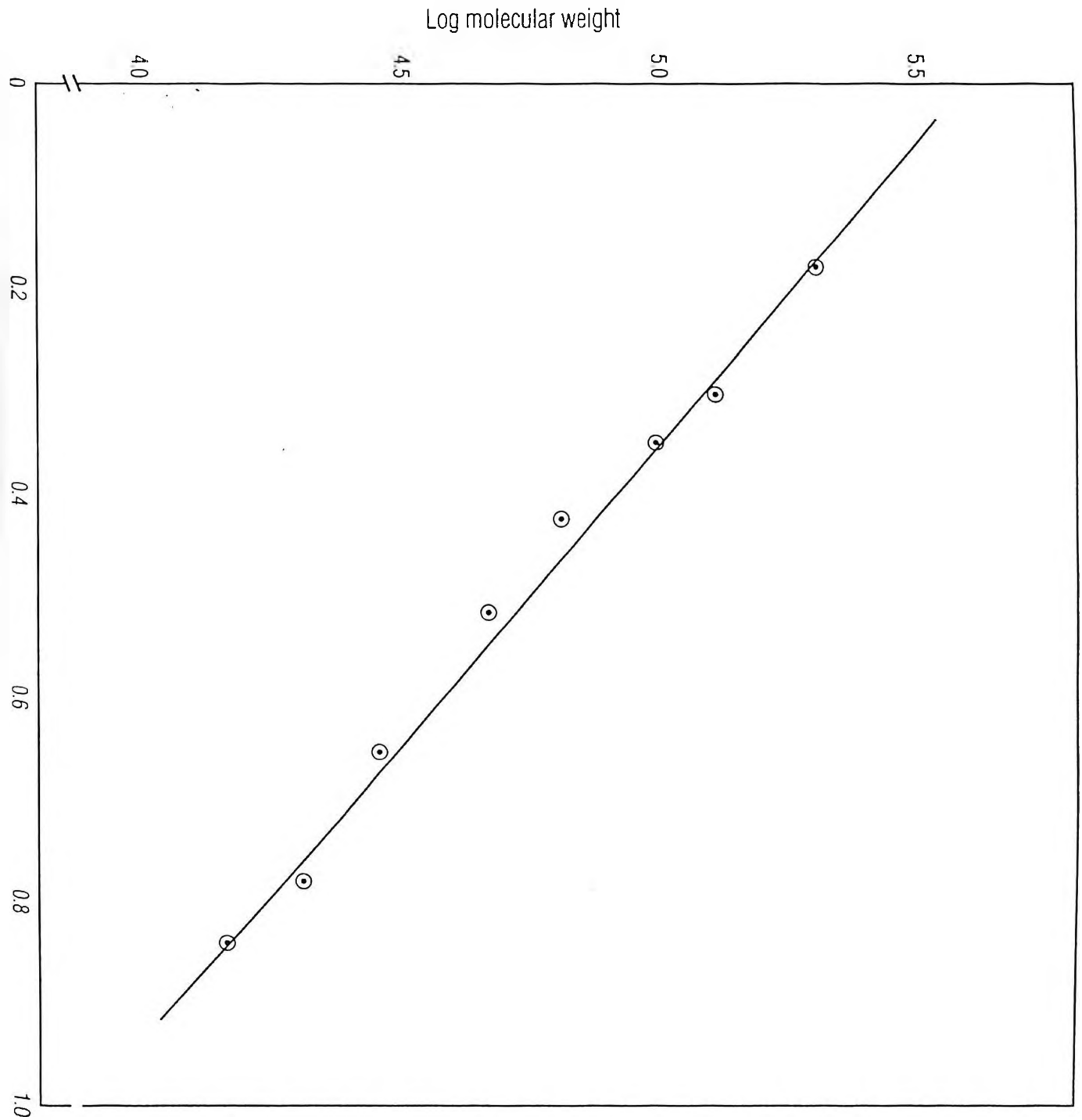

Log molecular weight
4.0
4.5
5.0
5.5
0
0.2
0.4
0.6
0.8
1.0

Table 1: Relative staining intensities of important bands in the larval, pupal and adult stages. L_1, larva (first instar); L_2, larva (second instar); LY_3 larva (third instar aborted 2 days to larviposition) LL_3, larva (third instar larviposited); P_1, pupa (five days old); P_2, pupa (ten days old); P_3, pupa (fifteen days old); A, adult; +++, major band; ++, intermediate band; +, minor band.

Dist	Mwt	Rf	L_1	L_2	LY_3	LL_3	P_1	P_2	P_3	A
2.2	200000	0.176	++	++	+	+++	++	+++	+++	+
4.0	112202	0.320				++	+	+		
4.3	97499	0.344				+				
4.4	97724	0.352					+++	+++	+++	+++
4.5	93325	0.360	++	++		++			+++	
4.8	85114	0.384			++					
4.9	83176	0.392					+++	+++		+
5.0	79433	0.400	++	++	+	+				
5.5	70795	0.440				++				
6.5	48978	0.520	++	++	+	+				
6.8	44668	0.544	+++	++	+++	+++	+++	+++	+++	+
7.3	38019	0.584				+++				
8.0	31623	0.640	+	+						
8.2	28840	0.656	+					+++	+++	++
8.3	28184	0.664				+++				
8.4	27542	0.672		+	+++					
8.6	25704	0.688				+++				
9.5	18197	0.760						+	++	
9.8	17378	0.784	++	+++	+++					
10.4	14454	0.832	+++	+++						
10.65	13804	0.852	++	+++	+++	+++				
11.3	10965	0.904		+		+++				

ranged in molecular weights from 31 KD to 200 KD (Fig. 1a, lane $P_{1,2,3}$). Of, these four bands (29 KD, 45 KD, 83 KD and 97 KD) were present throughout pupal and pharate adult although not in the same relative amounts. A heterogeneous collection of polypeptides of low molecular weights (10 KD, 12 KD and 14 KD) present in larval states disappeared at apolysis and did not show upto eclosion. A 45 KD protein present in all the stages in significant amounts was glycosylated as shown by Periodic acid Schiff reagent (PAS) staining (Figure not shown).

3.2.3. **Adult Cuticle**

In the adult cuticle extracts, five proteins were identified, ranging in molecular weights from 31 KD to 97 KD. The cuticle protein pattern became much simpler due to the disappearance of many of the protein bands (Fig. 1a, Lane A).

The major limitation of one-dimensional SDS-PAGE is the inability of the method to resolve proteins with the same molecular weights but different charge properties. In this case high-resolution two-dimensional PAGE was therefore used to achieve a more complete separation information than one-dimensional PAGE.

3.3. Analysis of Cuticle Proteins by Two-Dimensional Gel Electrophoresis

Proteins were separated into their component species using high resolution two-dimensional electrophoresis of proteins in polyacrylamide gels according to O'Farrel (1977) with a few modification. Proteins were first separated in cylindrical gels according to their charge properties. These gels were then laid across the top of an SDS slab gel and the proteins were separated according to their molecular weights. In this procedure, individual proteins appeared as discrete spots as visualized by silver stain.

3.3.1. First Dimension.

Two methods could be used for the first-dimensional separations, that is either IEF or NEPHGE. The IEF and NEPHGE methods were very similar, the only difference being in the composition of the Ampholines. IEF gels contained a 4:1 mixture of pH 5-7 : pH 3.5-10 Ampholines, while NEPHGE gels contained only pH 3.5-10 ampholines. Conditions of electrophoresis differed considerably between IEF and NEPHGE. In IEF gels, a stable pH gradient was established by the Ampholines, and proteins were electrophoresed to equilibrium, that was, until the proteins reached a position in the gradient where they were uncharged. The proteins were loaded at the cathode and NEPHGE gels were run in the reverse direction, so that the cathode (basic) end of the gel was at the bottom. NEPHGE was a non equilibrium electrophoresis system, where proteins were

loaded at the anode (acidic) and electrophoresed just long enough to allow the most basic proteins to reach the bottom of the gel.

The high-resolution two-dimensional gel demonstrated that individual bands within a molecular weight range on the one dimensional gels are generally composed of several proteins as seen in Fig. 2. For two-dimensional gels, extracts were run on isoelectric focusing (IEF) gels in the first dimension with a 4:1 ratio of pH 5-7 and pH 3.5-10 Ampholines. Two-thirds of the spots were not seen when non-equilibrium pH gradient gels (NEPHGE) were separated in the first dimension. IEF gels resolved proteins with isoelectric points between pH 4.5 and 7. This clearly indicated that the protein extracts were acidic. The NEPHGE gels resolved acidic as well as basic proteins. The NEPHGE system used resolved proteins with pI's between 4.5 and 9. It appeared that most of the proteins in the extract had pI values below 7 hence they spread out best in IEF than in NEPHGE gels.

3.3.2. **Second Dimension**

The second dimension SDS slab gels were made of 8.5% polyacrylamide or 6-20% gradient gels with a 3% stacking gel (Laemmli 1970) and silver stained. Since equal amounts of proteins were loaded in each case, any difference in the intensity of a spot among the patterns of proteins synthesized through the developmental series was taken to reflect an altered relative rate of synthesis.

3.3.3. Developmental Changes in Protein Synthesis

Analysis of protein synthesis by two-dimensional polyacrylamide gel electrophoresis showed many changes. Most striking was the disappearance of some polypeptides and the appearance of a few new ones as larva developed into adult. Figures. 2 through 11 demonstrated that protein synthesis varied during the larval-adult transformation. From these experiments, four types of cuticle proteins were identified: larval-specific, pupal-specific, adult-specific and proteins common to all cuticles. Few cuticle proteins were synthesized between the 1st and 2nd larval instars. 1st and 2nd instars had same electrophoretic profile except for differences in three proteins. New proteins 4, 15, 16 appeared by day one 2nd instar and disappeared by 5th day pupal stage (Fig. 2, 3a). When second and early third instar larval stages were compared using gradient gels (6-20%) proteins 53 and 54 (M_r ~ 21 KD) appeared by second instar (Fig. 3b). By early third instar the proteins increased very much in amounts (Figs. 3b and 4b). Very low molecular weight proteins , 58 (M_r ~ 18), 56, 57 and 59 (M_r ~ 13 KD) could not be seen by third instar. A number of low molecular weight proteins (60, 61, 62, 64, 65, 67, 69, 70) having molecular weights between 14 KD and 20 KD appeared by early third instar (Fig. 4b).

By third instar (2 days before larviposition), a large number of proteins were induced (Fig. 4a). Acidic proteins (16, 17, 18, 19, 20, 21, 26, $M_r \leq$ 30 KD) appeared in large amounts, persisted upto the brown pupal stage and showed a rapid decline thereafter. A number of proteins (26, 27, 28, 29, 30, 42) with

Fig. 2. Two dimensional separation of proteins extracted from first instar larval cuticle of tsetse fly. Separation in the first dimension was by electrophoresis in the pH 3.5 (left) to 10 (right), and the second dimension by SDS electrophoresis in 8.5% polyacrylamide gels and silver stained. First dimension was the same as above in all gels shown.

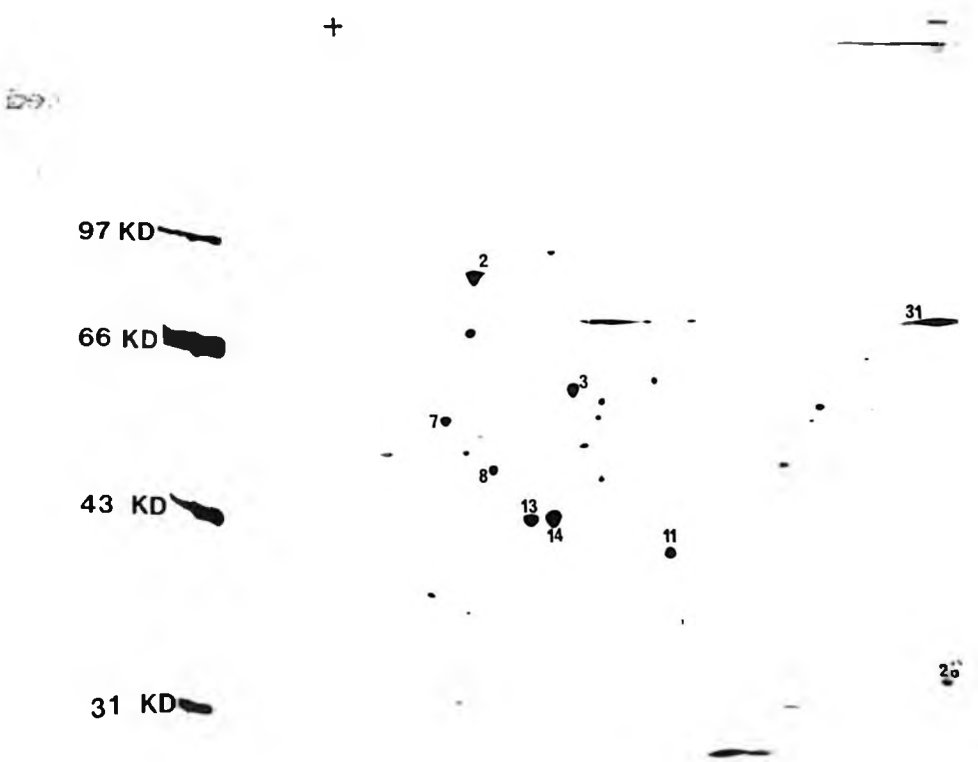
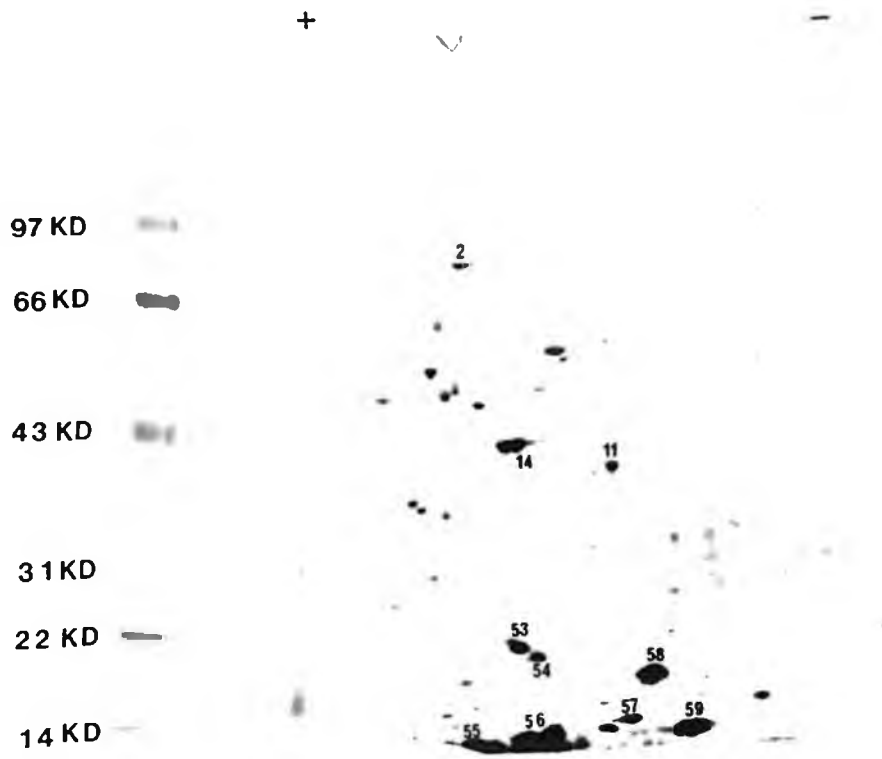

+
97 KD
66 KD
43 KD
31 KD
2
31
3
7
8
13
14
11
20

Fig. 3a. Two dimensional gel displaying cuticle proteins from second instar larvae. Second dimension was by SDS electrophoresis in 8.5% polyacrylamide gels. and silver stained. A, B, C and D represent low molecular weight markers similar to the ones in figure 2.

Fig. 3b. Two dimensional gel displaying cuticular proteins from second instar lavae. Second dimension was by SDS electrophoresis in 6-20% polyacrylamide gradient gels and silver stained.


+
−
97 KD
66 KD
43 KD
31 KD
22 KD
14 KD
2
14
11
53
54
58
55
56
57
59

molecular weights between M_r 45 KD and 66 KD appeared by early third instar. The proteins increased in amounts by third instar and the highest amounts was attained by brown pupal stage. The proteins then decreased in quantities during the development and were not visible by adult stage (Figs. 3a, 4a, 5, 6a). The late third instar (larviposited) larval cuticle proteins showed qualitative and quantitative changes when compared to early third instar. Proteins 22, 24, 31 and 34 appeared by late third instar and lasted only upto the brown pupal stage (Figs 4a, 5 and 6a). Protein 18 present in early third instar was not visible in late third instar. Decreased amounts of protein 17 was found in late third instar cuticle compared to early third instar in which large amounts was present. Protein 19 present in early third instar in very high amounts was only found in trace amounts in late third instar and was not detected by brown pupal stage. A protein 25 appeared by late third instar then declined in amounts and was not detected by adult stage. A majority of major cuticle proteins increased in amounts with maximum amounts being achieved by brown pupal stage (Fig. 6a). The proteins with molecular weight $M_r \leq 30$ KD were undetectable at apolysis (5 days after larviposition) (Fig. 7a). Proteins 5, 6 and 9 appeared by day one third instar larvae and persisted upto adult stage. A protein 34 appeared by day three third instar and lasted only upto the brown pupal stage. A protein 23 appeared by day three third instar and persisted upto the adult stage. Eight proteins (4, 16, 17, 18, 19, 20, 21, 34) were larval specific. Brown and 5 days old pupae were compared using gradient gels 6-20% in the second dimension. Very low molecular weight proteins (55, 62, 64, 65, 66, 67, 68, 70)

Fig. 4a. Two dimensional gel of cuticle proteins from third instar larvae (aborted 2 days before larviposition). Second dimension was by SDS electrophoresis in 8.5% polyacrylamide gels and silver stained.

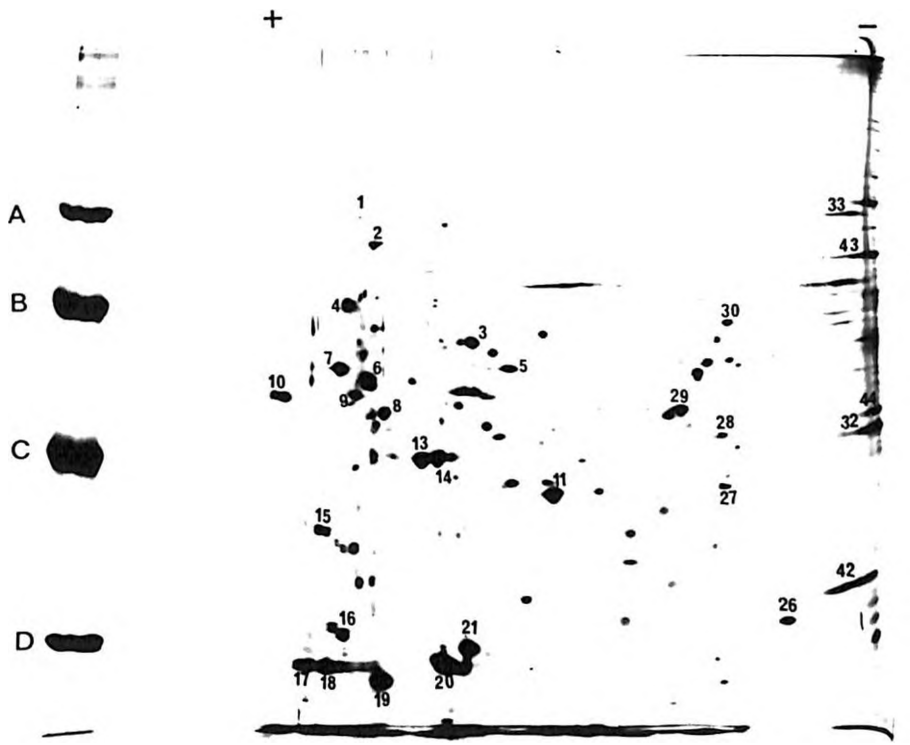

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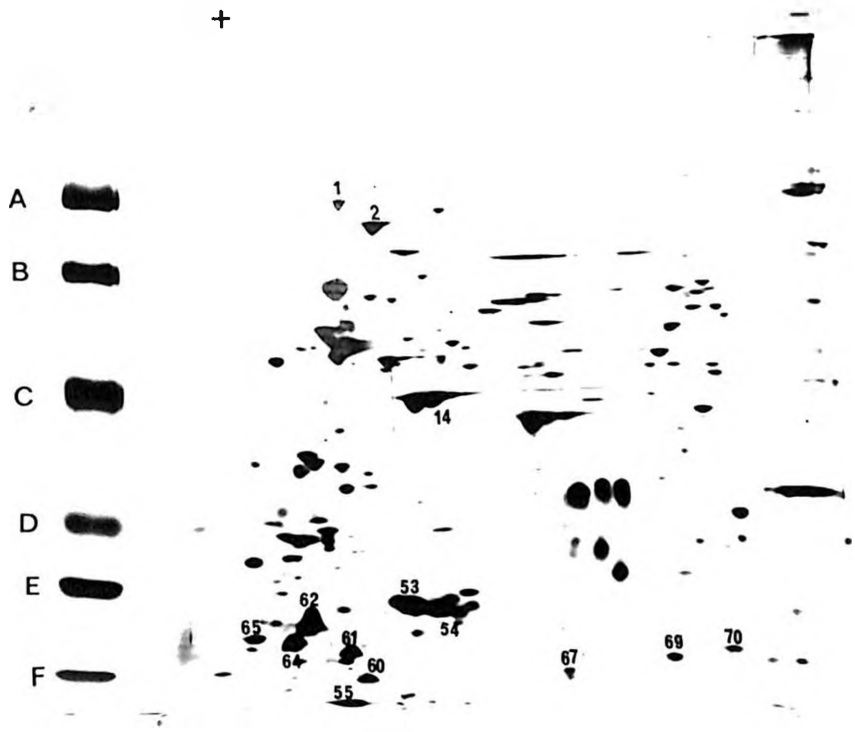
Fig. 4b. Two dimensional gel of cuticle proteins from third instar larvae (aborted 2 days before larviposition). Second dimension was by SDS electrophoresis in 6-20% polyacrylamide gradient gels A, B, C, D, E and F represent low molecular weight markers similar to the ones shown in figure 3b.


A
B
C
D
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1
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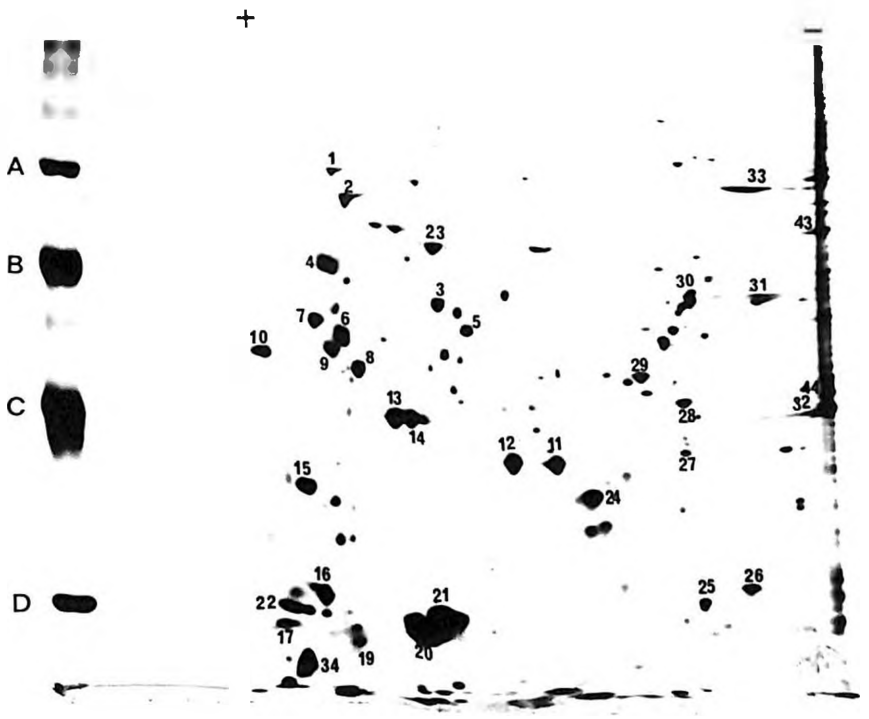
69
70

Fig. 5. Two dimensional gel of cuticle proteins from third instar larvae (larviposited). Second dimension was by SDS electrophoresis in 8.5% polyacrylamide gels.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44
32
13
14
28
12
11
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16
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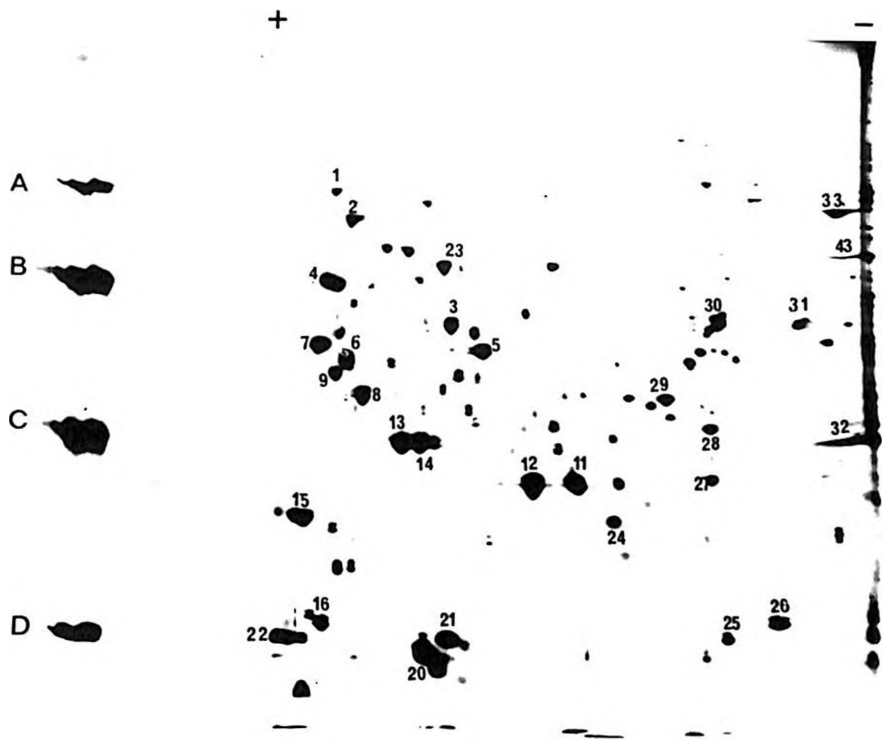
19
20
34

Fig. 6a. Two dimensional gel of cuticle proteins from brown pupae (2 hours after larviposition). Second dimension was in 8.5% polyacrylamide gels.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A
B
C
D
1
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23
4
3
30
31
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6
5
9
8
29
13
28
32
14
12
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15
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16
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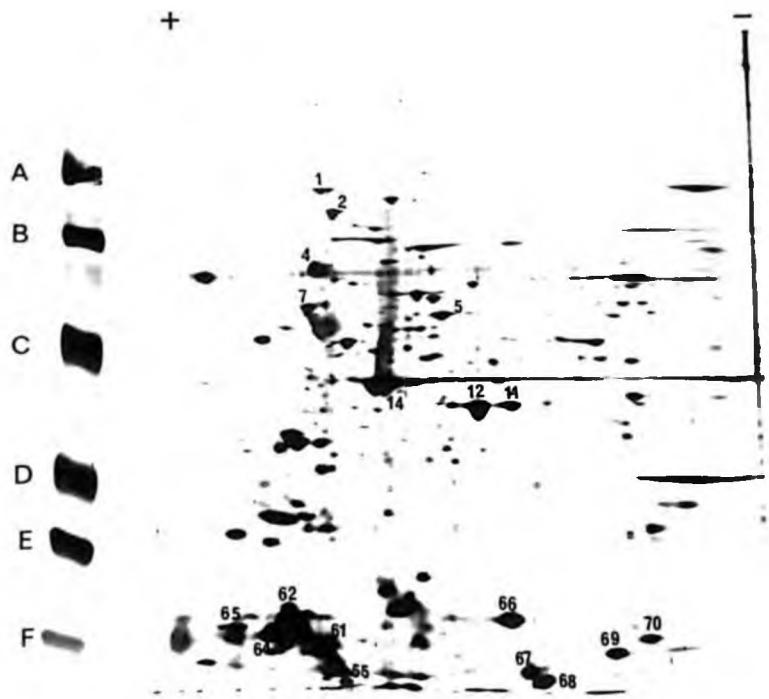
33
43

Fig. 6b. Two dimensional gel of cuticle proteins from brown pupae (2 hours after larviposition). Second dimension was by SDS electrophoresis in 6-20% polyacrylamide gradient gels.


+
−
A
B
C
D
E
F
1
2
4
7
5
14
12
11
62
65
64
61
55
66
69
70
67
68

with molecular weights between M_r 13 and 17, were present in brown pupae, but were missing in five-day old pupae (Figs. 6b and 7b).

Figures 7a, 8 and 9 showed proteins from the different pupal stages. Proteins 36, 37, 38, 40 with molecular weights between M_r 31 and 45 KD appeared by five-day old pupal stage and protein 40 lasted only upto ten-day old pupal stage. Protein 42 appeared by ten-day old pupal stage and was slightly visible by fifteen-day old pupal stage. The protein was not detected by adult stage. Protein 35 was unique to five-day old pupal stage. The electrophoretic profile of cuticle proteins during the pupal stage was relatively constant. By fifteen-day pupal stage, the number of cuticle proteins was very small. Five Proteins 37, 38, 39, 40, 41 were specific to pupal stages

Adult cuticles were removed from eclosed adults which had been kept at room temperature for 2 hours. Eclosed adult abdomens were examined (Fig. 10a). Major changes in the protein profile were observed. A series of proteins, relatively abundant in early pharate adult cuticle extracts, were found in diminished amounts in eclosed adult cuticle extracts. A cluster of proteins (52 M_r ~ 43 KD, and 47, 48, 49, 50, 51 M_r ~ 37 KD) relatively prominent appeared in the cuticle of newly emerged. Adult specific proteins were 47, 48, 49, 50 and 51.

3.3.4. **Ligated Adults**

Ligation of adults at eclosion resulted in notable changes of cuticle proteins. Proteins 10, 15 and 52 were undetectable in ligated adults. A cluster

Fig. 7a. Two dimensional gel of cuticle proteins from five-day old pupae. Second dimension was by SDS electrophoresis in 8.5% polyacrylamide gels.

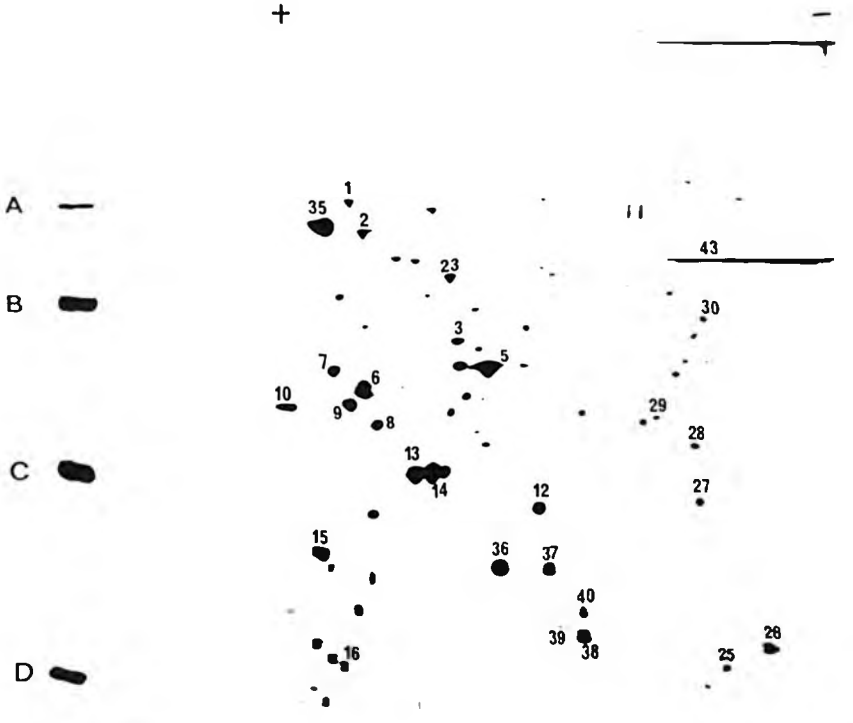

+
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Fig. 7b. Two dimensional gel displaying cuticle proteins from five-day old pupae. Second dimension was in 6-20% polyacrylamide gradient gels.

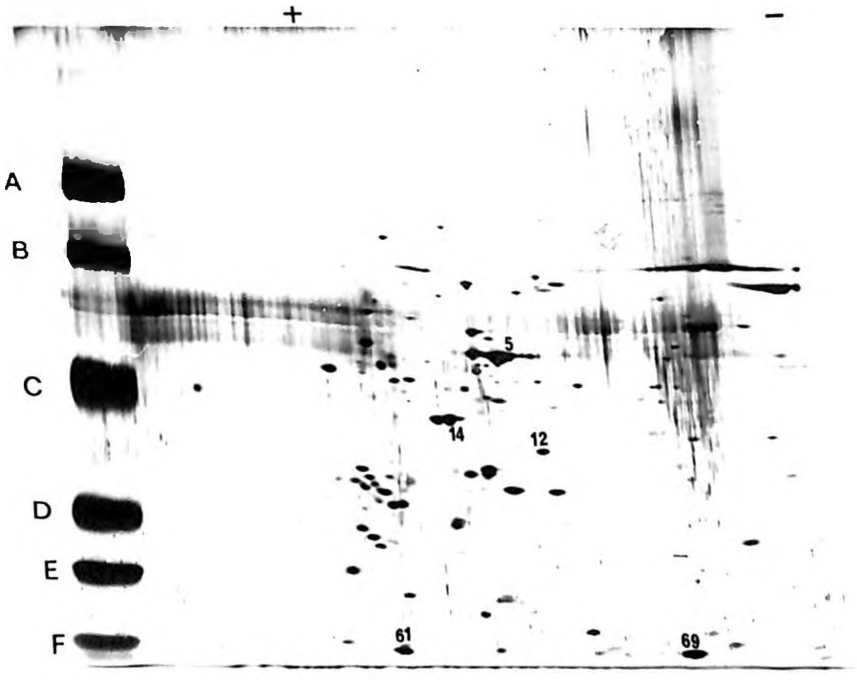
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Fig. 8. Two dimensional gel of cuticle proteins from ten-day old pupae. Second dimension was in 8.5% polyacrylamide gels.

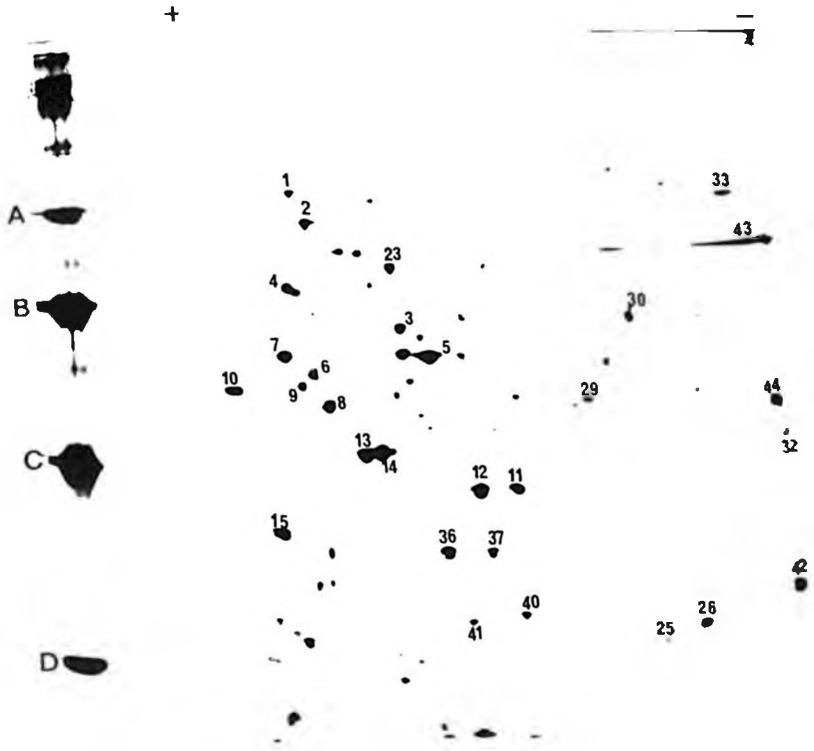
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Fig. 9. Two dimensional gel of cuticle proteins from fifteen-day old pupae. Second dimension was in 8.5% polyacrylamide gels.


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of acidic proteins (47, 48, 49, 50, 51 M_r ~ 37 KD) were also missing totally in ligated adults. A number of proteins present in ligated adults in diminished amounts were absent in adult control. Proteins 5, 9, 11 and 36, although very prominent in ligated adults, were not detected in control adult (Figs. 10a and 11a). Proteins 45 and 46 were present in both adult control and ligated adults. Cuticle proteins from ligated and normal adults were compared using 6-20% polyacrylamide gel electrophoresis gradient gels. Ligated adults showed numerous proteins (70, 71, 72, 74, 75, 76, 77, M_r ~ 17 KD) not present in adult control (Figs 10b and 11b).

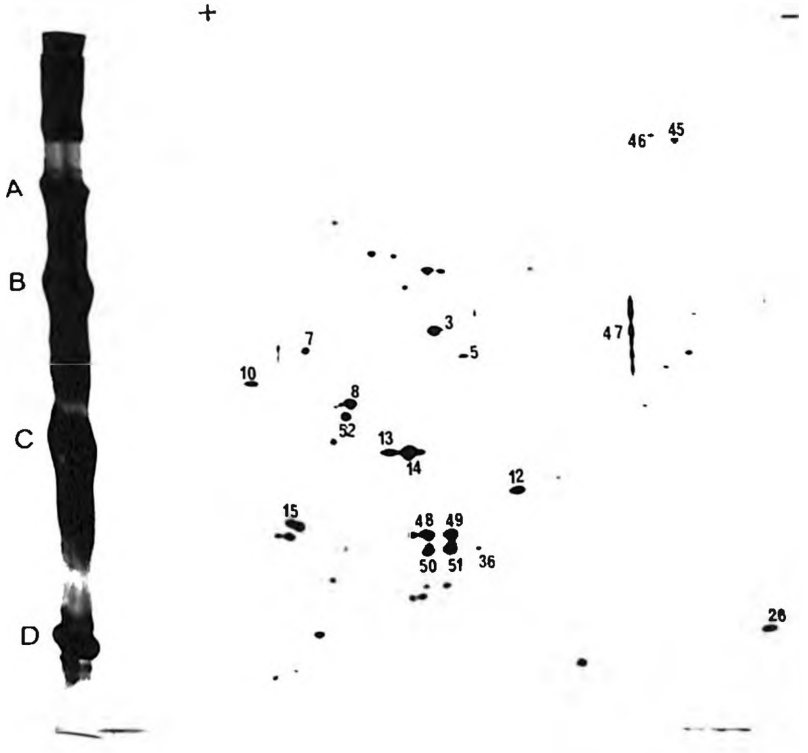
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Fig. 10b. Two dimensional gel of abdominal cuticle proteins from adult. Second dimension was by SDS electrophoresis in 6-20% polyacrylamide gradient gels.


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D
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51
69
61


+
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E
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10
8
52
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12
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49
50
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61
69

11a. Two dimensional gel of abdominal cuticle proteins from ligated adult. Second dimension was by SDS electrophoresis in 8.5% polyacrylamide gels


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36
26

11a. *Two dimensional gel of abdominal cuticle proteins from ligated adult. Second dimension was by SDS* electrophoresis in 8.5% polyacrylamide gels


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1a. Two dimensional gel of abdominal cuticle proteins from ligated adult. Second dimension was by SDS electrophoresis in 8.5% polyacrylamide gels


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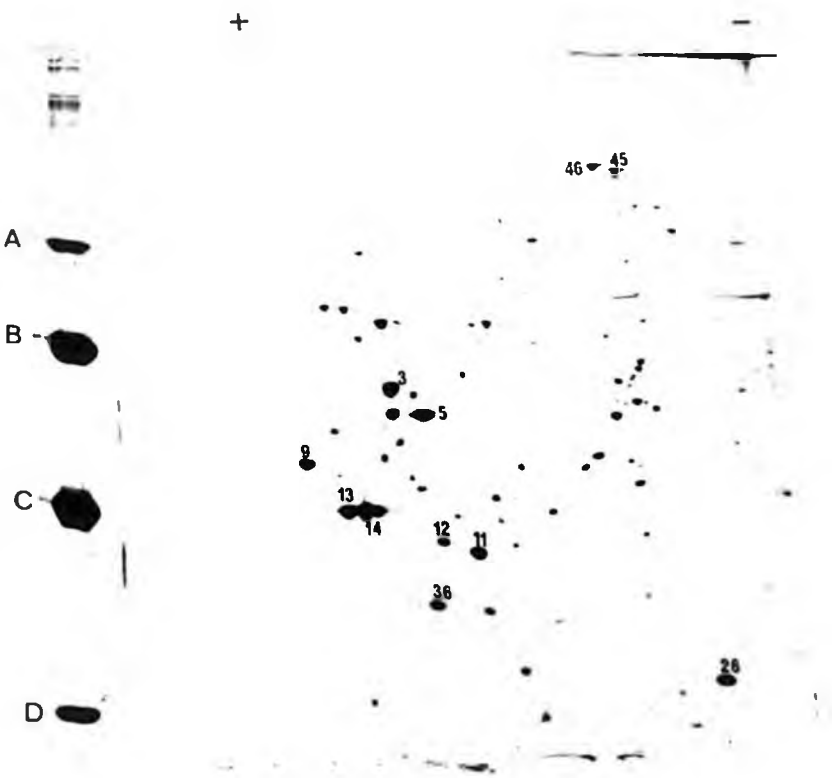
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Two dimensional gel of abdominal cuticle proteins from ligated adult. Second dimension was by SDS electrophoresis in 6-20% polyacrylamide gradient gels.


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CHAPTER 4

DISCUSSION

For the analysis of cuticle proteins, IEF is a sensitive and reproducible technique and less affected by minor fluctuations in gel components than SDS gel electrophoresis. Hence, this is an appropriate technique if one wants to compare a large number of cuticle protein samples from different regions, metamorphic stages or species (Cox and Willis, 1985). The combination of two electrophoretic separations in a two dimension electrophoretic system, offers significant information on the composition and properties of these proteins. It is absolutely necessary to control all possible variables, especially pH and urea concentration to obtain consistent results.

High quality water should always be used for optimal results since contaminating ions and organic compounds can interfere with IEF and gel polymerization as well as with staining reactions. For proper gel polymerization, tubes should be washed well with chromic acid and rinsed well using high quality water. Acrylamide solution should be adequately degassed, since oxygen interferes with polymerization. It is also important to degas solutions adequately to ensure uniform gel polymerization as well as to remove other nonamphoretic ions. Gel breakage during isoelectric focusing is a very common problem and is one of the most frustrating problems encountered with this technology. Several factors contribute to this problem: Samples contain

high salt concentrations such as lyophilized buffer salts. These will not only interfere with IEF but will also cause gel breakage. Gel breakage also occurs when protein concentration is too high for the gel. Since silver staining requires very little protein, this problem can frequently be overcome.

Occasionally, IEF gels are not readily removed from tubes, or may fall out of tubes either before or during IEF, a factor which is usually attributed to inadequately washed tubes. In addition, addition of too little N-P40 to the acrylamide solution, makes gels difficult to remove. On several occasions, the use of excessive detergent in the IEF gel resulted in gels falling out of tubes.

Two dimension gel analysis showed distinct qualitative and quantitative changes in the epidermal cell protein synthetic profile during larval development in the tsetse fly. Temporal change in the cellular protein synthetic profile has been reported in *Manduca sexta* during the larval-pupal transformation (Kiely and Riddiford, 1985). In addition, this temporal change in the cellular protein synthetic profile is concomitant with a loss of the translatable mRNAs for these proteins (Riddiford, 1982; Kiely *et al.*, 1985). The latter observation indicates control at the level of transcription and mRNA turnover and is in agreement with studies on *Drosophila* wing epithelial cell protein synthesis during the differentiation of hairs (Mitchell and Petersen, 1981).

The cuticle proteins observed in the *Glossina morsitans morsitans* differed from *Drosophila* (Chihara *et al.*, 1982) and *Tenebrio* (Roberts and Willis, 1980 a) in which none of the cuticle proteins observed had molecular weights greater than 30 KD. The pI range (4-6) of the cuticle proteins from *G. m.*

morsitans were of the same molecular weight range as the cuticle proteins observed in various other insects, including *Hyalophora cecropia* (Cox and Willis, 1985), *Locusta migratoria* adults (Andersen and Hojrup, 1987), *M. sexta* larvae (Wolfgang and Riddiford, 1986), and *Drosophila melanogaster* (Chihara *et al.*, 1982). In tsetse fly, proteins from first and second instar cuticles were identical except for minor differences. Each subsequent stage, third instar, pupa and adult had a unique set of cuticle proteins. Stage-specific proteins have been identified in *Drosophila* (Chihara *et al.*, 1982), *Lucilia cuprina* (Skelly and Howells, 1988). A number of other authors have also identified specific cuticle proteins (Sridhara, 1983; Kiely and Riddiford, 1985 a, b; Lemoine and Delachambre, 1986; Andersen and Hojrup, 1987).

Cuticle proteins extracted from tsetse fly larvae at different developmental stages (first, second and third instars) displayed quantitatively and qualitatively unique electrophoretic profiles. The proteins showed major differential deposition patterns in the third instar stage. Proteins 16, 17, 18, 19, 20 and 21 were deposited in relatively large amounts in the early third instar cuticle. Proteins from first and second instar cuticle were identical except for minor differences.

It is likely that the differences in the cuticle proteins are involved with differing functions of the various cuticles. For example, Willis *et al.*, 1981 found that guanidine HCl extracted proteins from flexible cuticle contain acidic polypeptides whereas more basic polypeptides were extracted from rigid cuticles. One major function of the insect cuticle is to protect the animal from

the environment. With the possible exception of L_1 and L_2, the environment of each stage is unique. Both L_1 and L_2, are spent feeding within the uterus. The first two days of L_3 are spent within the uterus and is then larviposited, and ultimately tans and sclerotizes in order to form the puparium. Thus, the third instar has an added function, providing the matrix for the formation of the puparium.

The term sclerotization describes the stabilization and stiffening of the arthropod cuticle. In puparia this involves the reorientation of the components of the cuticle, the addition of crosslinks between the components, a decrease in their solubility and the elimination of cuticular water (Lipke *et al.*, 1983). During sclerotization in *G. m. morsitans* the proteins of the third instar larval cuticle become stabilized by covalent linkage to chitin to form the stiff protective puparium. This cross linking of protein takes place within 2 hours of white puparium formation and was reflected in the lower amounts of proteins which was extracted with 1% N-P40 from older puparia. The absence of change in the pupal cuticle during pupation and subsequent adult development means that the pupal cuticle proteins are not reabsorbed upon apolysis and subsequent moulting events. This would be at variance with published descriptions of the moulting process (Locke, 1974; Jungreis, 1979) in which enzymes in the ecdysial fluid breakdown components of the old cuticle. If such a breakdown occurred at or around the time of pupal-adult moulting, its effects were undetected. The same major proteins were extracted from *G.m.morsitans* puparia at all time intervals except protein 35 that was only unique to five-day

old pupae. This indicated that the cross-linking of the proteins occurs in concert and that no single protein or group of proteins is preferentially cross-linked at any time.

A similar result in which the same major proteins are extracted from the larval cuticles and puparia was obtained with *Sarcophaga bullata* (Willis *et al.*, 1981, Lipke *et al.*, 1981) and *L. cuprina* (Skelly and Howells, 1988).

A feature of the pupal cuticle proteins of *L. Cuprina* (Skelly and Howells, 1988) and also of *D. melanogaster* (Chihara *et al.*, 1982) is that they change little during pharate adult development. This may be related to the fact that in dipterans, the pupal cuticle is enclosed within the puparium and remains unsclerotized. In other groups of insect in which the pupal cuticle becomes sclerotized, for example *Tenebrio molitor* and *Galleria mellonella*, the patterns of pupal cuticle proteins change markedly during development (Srivastava, 1970; Roberts and Willis, 1980).

At eclosion a number of previously prominent cuticle proteins could no longer be seen. The simple explanation for this observation is that these proteins are stabilized through sclerotization soon after emergence and cannot therefore, be extracted. The possession of a stiffened cuticle is of great importance for the new emerged insect. The inability to extract certain major pharate adult cuticle proteins soon after the final ecdysis, again presumably due to the cross-linking of those proteins, has also been recorded in the case of *Locusta migratoria*, *H. cecropia* and *D. melanogaster* (Anderson, 1973; Phillips

and Loughton, 1981; Chihara *et al.*, 1982; Cox and Willis, 1985; Roberts *et al.*, 1985).

Adults were ligated at eclosion to study the effect of bursicon on sclerotization. Proteins were extracted 2 hours after ligation. The results showed the presence of more proteins in the ligated fly than in the control which was left to develop normally. The terminal phases of the moulting process are regulated by two hormones, the eclosion hormone and bursicon. The cuticle of the newly ecdysed insects is usually soft so that it can be expanded to its proper size. Bursicon, which is released from the perivisceral organs in most insects, serves to regulate the postecdysial hardening and darkening of the new cuticle.

After ecdysis, the new cuticle of an insect is often soft and flexible so that it can be inflated into its proper shape. But before the animal can then begin its normal activities, the cuticle must become sclerotized, a process during which cross-linking between cuticle proteins occurs. The triggering of the sclerotization process is usually under hormonal control. Neck ligation of newly emerged tsetse flies served to prevent sclerotization of the thorax and abdomen. This is clearly indicated by more protein being present in the cuticle of ligated fly than in the normal fly. The tanning hormone (bursicon) has been found in the blood of tanning blow flies and in both the median area of the brain and the fused thoracic-abdominal ganglionic mass. It is released primarily from the latter ganglia (Riddiford and Truman, 1978).

From studies on cockroaches and *Pieris*, bursicon appears to increase the permeability of the epidermis to dopamine and possibly to tyrosine and/or dopa. The epidermal cells then convert the dopamine to N-acetyldopamine and tanning proceeds (Riddiford and Truman, 1978).

Conclusion

The study of cuticle proteins from tsetse fly has demonstrated that each cuticle contains its own set of proteins with the exception of cuticles from first and second instars which share a common set. It has also been shown in this study that cuticle proteins are developmentally modulated. A number of proteins (45 KD and 200 KD) are common to all cuticles of all stages of *G.m morsitans* indicating their essential nature in cuticle structure. Other proteins are found predominantly in a particular instar and it appears likely that these proteins are involved with specific differing functions of the various cuticles. The properties of the various highly specialized cuticles are due mainly to protein-protein interactions, which can be influenced by subtle changes in the surrounding medium. The main factors contributing to the mechanical properties can be assumed to be organization of chitin microfibrils, length of chitin microfibrils, chitin-protein interactions, protein conformations, and protein-protein interactions. The exploitation of cuticle proteins for selective management of insects which are vectors for various diseases might only be realized after determining the quantitative importance of the individual factors.

Several important aspects of cuticle proteins would form the subject of future investigations. Among these are the site of synthesis of cuticle proteins; determination of amino acid sequence of cuticle proteins and correlation of these results with biophysical properties; study of gene regulation by hormones in order to establish whether the proteins are the products of one or several gene families, and determination of whether any of these genes form a cluster. Another area of interest is to find out whether bursicon stimulates cAMP in the epidermal cells.

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