BIOCHEMICAL STUDIES ON THE ACCESSORY REPRODUCTIVE GLAND SECRETIONS OF THE MALE DESERT LOCUST, Schistocerca gregaria Forskäl

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This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as University supervisors.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABB	Amido black 10B
AcPase	Acid phosphatases
AlPase	Alkaline phosphatases
ARG(s)	Accessory reproductive gland(s)
ATP	Adenosine tri-phosphate
CBB-G	Coomassie billiant blue - G
Ch	Cholesterol
CE	Cholesterol ester
CTP	Cytidine tri-phosphate
DG	Diglyceride
fb	Paragonadal fat body
Gl(s)	Gland(s)
Gp-a-ARG mix	Guinea-pig anti-ARG serum mixture
Gp-a-Gl.1,2....12	Guinea-pig anti-serum to Gland 1,2.12
Gp-a-G.Cl	Guinea-pig anti-whole accessory gland complex (excluding seminal vesicle and fat body)
α-GPDH	α-Glycerophosphate dehydrogenase
He	Haemolymph
Ho	Homogenous glands
HVE	High voltage electrophoresis

IDH..... Iso-citrate dehydrogenase

MDH..... Malic dehydrogenase

ME..... Malic enzyme

MG..... Monoglyceride

N-Gp-S..... Normal guinea-pig serum

PAS..... Periodic acid-Schiff

SBB..... Sudan black B

♀ Sp..... Evacuated spermatophore

♂ Sp..... Unevacuated spermatophore

SV..... Seminal vesicle

TG..... Triglyceride

UTP..... Urdine tri-phosphate

XDH..... Xanthine dehydrogenase

LIST OF FIGURES

LIST OF TABLES

SUMMARY

The two initial objectives of this research project were (1) to obtain a detailed chemical description of secretions from the accessory reproductive glands (ARGs) of the male desert locust, Schistocerca gregaria Forskål, and (2) to identify the chemical nature of the spermatophore and seminal fluids in order to obtain a better understanding of the roles of individual ARG secretions in their formation.

The secretions of the 16 pairs of ARGs were subjected to disc gel electrophoresis and the gels were stained for proteins, glycoproteins and lipoproteins. Although a number of common protein bands were found in the secretions of some glands, the electrophoresed secretions of each gland showed distinct multiple protein and in some cases, multiple glycoprotein patterns. The number of protein fractions ranged from 10 in the seminal vesicle secretions to 26 in those from the homogenous glands. On the basis of histochemical and ultrastructural studies, Odhiambo (1969a, 1969b) indicated that the secretions of glands 3 and 5 were similar and were the only secretions containing lipoproteins. In this study, however, the electrophoresed secretions of these two glands showed distinct protein and glycoprotein patterns and

lipoproteins could not be detected with Sudan black B staining. Odhiambo (1969a) also reported that secretions of gland 2 and the homogenous glands were rich in mucopolysaccharides, but after electrophoresis the secretions from these glands produced single, narrow and slightly stained glycoprotein bands.

Immunochemical studies on the secretions of the various ARGs confirmed some of the conclusions drawn from the results of electrophoretic investigations. The secretions of glands 1,11 and 12 and the haemolymph contained one common antigen. Another common antigen was found in the secretions of glands 3,4,5 and 6. On the basis of similar electrophoretic mobilities, there appear to be more common proteins in the various gland secretions than were demonstrated by the immunochemical experiments. Some of these proteins may have been present in concentrations which were too low for antibody production or they may not have been antigenic in the guinea-pig immune system.

Eighteen free amino acids were detected in methanolic extracts of the various ARGs, the paragonadal fat body and the haemolymph by two dimensional ascending paper chromatography. Of these, α-alanine, methionine, phenylalanine, serine and aspartic acid were of quantitative importance. In descending order the highest concentrations of α-alanine were present in the secretions

of glands 3,5,6,1 and 2.

Trehalose and/or inositol, an amino sugar and an unidentified compound were demonstrated by descending paper chromatography in the secretions of all ARGs. Glucose was demonstrated in the secretions of gland 12, while fructose was present in the secretions of all other ARGs. The secretions of glands 3,4,11 and the seminal vesicle also contained sucrose. Spot tests for purines and pyrimidines and the Sakaguchi test on concentrated methanolic extracts of the unidentified compound indicated that it is probably a guanine derivative.

The neutral and phospholipid composition of the various ARG secretions, as revealed by thin-layer chromatography of chloroform-methanol (2:1 v/v) extracts were generally similar, but the secretions of gland 4 were distinguished by the absence of all neutral lipids. The phospholipid compounds of ethanolamine, serine, inositol and choline were also demonstrated in chromatographed secretions of the various ARGs. The phospholipid composition of secretions from glands 5,6,12 and the homogenous glands appeared to be identical.

Secretions of the various ARGs were tested for the presence of 8 enzymes. The levels of alkaline phosphatases were high in

secretions from glands 1-6 as compared to levels in secretions from glands 11,12, the homogenous glands and the seminal vesicle. Low acid phosphatase activity was detected in the electrophoresed secretions of glands 1 and 3-6. The secretions of all ARGs could be differentiated on the basis of electrophoretic patterns for alkaline phosphatases, acid phosphatases, malic dehydrogenase, α-glycerophosphate dehydrogenase and non-specific esterases. Malic enzyme, xanthine dehydrogenase and iso-citrate dehydrogenase activities were not detected in any of the ARG secretions.

Protein and glycoprotein patterns were determined for the electrophoresed washings of unevacuated spermatophores and supernatents of homogenized evacuated spermatophores. The distinct electrophoretic pattern of unevacuated spermatophores contained 20 protein bands, 2 of which were glycoproteins. Two precipitin arcs were obtained when guinea-pig anti-serum to homogenous glands was cross-reacted with the washings of unevacuated spermatophores, but no reactions were obtained when guinea-pig anti-sera to various ARG secretions were reacted against the supernatents of evacuated spermatophores. The electrophoretic pattern of evacuated spermatophores showed 14 protein bands, 4 of which were glycoproteins. Protein patterns of evacuated spermatophores appear to represent the polymerization products of ARG secretions which are used in spermatophore construction, while protein patterns from

evacuated spermatophore washings may come primarily from accessory seminal fluids which also appear to be polymerization products

The third objective of this research project was to determine whether the injection of antibodies raised against ARG secretions could be used to disrupt the normal growth and/or function of the ARG complex in sexually maturing adults of the male desert locust. When the injection of anti-serum against ARG secretions began as early as the third day of adult development a marked inhibition of ARG growth was accompanied by suppression of secondary sexual characteristics (yellow body colouration) and normal mating behaviour. Inhibition of ARG growth, while significant, was less pronounced when anti-serum injections began on the seventh day of adult development, but on day 19 the injected males did not exhibit normal mating behaviour when placed with virgin females. When single anti-serum injections were administered on day 10 of adult development the males had obtained their yellow body colouration by day 19 and copulated normally with virgin females, although their ARGs were only equivalent in size to those of 13 day old males. Since certain antigens were common to both the haemolymph and accessory gland secretions it is possible that ARG growth is slowed when these antigens are precipitated in the haemolymph prior to their transport to the ARGs. Another mechanism of inhibition might involve

the penetration of antibodies into the ARGs where they might precipitate gland antigens. These data indicate the great potential of immunological approaches to studies on arthropod reproductive processes.

CHAPTER I

INTRODUCTION

1.1 The Research Project

The biochemistry of the male accessory reproductive glands (ARGs) of terrestrial arthropods, which provide the materials necessary for the successful insemination of females, has not been adequately explored. Odhiambo (1969a, 1969b) used histochemical and ultrastructural methods to study the ARG secretions of male Schistocerca gregaria Forskäl. Based on these tests he demonstrated that the 16 glands in each bilobed gland mass could be classified into 9 different types. However, histochemical and ultrastructural studies only provide grossly qualitative information on the substances present in secretions. Secretions of the ARGs in male S. gregaria were found to contain both neutral and acidic mucopolysaccharides, and 2 of the glands were demonstrated to contain acidic lipoprotein complexes (Odhiambo, 1969a). Nothing else is known about the chemical nature of these ARG secretions.

The primary aim of this study was to characterise in detail the biochemical nature of the secretions of the various types of ARG. The techniques which were employed included disc gel electro-

phoresis and both paper and thin layer chromatography. These techniques were used in order to bring out any similarities and/or differences in the nature of the secretions of various ARGs, and hence to confirm or refute the classification of ARGs in S. gregaria established by Odhiambo (1969a).

Tatchell (1962) used histochemical tests to demonstrate the presence of non-specific esterases in one of the lobes of the ARGs of the male tick Argas persicus. Besides Tatchell's work, enzymic studies on ARG secretions in arthropods have not been performed. In this study the presence of enzymes was also investigated as another parameter for differentiating between gland secretions. Such data was also expected to give an indication, from the enzymes tested, as to which glands might be involved in spermatophore production. To further differentiate between the glands, immunochemical studies were carried out in order to determine which glands have common antigens. Immunochemical studies in combination with electropheretic and chromatographic techniques would not only give qualitative but also semi-quantitative information on the chemical constituents of the ARG secretions.

One known function of the ARG is to provide secretions for spermatophore formation. However, in cases where the ARG are heterogenous unpaired structures as in Dictyoptera (Roth, 1967),

or are multipaired structures as in Orthoptera (Uvarov, 1966), information is lacking as to which of the ARGs are doing what. There is extensive data on several functions of ARGs in controlling the physiology of mated females (Hinton, 1974; Leopold, 1976). One such function demonstrated in two Orthoptera species, is the enhancement of oviposition (Pickford et al,1969; Leahy, 1973a, 1973b). By implanting selective male ARG into virgin females of Melanoplus sanguinipes, Pickford and co-workers (1969) found that the secretions of the short hyaline ARG had the most pronounced effect, whereas the "white glands" also stimulated egg deposition but to a much lesser extent.

Male S. gregaria produce relatively many simple looking spermatophores (Pickford and Padgham, 1973) in comparision to the elaborate and complex spermatophore produced by Locusta migratoria (Gregory, 1965), even though the ARGs in the two insects have the same morphological characteristics. Considering this fact and the many documented functions of male ARGs it seems reasonable to assume that individual ARGs in male S. gregaria have different functions, and possibly more than one such function per gland. Whatever functions the individual glands may be performing, it is certain that the active factor(s) effecting the physiology of the female is (are) transferred in the spermatophore along with sperm.

Several experimental approaches could be used to eliminate some of the above mentioned gaps in our knowledge on the function of the various ARGs in males S. gregaria. One such approach would be to selectively ablate various ARGs and to observe if the males can still produce normal appearing spermatophores. Such results would provide direct evidence as to which of the ARGs are involved in spermatophore formation, and this approach has been successfully used by Hartmann (1970) for Gomphocerus rufus. The glands which do not seem to be involved in spermatophore production could then be tested for their possible involvement in other known functions of the ARG. A second approach would be to investigate the chemical nature of the spermatophore in different stages of spermatophore transfer. These data could then be compared with those obtained from the chemical analysis of various ARG secretions to determine which of the glands might be involved in the process of spermatophore formation. Relying on histochemical methods, this indirect approach has been used by various workers (Tatchell, 1962; Gregory, 1965; Gerber et al, 1971b; Legg, 1973) on a wide variety of arthropods. A biochemical analysis could also be coupled with immunochemical studies like those performed by Frenk and Happ (1976) who traced the origin of chemical components of the spermatophore to the bean-shaped ARG of Tenebrio molitor.

The first approach to the problem of ARG function in S.

gregaria (Dhadialla, 1974) proved to be difficult. That study showed that selective ablation was possible, but it could not be used without damaging the remaining glands and disrupting their positions within the paird ARG complex. Hence the second approach has been used in this study in combination with the immunochemical approach. Additionally, specific antibodies raised to the ARGs of S. gregaria have been employed to inhibit the growth and disrupt the function(s) of the ARGs. To date, this is the first time such experiments have been performed on an insect.

1.2 General Functions of Accessory Reproductive Glands

The various known functions of ARG secretions have been reviewed by Hinton (1974) and Leopold (1976) and the following effects, other than those involved in spermatophore formation, have been attributed with certainty to the secretions of male ARGs in some arthropods : (1) stimulation of oviposition (Pickford et al, 1969; Riemann and Thorson, 1969; Chen and Bühler, 1970; Hiss and Fuchs, 1972; Leahy, 1962, 1967, 1973a, 1973b; Leahy and Craig, 1965; Leahy and Galun, 1972, Baumann, 1974b); (2) acceleration of oocyte maturation (Leahy and Galun, 1972; Aeschlimann and Grandjean, 1973; Khalil and Sharbaky 1975); (3) prevention of subsequent copulation through control of female receptivity (Craig, 1967; Riemann et al, 1967; Fuchs et al, 1968, 1969;

Adams and Nelson, 1966; Nelson et al, 1969; Gwadz, 1972; Baumann, 1974b); (4) stimulation of genital duct contractions which are necessary for translocation of sperm (Davey, 1958, 1960, 1965; (5) activation of sperm (Shepherd, 1974a, 1974b, 1975) and sperm capacitation (Wagner-Jevseenko, 1958; Shepherd, 1974c); (6) involvement in the fertilization process (Adlakha and Pillai,1975); and (7) stimulation of feeding by females (Pappas and Oliver, 1972; Aeschlimann and Grandjean, 1973; Dhadialla, 1975). The ARG secretions which effect the physiology of the female may be transferred with the sperm either directly to the female, as in Drosophila melanogaster (Lefevre and Jonsson, 1962), or they may be transferred within the spermatophores (Khalifa, 1949; Davey, 1960, 1965; Wigglesworth, 1965; Chapmann 1969; Engelmann, 1970; Pickford and Padgham, 1973).

It is generally assumed for all arthropods that the spermatophore is derived from the paraseminal secretions of the ARGs and this has been confirmed in some species. Hartmann (1970), Loher and Edson (1973), Leahy (1973b) and Chaudhury and Dhadialla (1976) by removal of ARGs of males have shown that the secretions of those glands contribute to the formation of spermatophores. Other workers have used histo- and immunochemical methods to trace the origin of spermatophore components to the accessory glands (Tatchell, 1962; Gregory, 1965; Gerber et al, 1971b;

Legg, 1973; Gadzama and Happ, 1974; Frenk and Happ, 1976). In spite of these published works, our knowledge on the biochemical composition of the male ARG secretions and their involvement in spermatophore formation remains fragmentary.

1.3 Morphology of the Male Accessory Reproductive Glands

Among the Insecta, the morphology of the male ARGs is as diverse as the morphology of the insects themselves. The glands may be totally lacking, as in most of the Apterygota and Paleoptera (Imms, 1960), or they may occur as heterogenous unpaired structures, as in the Dictyoptera (Roth, 1967) and in Arachnida such as the ticks (Balashov, 1972). The ARGs are multipaired structures in Thysonoptera (Sharga, 1933) and Orthoptera (Uvarov, 1966), but it is common for them to occur as single paired structures as in the Glossinidae (Buxton, 1955). In a number of muscoid flies distinct accessory glands are lacking, but the anterior one-third to one-half of the ejaculatory duct is usually enlarged in these insects. In Musca domestica this portion of the ejaculatory duct was found to be glandular (Leopold, 1970; Riemann, 1973). In many Lepidoptera a pair of unusually elongate accessory glands arise from the seminal vesicles or "duplex", while a rather complex glandular region (the unpaired gland system or "primary simplex") lines the anterior portion of the genital

canal (Callahan and Cascio, 1962).

Odhiambo (1969a) and Cantacuzene (1967) have detailed the morphology of the various accessory glands of S. gregaria. In brief, the internal reproductive organs of the male consist of the paired testes and seminal ducts which open into the ejaculatory duct. In turn, numerous accessory glands open into the ejaculatory duct which terminates in the phallic complex. These accessory glands are in the form of blind-ended tubules (Figures 1 and 2) which form two masses, each about 6mm long and 2.5mm wide, lying in the abdomen on either side of the hind gut. Each accessory gland mass consists of a long centrally located gland and 15 peripheral interwined glands. They are held together by fine tracheae and a sheet of fat body, the so-called paragonadal fat body (Odhiambo, 1967). Odhiambo (1969a) has grouped the ARGs, from the appearance of their contents, into four types :

1.3.1 The "Opalescent" Gland

Only one such gland is present in each gland mass. It is long and convoluted and is located on the dorso-lateral side of each gland mass. It is referred to as gland number 1.

FIGURE 1

Diagrammatic representation of the accessory reproductive gland complexes of the sexually mature adult male desert locust showing the disposition of the first 6 glands as seen from a dorsal dissection. The glands are embedded in the paragonadal fat body (fb). ae-aedeagal apparatus; Gl-gland; tr-trachea; Vd-vas deferens.

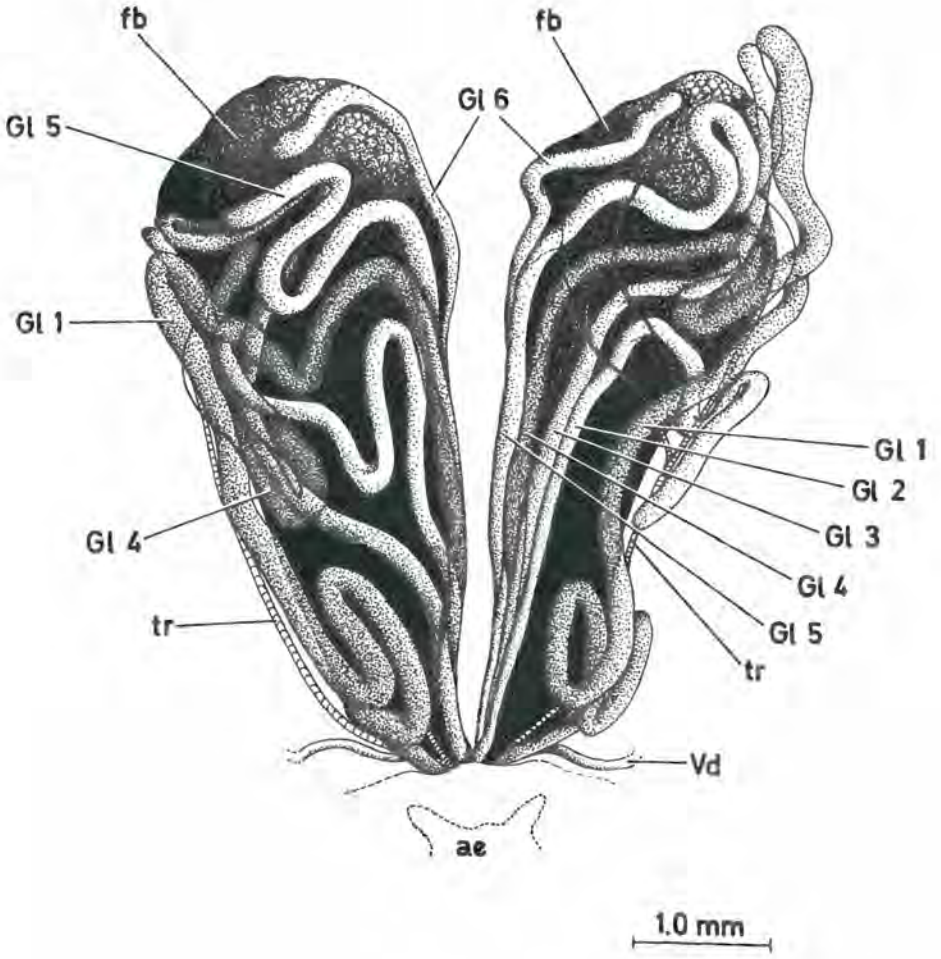


FIGURE 1

1.3.2 The "White" Glands

The "white" glands lie on the dorsal side of each gland mass and there are 4 such glands in each gland mass which are numbered 2,3,5 and 6. The colour of the gland contents varies from a pale white to an opaque or milky white. Of these, gland 6 is a nearly straight gland, containing a milky white secretion, and is located on the median edge of the gland mass. Gland 2 contains an opaque sometimes glistening, white secretion, and glands 3 and 5 contain a pale, whitish secretion.

1.3.3 The "Hyaline" Glands

These 10 transparent or "hyaline" glands are located ventrally in each gland mass, except gland 4 which is present dorsally. Gland 4, like glands 11 and 12, is long and convoluted. The other glands in this group vary in length, but are comparatively short. Glands of this latter group (assigned to numbers 7,8,9,10,13,14 and 15) have been named the "homogeneous glands" (Odhiambo, 1969a).

FIGURE 2

Diagrammatic represention of a dissection of the accessory reproductive gland complexes of the adult male desert locust showing the disposition of individual glands in a ventral view. The hyaline glands (7-15) are more deeply embedded within the fat body (fb) than the first six glands (Figure 1). Ed-Ejaculatory duct; Gl-gland ; tr-trachea; Vd-vas deferens.

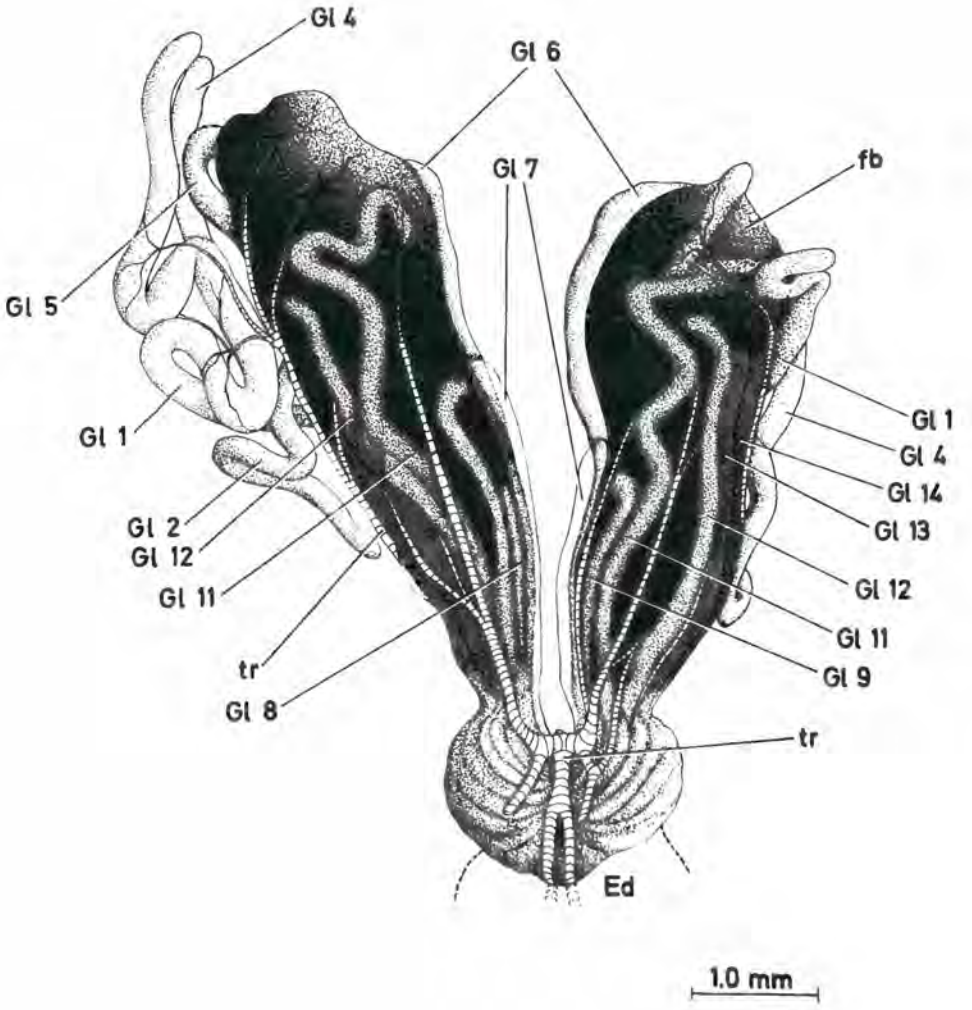

Gl 4
Gl 6
Gl 7
fb
Gl 5
Gl 1
Gl 2
Gl 12
Gl 11
tr
Gl 8
Gl 1
Gl 4
Gl 14
Gl 13
Gl 12
Gl 11
Gl 9
tr
Ed
1.0 mm

FIGURE 2

1.3.4 Seminal Vesicle

Gland 16, or the functional seminal vesicle, has an iridescent appearance (due to the quantity of contained sperm) and is highly convoluted and enveloped by the fat body. One seminal vesicle is present in each gland mass where it functions in the storage of sperm produced in each testis.

1.4 Histo- and Biochemical Studies on the constituents of Accessory Glands

The chemical nature of the ARG secretions in arthropods have been mainly investigated by histochemical means, although in a few notable exceptions biochemical techniques have also been used. On the basis of histochemical and ultrastructural studies on ARG secretions, Odhiambo (1969a, 1969b) classified the 16 pairs of accessory glands of male S. gregaria into 9 distinct types. The secretions of all ARGs, with the exception of those from glands 3,5 and 16 (the functional seminal vesicle), were found to be mucopolysaccharides. The secretions from glands 1 and 2 were acid mucopolysaccharides, but the secretions of all the hyaline glands were neutral mucopolysaccharides. In the same study, Odhiambo found that the secretion of gland 6 was a globular mucoprotein.

Acid and neutral mucopolysaccharides have also been found in the ARG secretions of Periplanetta americana(Vijayalekshmi and Adiyodi, 1973). Proteins and carbohydrates also occur in the ARG secretions of Rhodnius prolixus (Davey, 1958), some Coleoptera (Anderson, 1950; Gerber et al., 1971b; de Loof and Lagasse, 1972) and some Diptera (Leopold, 1970). Among the Arachnida, protein and carbohydrates have been found in the ARGs of the soft tick, A. persicus (Tatchell, 1962) and the pseudoscorpion, Chthonius ischnocheles (Legg, 1973). Gerber et al., (1971a, 1971b) have identified at least 8 secretory components from the 3 pairs of ARGs of Lytta nuttalli. They observed that the first pair of glands secrete at least 6 different components, 5 of which were proteinaceous. Polysaccharides were also detected and one component was shown to be a glycoprotein complex.

Leopold (1970) investigated M. domestica with cytochemical methods and reported that the secretion within the ejaculatory duct was a homogenous proteinaceous material probably composed of highly basic polypeptides. Electrophoresis of homogenates taken from the ejacualtory ducts of male M. domestica revealed 20 stainable protein fractions (ranging from 650-4150 in molecular weight). Eight of these bands were, however, presumed to result from contamination with haemolyph (Terranova et al., 1972). Similarly, preliminary electrophoresis of the proteins from the mushroom glands of

P. americana on polyacrylamide gel has shown 5-7 fractions (Adiyodi and Adiyodi, cited by Vijayalekshmi and Adiyodi, 1973). Electrophoresis of homogenates from the paired ARGs of the tsetse fly, Glossina morsitans, revealed 12 stainable protein fractions, 3 of which appeared to be glycoprotein complexes (Dhadialla, 1973). Using different polyacrylamide gel systems, von Wyl (1976) separated the paragonial gland homogenates of D. melanogaster adult males into 12 protein fractions. Only one of these 12 bands was absent in the protein pattern obtained from the actual paragonial secretions. The molecular weight range of the paragonial proteins was between 12,000 and 122,000 daltons (von Wyl, 1976). He also identified 4 to 5 glycoprotein bands the most intensely staining of which had a molecular weight of about 61,000 daltons.

The polysaccharide chitin has not been detected in any of these studies on ARGs or their secretions (cited above). There is evidence, however, that it may be produced by the ARG of Heliothis zea, since Callahan (1958) demonstrated its presence in the outer shell of the spermatophore of that insect. Glycogen, like chitin, does not appear to be a common constituent of male ARG secretions. Thus far Landa and Riemann have found glycogen in the secretions of Melolontha and Anagasta (Leopold, 1976). Vijayalekshmi and Adiyodi (1973) demonstrated that inositol was the most prominent carbohydrate reserve in the ARG complex of P. americana. They also

found traces of glucose in the mushroom glands of P. americana. Baumann (1974a) indicated that the reducing sugars, glucose and xylose were important components of the active factor from the paragonial secretions of Drosophila which stimulate egg maturation and oviposition.

There are few studies on the amino acid composition of the proteins and smaller peptide components of the accessory secretions. Tyrosine was found to be a major amino acid in the secretions of P. americana (Vijayalekshmi and Adiyodi, 1973) and L. nuttalli (Gerber et al, 1971b). Whereas, arginine, asparagine and lysine were found to be the most prominent amino acids in the polypeptides forming the bulk of the accessory secretions of M. domestica (Leopold, 1970; Leopold et al, 1971; Terranova et al, 1972). Trace amounts of phenylalanine, methionine, glycine, cysteine, tyrosine, valine, threonine, leucine/isoleucine, histidine, serine, proline, tryptophan, aspartic acid, alanine, glutamic acid and glutamine were also found in homogenates of the ejaculatory ducts of Musca (Terranova et al, 1972). Frenk and Happ (1976) used an amino acid analyser to detect the presence of sixteen amino acids in the hydrolysates of the bean-shaped accessory glands of T. molitor. Quantitatively, the most important amino acids were glutamic acid, aspartic acid, alanine, glycine, proline and serine. With histochemical techniques Legg (1973) demonstrated proteins containing

-SH groups, tyrosine, arginine and traces of cystine in the secretions of the anterior glands and outer lumena of the posterior dorsal gland of the pseudoscorpion, C. ischnocheles.

Extensive studies on the paragonial gland of Drosophila have revealed that the secretions contain large numbers of ninhydrin-positive components including proteins, peptides, amino acids and related compounds (Chen and Diem, 1961; Chen and Bühler, 1970; Baumann, 1974a). Baumann (1974a) has determined the amino acid composition of the accessory secretion of D. funebris. He found that the active factor which reduces mating receptivity (PS-1 fraction) was composed of 27 amino acid residues, of which alanine, aspartic acid, glutamic acid and serine were the most abundant. Moreover, the substance which enhances oviposition (PS-2 fraction) was found to be glycine derivative. Recently, Baumann and co-workers (1975) have determined the complete amino acid sequence of the PS-1 fraction from the paragonial gland of adult male D. funebris. Further work on the paragonial gland of D. nigromelanica (Chen and Oechslin, 1976) has revealed the presence of large quantities of glutamic acid in the secretions. These authors suggested that glutamic acid may be present in both the free form and bound to peptides and related derivatives, since acid hydrolysis of the paragonial gland extract resulted in a 40% increase in glutamic acid. Chen and Baker (1976) found the

activity of L-alanine amino-transferase to be 2-3 times higher in D. nigromelanica than in D. melanogaster which does not accumulate glutamic acid.

Studies on the ARG secretions have also revealed the presence of lipids, primarily in the form of neutral and phospholipids. Vijayalekshmi and Adiyodi (1973) used histochemical and chromatographic methods to demonstrate the presence of neutral and phospholipids in the ARG secretions of P. americana. Among these phospholipids, phosphatidylcholine predominated over phosphatidylethanolamine. The presence of sterols and/or their esters was also demonstrated.

Three of the secretory components from the first pair of male ARGs in L. nuttalli contained phospholipids (Gerber et al, 1971b), while neutral lipids were present in two other substances from the same glands. These authors also demonstrated the presence of phospholipo-protein granules in a carbohydrate-phospholipid complex matrix from the second pair of accessory glands of L. nuttalli. Similarly, Tatchell (1962) demonstrated the presence of a phospholipoprotein, rich in phenolic groups, and a polyphenol oxidase in the median granular lobe of the ARG of male A. persicus. Secretions from two of the ARGs of male S. gregaria were charecterized as acidic lipoproteins (Odhiambo 1969a). Cyclical increases

in both lipid and protein content of the posterior dorsal gland secretions have been observed to correspond to breeding activity of C. ischnocheles (Legg, 1973).

Roth and Dateo (1964) found that uric acid was secreted by the accessory glands of cockroaches and that it covered the outside of spermatophores of 7 of the 39 species examined. They suggested that mating may be one of the means by which the male eliminates this end product of protein metabolism in cockroaches. The ARGs of other insects may play similar roles. One other role of guanine-based compounds may be as intermediates in hormone action. Guanylate cyclase activity has been demonstrated in the accessory glands of the male cricket, Acheta domesticus, by Fallon and Wyatt (cited by Filburn and Wyatt, 1976).

Tatchell (1962) proposed that the white material secreted by the third lateral granular lobe of the ARG of A. persicus was analogous to the secretions of the opaque accessory glands of Rhodnius (Davey, 1958, 1960). In Rhodnius this secretion promoted the rhythmic contractions of the female tract which transported the semen to the spermatheca and was tentatively identified as an indolamine. A similar physiological effect was obtained with homogenates of third lateral granular lobes of A. persicus ARG, which increased the beat of an isolated cockroach heart pre-

paration from 75-83 contractions per minute (Tatchell, 1962). Galun and Warburg (1967) found that the ARG of male Ornithodoros tholozani contained several pharmacologically active compounds (DOPA, dopamine, epinephrine and norepinephrine). These catecholamines were also assumed to be responsible for sperm relocation by stimulation of peristalsis in the ovaries and oviducts of mated females.

Biochemical studies on these factors from the male ARGs which influence sperm transfer, fecundity and/or mating behaviour indicate that they are relatively low molecular-weight substances. Shepherd (1974a) conducted studies on sperm activation in saturniid moths and found that upon ejaculation the sperm are mixed with a secretion from the male's common duct which renders them vigorously motile. Shepherd (1975) also concluded that the activating factor probably functions as a catalyst rather than as an exogenous source of energy. The activator has been characterized as a polypeptide with a molecular-weight falling between 1600-4500 (Shepherd, 1974b, 1975). Polypeptides in the PS-1 fraction of D. funebris ARGs secretions have a molecular-weight of 2700 (Baumann, 1974a) although dimerization may generate polypeptides in the range of 5400. Terranova et al (1972) used electrophoretic methods and showed the molecular-weight of the polypeptide fractions in the secretions of Musca ranged from 600-1400. The active factor

which induced non-receptive behaviour in mated female Musca appeared to be a peptide with a molecular-weight of 750 or less (Nelson et al, 1969). Fuchs et al (1968) and Fuchs and Hiss (1970) purified the substance "matrone" from the accessory secretions of male Aedes aegypti and concluded that it was composed of two proteins with molecular-weights of 30,000 and 60,000 respectively. It should be noted that molecular-weights of the matrone subunits are considerably higher than those of the active factors in accessory secretions of other insects.

1.5 Functions of Accessory Reproductive Glands in Relation to Spermatophore Formation and the Chemical Nature of Spermatophores

Many terrestrial arthropods use a spermatophore for the transfer of sperm and seminal fluid from the male to the female. In such cases, a spermatophore is derived from the secretions of male ARG. Spermatophores of various shapes and complexities have been described for various arthropods (Khalifa, 1949; Tatchell, 1962; Davey, 1965; Gregory, 1965; Feldman-Muhsam, 1967, 1969; Legg, 1973; Pickford and Padgham, 1973; Gadzama and Happ, 1974). Among the Acridoidea the general morphology of male ARGs is similar (Uvarov, 1966), but there is still some considerable variation in the shape and number of spermatophores produced per

mating. Detailed investigations have been performed on the formation and fate of the spermatophore in L. migratoria (Gregory, 1965). In this species the fully formed spermatophore is a thin walled, dilated reservoir which remains within the male copulatory organ during copulation. From the spermatophore a long blind ended tube passes into the spermatheca of the female. In M. sanguinipes (Pickford and Gillot, 1971) and S. gregaria (Pickford and Padgham, 1973), however, males produce small, relatively simple, tubular spermatophores. As many as 14 spermatophores may be produced during a single copulation in M. sanguinipes and S. gregaria but only a single spermatophore is produced by L. migratoria during each copulatory period.

Gregory (1965) was able to trace strands of secretion from the developing spermatophores of L. migratoria back to the gland tubules which produced them and discovered the types of accessory glands which contributed to the formation of different parts of the spermatophore. In this same line of research Hartmann (1970) has thoroughly studied spermatophore formation in G. rufus and has clarified the function of different ARGs during the process. He showed that the structural part of the spermatophore was formed by secretions of the translucent glands, while the extrusion of the spermatophore was dependent on the seminal fluid and secretions of the type 1 white glands. For-

mation of the spermatophore lumen was effected by passing the secretions of the type 2 white glands and opalescent gland into the proximal tubule of the developing spermatophore. Davey (1959), removed one or more lobes of the transparent accessory glands of R. prolixus and demonstrated that the spermatophores which were produced were smaller but normal. These data implicated the transparent glands as the primary source of the material which contribute to the main body of the spermatophore. In the same study, ablation of the opaque lobes of the accessory glands did not prevent spermatophore formation, but did prevent the transfer of sperm within the female from the bursa copulatrix to the spermatheca. Davey (1959) also demonstrated that solidification of the fluid secretion of the transparent glands results from the lowering in pH brought about by secretions from the bulbus ejaculatorius.

The complex spermatophore of L. nuttalli consists of a unique tubular structure and a mass of jelly-like material which contains the spermatozoa (Gerber et al, 1971b). They found that the wall of the spermatophore tube was composed of 3 layers of different substances which were produced by the first pair of male ARGs. The outer layer and the lateral mass of the wall were composed of a carbohydrate-lipid complex with phospholipoprotein crystal-like inclusions. The middle layer contained a phospholi-

poprotein, while the inner layer of the wall consisted of a lipoprotein with a neutral lipid moiety. The jelly-like material, which enclosed the spermatozoa, contained carbohydrate (not glycogen or chitin), protein and phospholipid and was derived from the vasa deferentia. Similarly, Gadzama and Happ (1974) showed that the wall of the highly organized spermatophore of T. molitor consisted of an outer tube and an inner core, both containing lipoprotein and protein fractions.

Frenk and Happ (1976) were the first to combine biochemical and immunological methods to further our knowledge on the chemical composition of insect spermatophores. Acid hydrolysis of spermatophores from male T. molitor yielded 16 amino acids of which proline, glutamic acid, aspartic acid, alanine and glycine had quantitative importance. A similar amino acid composition was found in acid hydrolysis of the bean-shaped accessory gland of T. molitor. These biochemical data and similarities in immunological characteristics led Frenk and Happ (1976) to suggest that the spermatophore is derived from the bean-shaped accessory gland.

Tatchell (1962) studied the intimate morphology and histochemistry of both the spermatophore and the male accessory glands in the soft tick, A. persicus. He found that the spermatophore was coated with a thin layer of acid mucopolysaccharide secreted

by the anterior and posterior spongy lobes of ARG. The main wall of the ectospermatophore was formed from lateral granular lobe which interacts with the phospholipoprotein secretions of median granular lobe. Tatchell (1962) also found that the tip of the spermatophore was derived from an acid-mucopolysaccharide-protein complex which was secreted by the anterior dorsal lobe of the ARG. The role of ARG in the male pseudoscorpion, C. ischnocheles, in relation to spermatophore formation has been ellucidated by Legg (1973). He showed that the spermatophore was largely proteinaceous and that the stalk protein contained arginine, traces of -SH and tyrosine. The viscous sperm fluid packet and sperm cyst was shown to contain polysaccharide, acidic and neutral mucopolysaccharides and traces of alpha glycerol. Lipids were demonstrated only in the spermatophore stalk and viscous sperm packet (Legg, 1973). Amino acid analysis of the spermatophore revealed 17 amino acids with high levels of glycine, glutamic acid, serine and aspartic acid (Hunt and Legg, 1971). Legg (1973) proposed a new function for the secretion of the lateral accessory gland C. ischnocheles. This secretion forms an oily liquid droplet on the completed spermatophore; and it was suggested that it contains a substance (possibly a pheromone) which enables the female to locate the spermatophore.

Studies on the enzymatic composition of ARG secretions

and/or spermatophores are few (Feldman-Muhsam et al, 1973; Mann et al, 1973). In their work on unevaginated spermatophores of O. savignyi (Feldman-Muhsam, 1967, 1969), Feldman-Muhsam and co-workers (1973) indicated the presence of a non-specific esterase in the endospermatophore membrane and an alkaline phosphates in the neck region of the endospermatophore and in the spermiophores. Tatchell (1962) demonstrated the presence of a high non-specific esterase activity in the mid-region of the posterior dorsal granular lobe of A. persicus. The presence of highly active glycosidases in the spermatophores and male accessory secretions of the mollusc, Octopus dofleini martini, was discovered by Mann and co-workers (1973). They showed that α-mannosidases and β-N-acetylglucosaminidase activity was located within the spermatophore.

1.6 Immunological Studies with Accessory Reproductive Glands

Considerable interest has been shown in the use of immunological methods in studies on verebrate reproduction (Piko, 1967; Shivers and Dudkiewicz, 1974). However, these methods have not yet been fully exploited in the study of arthropod reproductive mechanisms. Frenk and Happ (1976) used rat-anti-spermatophore and rat-anti-bean-shaped ARG sera to identify immunologically similar components in the spermatophore and bean-shaped ARG in

T. molitor. Their immunological data was supported by biochemical data which strongly suggested that the spermatophore was derived from the bean-shaped accessory gland, at least in part. In a different study, Friedel and Gillot (1976) demonstrated the extra glandular synthesis of ARG components in male M. sanguinipes. With immunochemical and radio-tracer methods they showed that the fat body synthesizes certain proteins which are released into the haemolymph and accumulated within the tissues of the ARG.

1.7 Summary of Research Project Objectives

In consideration of the previously existing data on the anatomy and function of the ARGs of S. gregaria in relationship to what was known about the ARGs of other arthropods, two principal goals were set for this research project. The first goal was to obtain a chemical characterization of the secretions of individual ARGs by electrophoretic, immunochemical and chromatographic methods. The results of these investigations would then be used to confirm, refute or expand Odhiambo's (1969a, 1969b) classification system for the ARGs of male S. gregaria. The second goal of this project was to use these same electrophoretic and immunochemical techniques to charactertize the chemical nature of the spermatophore and the seminal fluids and to obtain a better understanding of the roles of individual ARG secretions

in their formation. Following initial successes in the raising of antibodies in guinea-pigs to components of ARG secretions a third goal was formulated. This goal was to determine if the injection of antibodies raised against ARG secretions could be used to disrupt the normal sexual maturation and/or the functions of the ARGs in male S. gregaria.

CHAPTER II

MATERIALS AND METHODS

2.1 Source and Breeding Conditions of the Experimental Material

The insects used for this study were the male desert locust, Schistocerca gregaria Forskäl belonging to the family Acrididae and sub-family Cyrtacanthacridinae. The locusts were obtained from a breeding stock maintained in the Department of Zoology, University of Nairobi.

The breeding methods of Hunter-Jones (1966) were followed and the locusts were kept in a crowded condition. The mean temperature inside the cages was 30±4°C under a 12 hour light and 12 hour dark photoperiods. During the course of the study the locusts were fed fresh grass daily.

All biochemical investigations were performed on 19-day-old sexually mature, unmated males. The sexually mature males have attained their yellow body colouration by this age (Norris, 1954; Penner, 1967) and ARGs are fully developed (Odhiambo,1966b). Under the described breeding conditions, copulation occurred as early as the twelfth day after the adult moult. Therefore, males

were maintained in the unmated state by separating the sexes on the tenth day after adult ecdysis.

2.2 Dissection and Collection of Samples

Prior to dissection, 60 to 100 µl of haemolyph was collected from each locust through a cut on the dorsum of the third abdominal segment. This haemolymph was pooled in small plastic tubes, containing a few crystals of phenylthiourea, and stored at -20°C until needed for analysis.

Immediately after collecting haemolymph, the adult male was dissected in 0.9% NaCl (normal saline). Each ARG was carefully pulled from the proximal end, rinsed in normal saline and then collected in little ampoules, containing 50 µl saline, stored over ice. Care was taken not to puncture or break the glands and to remove as much of the adhering fat body as possible. The landmarks described by Odhiambo (1969a) were used to identify the various glands.

Each of the gland types from 23 pairs of ARG were individually squeezed to collect their secretions. The secretions were immediately transferred into small plastic tubes and stored at 4°C. Pooled secretions from each gland type were then stored

at -20°C. The milky white washings of the paragonadal fat body were also collected and stored at -20°C.

2.3 Protein and Free Amino Acid Analysis

2.3.1 Disc Electrophoresis

Disc electrophoresis, modified from the method of Davis (1964), was employed to separate proteins, glycoproteins and lipoproteins in secretions of various glands, fat body and the haemolymph. Sample and spacer gels were prepared as described by Davis (1964), but the separation gel used in this experiment consisted of 7.5% Cyanogum "41" (BDH Chemicals Ltd., Poole, England) and 10% ammonium persulphate in Tris-citrate buffer (.076M- .005M) pH 8.6, and N,N,N',N'- Tetramethyl-ethylenediamine (TEMED, BDH, Chemicals Ltd., Poole,England). When 19 ml 7.5% Cyanogum '41', 0.1 ml 10% ammonium persulphate and 50 µl TEMED, were used, the chemical polymerization of the gel proceeded at a favourable rate which allowed water layering of the gels. It was necessary, however, to de-aerate the mixture before filling the glass tubes. This allowed the preparation of polymerized gels which were free of air bubbles. Glass tubes with an inner diameter of 2.5 mm and a length of

8 cm were used on a modified "Canalco" disc electrophoresis apparatus, model 1200 (Canalco Industrial Corporation, Rockville, Maryland, U.S.A.) and rubber grometts to fit the glass tubes were inserted into original rubber grometts in the upper chamber of the apparatus. Electrophoresis was carried out in 0.3M Boric acid buffer adjusted to pH 8.6 with NaOH. A current of 1.5mA/gel was applied for 35 minutes.

2.3.2 Staining Methods

2.3.2.i Proteins

Following electrophoresis, proteins were stained with Coomassie brilliant blue-G (CBB-G) (Diezel et al., 1972). Bands positve for proteins appeared as light to dark blue bands.

2.3.2.ii Glycoproteins

Following electrophoresis, the gels were fixed overnight in 7.5% acetic acid at $4^{o}C$. They were then washed in distilled water and oxidized in 1% aqueous periodic acid for one hour at $4^{o}C$. Excess

periodic acid was removed by washing the gels for 3-4 hours with distilled water which was changed every half hour. The gels were then immersed in Schiff's reagent (Pearse, 1960) for 1-2 hours. Carbohydrates appeared as pink to red coloured bands. To confirm whether any of the Periodic acid-Schiff (PAS) positive bands are formed by glycoproteins, the gels were destained in sulphite water (Olembo, 1972) for 2-3 hours and then transferred into 50 ml 7.5% acetic acid. Three drops of 1% Amido black 10B (ABB) in 7.5% acetic acid were added to the staining dish and PAS-positive bands containing glycoproteins turned blue after 16 hours. With this method care was taken (i) to note the position of PAS-positive bands before adding Amido black 10B solution, and (ii) not to add an excess of Amido black staining solution, which would then stain some of the non-glycoprotein bands.

2.3.2.iii Lipoproteins

The method of Maurer (1971), using Sudan black B, was used to locate lipid or lipoprotein bands on gels. The lipoprotein bands detected by this method were fixed and counterstained permanently with iodine solution, which was prepared as cited by Maurer (1971).

2.3.3 Immunoelectrophoresis Analysis

2.3.3.1 Preparation of test antisera

Out-bred guinea-pigs, either sex, weighing 0.5 to 1 kg, were used. They were maintained at the East African Veterinary Research Organization, Muguga, on a free choice of cabbage, rabbit chow and water.

Prior to injection, the protein concentration of the various ARG antigens to be used was determined by the the method of Lowry et al., as cited in Bailey (1968). A protein standard solution of crystalline bovine albumin was used to make the standard curve. Immediately prior to injection, a dilution of each antigen was prepared in sterile, 0.85% saline solution to give approximately 200 to 300 µg protein in 2 ml final volume. An equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) was added and the mixture emulsified by repeated aspiration into a syringe.

At injection, each antigen mixture was administered to groups of two guinea-pigs. Each animal received 0.1 ml in two intramuscular sites, and 1.6 to 1.8 ml in 5-8 subsutaneous sites. The injections were repeated at approximately two week intervals for a total of 3 injections. Approximately 8 weeks after the initial injection, blood was collected by intracardiac puncture and the serum collected from the clot after 18-24 hours at ambient temperature.

Antisera were prepared against the following antigenic materials :

1 Homogenate of ARGs 1,2,3,4,5,6,11,12 and homogenous glands

2 Homogenate of ARG 1

3 Homogenate of ARG 2

4 Homogenate of ARG 3

5 Homogenate of ARG 4

6 Homogenate of ARG 5

7 Homogenate of ARG 6

8 Homogenate of homogenous glands

9 Homogenate of ARG 11

10 Homogenate of ARG 12

11 Haemolymph after centrifugation to to remove haemocytes

2.3.3.ii Immunoelectrophoresis

Immunoelectrophoresis (IE, Scheideggar, 1955) was carried out in 1% Noble agar in pH 8.2 barbital- HCl buffer (0.05M) on glass microscope slides using the "Shandon" apparatus (Shandon Southern Products Limited, Cheshire, England). A gel cutter was used to cut 60 mm troughs and 2mm wells in various configurations, depending on the experiment. The wells were aspirated, the material to be electrophoresed added, and separation carried out for 45 minutes with a potential of 30 volts across the slides. Following electrophoresis the troughs were removed by aspiration and filled with guinea-pig antiserum to develop the precipitin arcs. The slides were incubated in a humid chamber at ambient temperature for 24 hours. Occasionally during incubation, the troughs were refilled with antiserum. The slides were then

submersed in distilled water for 24-48 hours to remove unreacted protein, dried, and stained with 0.1% Amido black B in 9:1 methanol: acetic acid solvent. Excess stain was removed by 3 washings in methanol:acetic acid (9:1) solvent.

2.3.4 Free Amino Acid Analysis

Free amino acids were extracted from individual gland secretions of 46 glands of each type using 4 ml 80% methanol (MeOH). The methanolic extracts were centrifuged after 24 hours, at 4°C, and the supernatents were evaporated to dryness under vaccum at 50°C using a rotary evaporator. The residue in a flask was weighed and redissolved in 200 µl 80% MeOH. These were then stored at -20°C until used for paper chromatography and high voltage electrophoresis.

2.3.4.i Paper chromatography

Two dimensional chromatograms were prepared by an ascending technique (Smith, 1969) on Whatman No. 1MM paper (25x25 cm). Ten microlitres of each extract was applied on a chromatogram. The

first separation was performed with a solvent mixture of η-butanol-acetic acid-water (12:3:5 v/v/v), giving a migration time for 23 cm of about 15 hours. After air drying for 9-10 hours, the chromatograms were run in the second direction with a phenol-water (4:1 v/v) solvent mixture, also giving a migration time for 23 cm of 15 hours. During the second run a beaker containing a saturated solution of KCN was placed in the tank to suppress the oxidation of phenol on the paper. The chromatograms were air dried for 24 hours to remove residual phenol, before dipping in 0.2% ninhydrin in acetone solution. They were developed by heating in an oven at 105^{o}C for about one to two minutes.

2.3.4.ii High voltage electrophoresis (HVE)

Using methanolic extracts (Section 2.3.4) of individual gland secretions, paragonadal fat body and haemolymph, HVE was performed on Whatman No.3 MM paper on a Savant instrument (plate type) with 5,000 volt power supply. A potential difference of 60 V/cm was applied for 1 hour.

Buffers used were of pH 1.9 (formic acid -acetic acid-water; 33:147:1820 v/v/v), pH 3.6 (pyridine-acetic acid-water ; 10:100:1900 v/v/v) and pH 6.5 (pyridine-acetic acid-water; 100:4:1900 v/v/v). Papers were dried in an oven at 105°C for 10 minutes before dipping in 0.2% ninhydrin in acetone solution. They were then developed in an oven at 105°C for about 2 minutes.

2.4 Paper Chromatography of Free Sugars

Descending paper chromatography of methanolic extracts of individual gland secretions, paragonadal fat body and haemolymph (Section 2.3.4) on Whatman No. 1 MM (46x57 cm) was carried out with n-butanol-acetic acid-water (4:1:1 v/v/v) solvent mixture, giving a migration time of approximately 18 hours.

After air drying the chromatograms, the free sugars were detected by the following reagents :

1. The paper was dipped in an acetone silver nitrate solution (5g $AgNO_3$ in 400 ml acetone), air dried and dipped in an alcoholic solution of sodium hydroxide (0.5% NaOH in 95% ethanol). The chromatograms were

finally fixed by dipping in a 5% sodium thiosulphate solution. Sugars and amino sugars gave black and brown spots respectively (Trevelyan et al., 1950).

2. Alternatively, sugars and amino sugars were detected by dipping the air dried chromatograms in aniline pthalate reagent (Harbone, 1973) and drying the chromatograms in an oven at 105^{o}C for 2-3 minutes.

3. The amino sugars or compounds containing an amino group were also detected on the chromatogram after exposure to ninhydrin solution (Section 2.3.4.i).

2.4.1 Spot Tests for Purines and Pyrimidines

A compound which did not react with alkaline silver nitrate, leaving a white spot on the chromatogram, was eluted and spot tests were performed on the eluant. Extracts were applied across the full width of the chromatogram. After chromatography, the band of the unknown compound was located by cutting off edges of the paper which were then developed with alkaline $AgNO_3$. The slip of chromatogram which contained the unknown band was cut out, wrapped in parafilm and eluted with 80% methanol by capilarity.

From 12-20 µℓ of the concentrated eluant was spotted on a strip of Whatman No.1 MM paper which was also spotted with adenosine, adenosine triphosphate (ATP), cytidine triphosphate (CTP), urdine triphosphate (UTP) standards and 80% methanolic extract of tick excreta, which contains guanine (Balashov, 1959; Hamdy, 1972).

Spot strips were exposed to the following tests : (a) eosine-mercury stain for purines; (b) silver nitrate-dichromate stain for purines; (c) Wood's reagent for purines and pyrimidines (Merck, 1967); (e) Sakaguchi test for arginine and guanidines (cited in Dawson et al., 1972) (f) ninhydrin stain for amino or imino containing compounds; and (g) alkaline silver nitrate for sugars (Trevelyán et al 1950).

2.5 Thin Layer Chromatography of Lipids and Phospholipids

Lipids and phospholipids from individual gland secretions, paragonadal fat body and haemolymph were extracted with chloroform : methanol (2:1 v/v). The samples were left overnight in chloroform : methanol and then centrifuged in a clinical centrifuge for 10 minutes. The supernatent from each sample was evaporated to dryness at 50°C using a vaccum rotary evaporator. The

dried samples were weighed and redissolved in 0.2 ml chloroform : methanol (2:1 v/v) and stored at -20°C until needed for thin layer chromatography (TLC). The method of Folch et al., (1957) for lipid extraction was modified due to the small size of samples.

2.5.1 Neutral Lipid Separation

Glass plates (20x20 cm) coated with 0.25 mm thick silica gel 'G' were prepared from a mixture of 40g silica gel 'G' and 80 ml distilled water (Stahl, 1969). Plates were activated in an oven at 110°C overnight before use. Fifteen microlitres of each sample and known standards of various neutral lipid classes were applied in a row 2 cm from the bottom of each plate.

In a solvent system of petroleum ether (b.pt. 60-80°C) : diethyl ether-acetic acid (60:40:1) (Stahl, 1969) it took 25 minutes for the solvent front to migrate 15 cm beginning at the point of sample application. All solvents used were of analytical grade and had been redistilled.

The plates were air dried and the separated neutral lipid classes were visualized with iodine vapour. The visible spots were marked when the plates were removed

from the iodine tank.

2.5.2 Phospholipid Separation

Glass plates were coated with 0.25 mm thick layer of 40 g silica gel 'G' in 80 ml 0.001N Na_2CO_3 (Randerath, 1968). The plates were activated at 100°C for 1 hour before use. Each TLC plate was spotted with known phospholipid standards and with 15 µl of each sample.

Solvent system of chloroform-methanol-acetic acid-water (60:30:10:3.5 v/v/v/v) was added to chromatography tanks lined on 3 sides with blotting paper. The tanks were saturated for 5 hours. The running time was 45 minutes for 15 cm. After 13 cm of solvent migration, the solvent moved slower in the centre than on the sides.

The spots were visualized as for neutral lipids in Section 2.5.2.

2.6 Enzyme Studies

The disc electrophoresis method described in Section 2.3.1 was modified by mixing the samples with 40% sucrose

(1:1 v/v) and applying them on top of the spacer gel. Ten microlitre of each sample was used. Because of the slow electrophoretic mobility of enzymes, the running time was 40 minutes with a current of 1.5 mA/tube.

Enzyme patterns were compared with the protein patterns of each sample, which were obtained after electrophoresis with the above modifications and stained with CBB-G (Diezel et al., 1972). A further modification of the method was necessary in the case of the haemolymph where only 2 µl of the sample was used.

Detection methods for alkaline phosphatases (AlPase), malic dehydrogenase (MDH), malic enzyme (ME), α-glycerophosphate dehydrogenase (α-GPDH), iso-citrate dehydrogenase (IDH) and xanthine dehydrogenase (XDH) were those of Toledo and Magalhães (1973). The technique for detecting acid phosphatase (AcPase) was that of Terranova and Leopold (1973). Non-specific esterases were detected by the method of Whitemore and Gilbert (1974).

Stained gels were thoroughly washed in distilled water and stored in 7.5% acetic acid.

2.7 Involvement of the Glands in Spermatophore Formation

2.7.1 Sequence of Events During Sperm Transfer

In order to study the sequence of events during sperm transfer, adult unmated males and virgin females were separated on tenth day after adult moult. Three days later mating was initiated by introducing virgin females into cages (27x27x16 cm) containing males. After pairs had been in copula for 5-7 minutes, they were gently removed from the cage and transferred into 90x80 mm Kilner jars. At various time intervals, locusts were transferred into a deep freeze to virtually stop the process of sperm transfer. When present, spermatophore(s) and sperm were collected from the male aedeagus and from between the female ovipositors and were observed under a compound phase contrast microscope. Female spermathecal duct and spermatheca were dissected and the presence or absence of sperm was observed.

2.7.2 From results of experiments in Chapter 7.1 the time taken for the formation of the first spermatophore was known. Within 15-30 minutes of the onset of copulation, copulating pairs were separated and the spermatophore plus

seminal fluid was collected from the male aedeagus. Sperm and spermatophores from various males were collected in a small plastic tube containing 50 µℓ normal saline, centrifuges for 30 seconds in a Beckman spincho(R) microfuge and stored at -20°C.

Evacuated spermatophores were collected from inseminated females. Following a normal copulation period (as long as 6 hours) at least 6 spermatophores could be collected from each female. These pooled spermatophores were briefly homogenized in an all glass homogenizer containing 20 µℓ normal saline, centrifuged for 3 minutes in a Beckman spincho(R) microfuge and the supernatent stored at -20°C.

2.7.3 Disc Electrophoresis

The disc electrophoresis method used for analysing the washings from intact spermatophores obtained from males and the homogenates of evacuated spermatophores from females was that described in Section 2.3.1. After electrophoresis, the gels were stained for proteins, glycoproteins and lipoproteins as in Section 2.3.2.

2.7.4 Immunoelectrophoresis

Immunoelectrophoretic procedures Section 2.3.3.ii were used to identify the probable origin of spermatophore antigens from the various gland secretions. Two microlitre samples of intact spermatophore washings and supernatents from homogenised evacuated spermatophores were electrophoresed in 1% Noble agar on glass microscope slides. Electrophoresed samples were cross-reacted with various guinea-pig-anti-individual ARG sera using the technique discussed in Section 2.3.3.ii.

2.8 Effects of Specific Antibodies on the Development and Function of Accessory Reproductive Glands

Adult male locusts, 3 days (series I), 7 days (series II) and 10 days old (series III) were injected with guinea-pig-anti-ARG serum mixture (Gp-a-ARG mix). Injections were made into the haemocoel of the locusts between the lateral margins of 3rd and 4th abdominal segments using a 26 gauge needle. Each injection consisted of 0.1 ml of the Gp-a-ARG serum mixture. Series I was given a total of 3 injections (on 3rd, 7th and 10th days respectively), series II two injections (on 7th and 10th days respectively) and series III one injection on the 10th day. Control

males of series I and II received 0.1 ml normal guinea-pig serum injections on the same days. On the 10th day following adult moult both experimental and control males were separated from females. After 3 days they were re-introduced into the original cage to observe the occurrence and relative success of matings.

All experiments were terminated when males (experimental and controls) were 19 day old. At this age the male were dissected and measurements of length and width of the ARG complex was taken in order to access effects of antibody injections on their development.

Similarly 3,7,10 and 19 day old normal untreated males were dissected and measurements of their ARG complex were taken to form a basis for comparison with the results from experimental and control male locusts.

Normal guinea-pig serum was electrophoresed using the methods described in Section 2.3.1 to determine if any guinea-pig serum proteins are similar to locust haemolymph and individual ARG gland proteins.

CHAPTER III

PROTEIN AND FREE AMINO ACID ANALYSIS

Polyacrylamide gel electrophoresis and/or immunoelectrophoresis have rarely been used to analyse and characterise ARG secretions from males of arthropods. Terranova and co-workers (1972) and von Wyl (1976) have analysed ARG secretions from male dipterans using electrophoresis and discerned that the secretions had multiple protein patterns, even though, at least in the case of M. domestica, the secretory cells appear to be histochemically and ultrastructurally the same (Leopold, 1970; Riemann, 1973). The unpaired gland of several lepidopterans has been variously reported to produce 4-8 secretions (Callahan and Cascio, 1962; Khalifa, 1950; Shepherd, 1974a). Contrary to the rather simple anatomy of the ARGs in Lepidoptera, that of both ARG masses in S. gregaria is very complicated. In this species both ARG masses contain 16 tubular glands each, and have been grouped into 9 different types (Odhiambo, 1969a). Each type has a characteristic anatomy and secretes a product with distinctive morphological, histochemical and ultrastructural properties (Odhiambo, 1969a, 1969b). The results obtained by Odhiambo will be brought in the text as the electropherogram for each gland is described.

3.1 Disc Gel Electrophoresis

In the present study one of the primary objectives has been to confirm or find other differences in the classification of the ARGs of S. gregaria, established by Odhiambo (1969a). Using disc electrophoresis, secretions of the various ARGs of male S. gregaria have been found to have multiple protein patterns and in some cases even multiple glycoprotein patterns. None of the individual glands and the homogenous gland secretions analysed have revealed identical protein composition though there are a number of protein bands common to some glands.

In this section, description of the protein and glycoprotein patterns for the secretions of individual male accessory glands, paragonadal fat body and haemolymph has been followed by a comparison of the protein patterns of all glands. The protein electropherograms have been described using two systems. For the first system a lettering convention has been used assigning the letter A to the fastest moving band and the subsequent alphabet letters to the slower bands as they appear. Consequently, in the text, band 1-A represents the fastest moving band of gland 1, and band Ho-Z represents the slowest moving band from the secretions of the homogenous glands. For comparison of the protein patterns a numbering convention has been used, assigning a number to

protein bands that have a similar or identical electrophoretic mobility. Once again the number one has been assigned to the fastest moving band. Protein patterns from the various samples have been arbitrarily divided into fast, medium and slow zones based on the relative electrophoretic mobility of the various proteins. Periodic acid-Schiff-positive bands in a sample have been designated by numbering the fastest moving band with the Roman number one (I).

3.1.1 Gland 1

Viewing from a dorso-lateral position on the paired gland complex, this gland is long and opalescent. It has previously been shown to contain an acid muco-protein or mucopolysaccharide secretion (Odhiambo 1969a). In this study, 20 fractions were obtained when the electrophoresed secretions of gland 1 were stained with CBB-G. Three faintly staining bands were obtained in the fast zone, whereas 10 bands appeared in the medium zone (Figure 3). Of these, the wide and intensely stained band designated as 1-L consisted of 2 overlapping bands. This was revealed by electrophoresing dilute samples of the secretion for 40 minutes instead of the usual 35 minutes. Protein band 1-I also stained intensely. In

the slow zone 7 bands were present. The high intensity of the staining reaction of the wide band designated as 1-O was once again due to the presence of two overlapping bands. Since a PAS-positive band (1-I, Figure 3) appeared in this same region (1-O, Figure 3), the bands were probably formed by a glycoprotein complex. A similar situation existed for a second PAS band (1-II, Figure 3) which corresponded to the 1-P protein band.

3.1.2 Gland 2

The fibrous mucopolysaccharide secretion of this white opaque gland (Odhiambo, 1969a) could be separated into 18 protein fractions (Figure 3). The faint histochemical reactions of the various protein fractions indicated the low proteinacous content of this gland secretions. A PAS-positive band (2-I, Figure 3) appeared in the same region as protein band 2-P (Figure 3) indicating the glycoprotein nature of the bands.

3.1.3 Gland 3

This is a long pale white gland lying ventral to gland 2 on the dorsal side of the accessory gland complex.

FIGURE 3

Electrophoretic patterns of proteins and glycoproteins from the secretions of accessory reproductive glands 1,2 and 3; CBB-G, gels stained for proteins with coomassie brilliant blue-G; PAS, gels stained for carbohydrates with per-iodic acid Schiff's reagents, See text for explanation of electrophoretic band labelling and numbering systems.

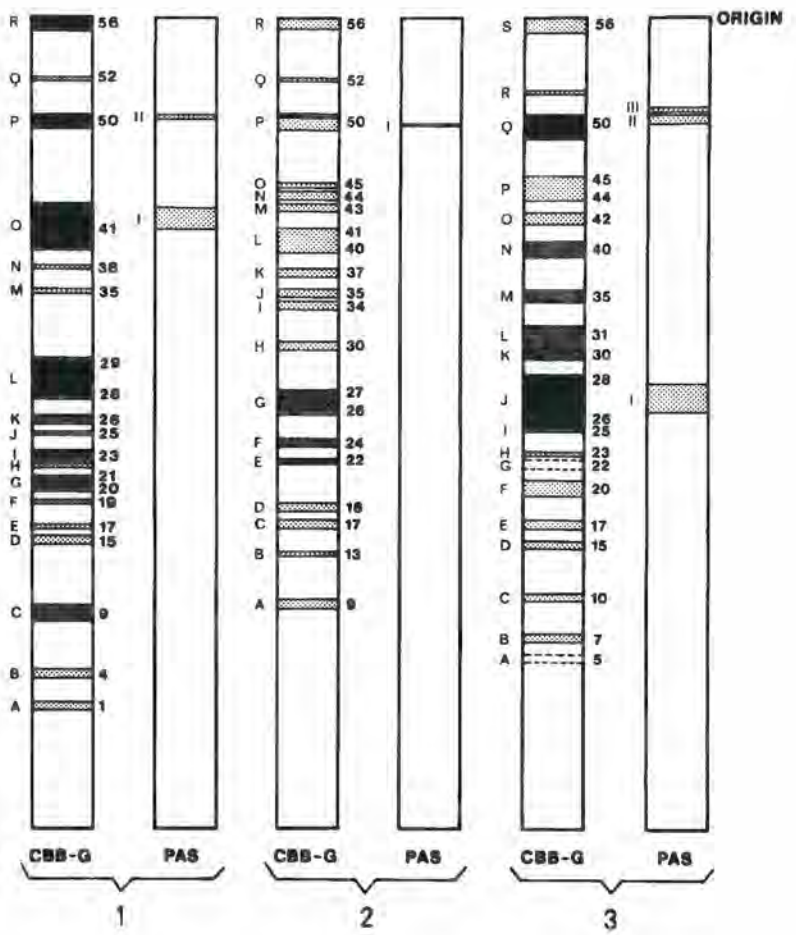


FIGURE 3

The secretions of this gland have been shown to consist of acidic lipoproteins (Odhiambo, 1969a). Electrophoresis of the gland 3 secretion revealed 19 protein bands (Figure 3). Maximum concentration of protein bands was in the slow and medium zone. Of these, band 3-J (Figure 3) consisted of two protein bands. Three positive stained bands were obtained after PAS staining (Figure 3). The PAS-positive band 3-I corresponded to protein band 3-J, indicating that this may be a glycoprotein complex. This was confirmed by purplish counter staining of the PAS-positive bands with ABB. While band 3-II appeared in the same region as the protein band 3-Q (Figure 3), band 3-III did not correspond to any protein band.

3.1.4 Gland 4

When viewed dorsally, lying inbetween gland 3 and 5, is the long convoluted hyaline gland 4. Secretions of this gland have been shown to be minutely granular, consisting of mucopolysaccharides (Odhiambo, 1969a). Twenty one protein fractions appeared after staining the electrophoresed secretions of gland 4 with CBB-G (Figure 4). The secretions of gland 4 always resolved into clear and sharp protein bands, more so than the protein patterns from other

gland secretions. There was a major protein band (4-J, Figure 4) in the medium zone. This band was wide and stained intensely with CBB-G. There were six other protein bands (bands 4-A, 4-C, 4-E, 4-F, 4-H and 4-T, Figure 4) which also stained intensely with CBB-G. Four bands appeared when the electrophoresed secretions were stained with PAS reagent. A major PAS-positive band (4-II, Figure 4) corresponded to protein band 4-J. The PAS-positive band, 4-I appeared in the same region of protein band 4-I, whereas bands 4-V and 4-V1 corresponded to protein band 4-T (Figure 4). The other bands which stained faintly with the PAS reagent appeared between protein bands designated as 4-R and 4-S.

3.1.5 Gland 5

The pale white acidic-lipoprotein secretion of this long opaque white gland (Odhiambo, 1969a) was separated into 17 protein fractions. Four major protein bands appeared in the medium zone. These bands are designated as 5-J, 5-K, 5-L and 5-M (Figure 4). In the slow zone, band 5-P was the only major protein band. PAS-positive bands 5-1V and 5-V appeared in the region of the single protein band 5-P (Figure 4), indicating, not only, that the protein band

FIGURE 4

Electrophoretic patterns of proteins and glycoproteins from the secretions of accessory reproductive glands 4,5 and 6; CBB-G, gels stained for proteins with coomassie brilliant blue-G; PAS, gels stained for carbohydrates with per-iodic acid Schiff's reagents. See text for explanation of electrophoretic band labelling and numbering systems.

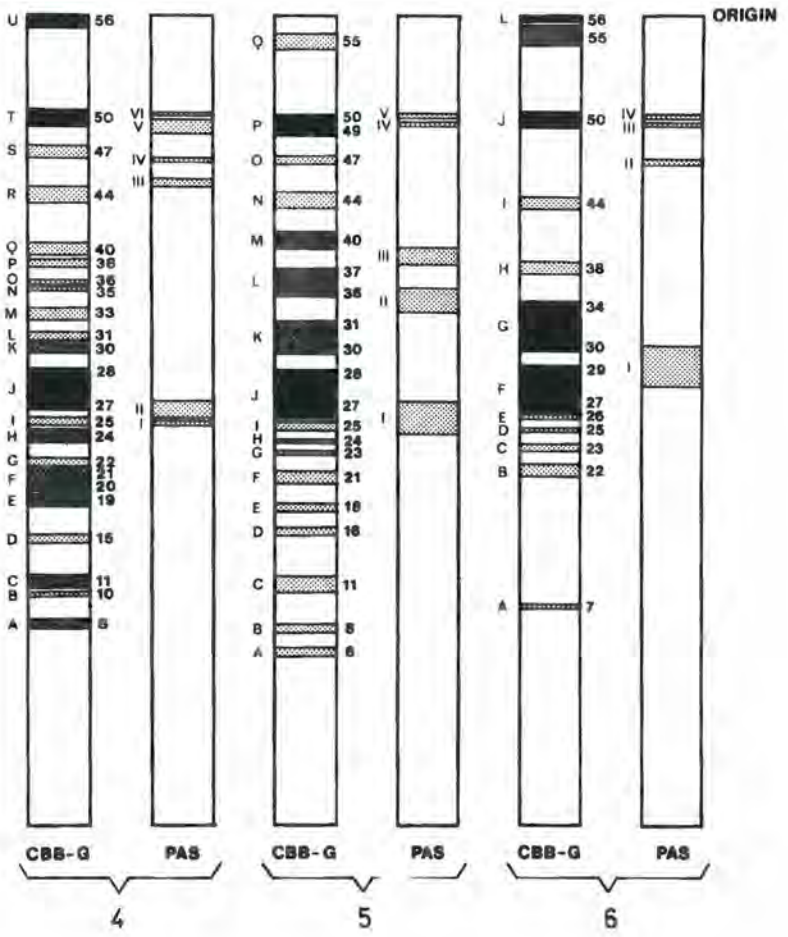


FIGURE 4

5-P may be composed of two protein bands but also that these bands are glycoprotein complexes.

3.1.6 Gland 6

This gland is an almost straight gland, containing a milky white mucoprotein secretion (Odhiambo, 1969a). This gland is present on the median edge of the gland mass. Twelve protein fractions were obtained after electrophoresis of gland 6 secretions. Two major protein bands, 6-G and 6-F, were intensely stained with CBB-G (Figure 4). Both bands appeared to be composed of two bands (as indicated by electrophoresing dilute samples). Like the protein bands 3-O (Figure 3) and 5-P (Figure 4) from secretions of glands 3 and 5 respectively, band 6-J of gland 6 (Figure 4) stained intensely for proteins. Four bands appeared with the PAS test. The major PAS-positive band, 6-I (Figure 4) appeared in the region between bands of 6-F and 6-G of the protein pattern (Figure 4). Infact this PAS-positive band is overlapped at its edges by the two protein bands, more so by the 6-F protein band. The PAS-positive band is either a glyco-6-F or a glyco-6-G protein band. Two PAS-positive bands, 6-III and 6-IV, also appeared in the region of protein band 6-J. The fourth PAS-positive

band 6-II did not correspond to any of the protein bands, indicating that it is probably not a glycoprotein

3.1.7 Homogenous Glands

These groups of glands have a homogenously minutely fibrous secretion which stains intensely for neutral mucopolysaccharides (Odhiambo, 1969a). The combined secretions from these glands were separated into 26 protein bands (Figure 5). These bands were numerous than those obtained from any of the other single gland analysed. Like the secretions of gland 2 (Figure 3) the secretions of the homogenous glands contained no major protein bands. The only faintly PAS-postive band corresponded to band H-X of the protein pattern (Figure 5).

3.1.8 Gland 11

This gland is the longest hyaline gland on the ventral side of the gland mass. The secretion of this gland has been shown to be coarsely granular containing neutral polysaccharides. (Odhiambo, 1969a). Secretions of gland 11 revealed one major protein band (11-0) and 20 other bands (Figure 5). The major protein band is

FIGURE 5

Electrophoretic patterns of proteins and glycoproteins from the secretions of the homgenous accessory reproductive glands (Ho) and glands 11 and 12; CBB-G, gels stained for proteins with Coomassie brilliant blue-G; PAS, gels stained for carbohydrates with per-iodic acid Schiff's reagents. See text for explanation of electrophoretic band labelling and numbering systems.

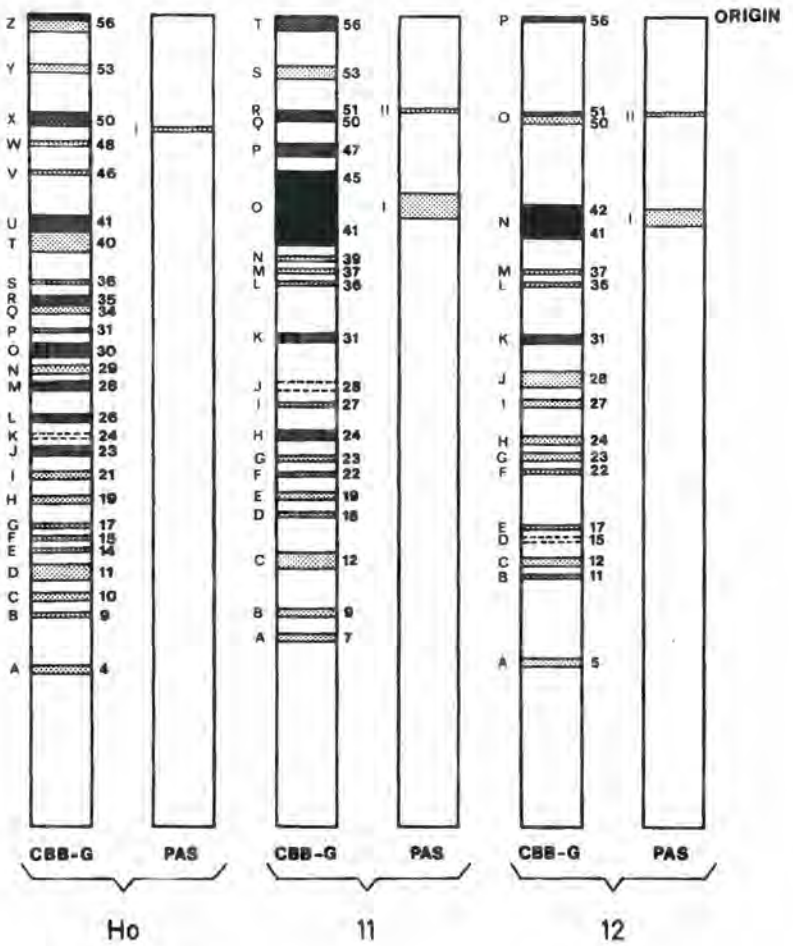


FIGURE 5

composed of at least two adjacent protein fractions (as indicated when dilute sample was electrophoresed) but appeared as a single protein band due to intense staining reaction. A PAS-positive band 11-I (Figure 5) appeared in the region of protein band 11-O. Another PAS-positive band 11-II did not correspond to any of the protein bands.

3.1.9 Gland 12

This is another hyaline gland on the ventral side of the gland mass. Like the secretions of gland 11, it also contains neutral polysaccharide in the secretion. Electrophoretic analysis of the secretions of gland 12 revealed a protein pattern which partially resembled that of gland 11 secretion (Figure 5). Some protein fractions of the gland 11 secretion are missing in the gland 12 secretion, however. The major protein band of the gland 12 secretion, designated as 12-N (Figure 5) had, however, a lower protein concentration as indicated by the width and intensity of staining of the band. Two PAS-positive bands, 12-I and 12-II (Figure 5) corresponding to protein bands 12-N and 12-O respectively, indicate that these may be glycoprotein complexes.

3.1.10 Gland 16

Gland 16 is the very long and convoluted gland in the centre of each of the two masses. This gland is the functional seminal vesicle. Nineteen protein bands were obtained after electrophoresis of the contents of these glands. All bands stained faintly (Figure 6). It was not certain if all the 19 bands represented the proteins of secretions of gland 16. In order to investigate this, broken seminal vesicle and sperm were washed with 0.9% NaCl followed by centrifugation. The washing procedure of the seminal vesicles was repeated three times; electrophoresis of the supernatent then revealed no protein band. After the third washing the sperm and seminal vesicle walls were electrophoresed. Nine protein bands were obtained (Figure 6). This indicated that 9 of the first protein pattern bands were mainly from the sperm. The other 10 bands probably represented apocrine secretions (Odhiambo, 1969a). A PAS-positive band (Figure 6) corresponding to band SV-R of the protein pattern indicated the presence of a glycoprotein.

3.1.11 Paragonadal Fat Body

Washings of the paragonadal fat body revealed 16 protein bands. The latter stained faintly with CBB-G with

FIGURE 6

Electrophoretic patterns of proteins and glycoproteins from the contents of the seminal vesicles (mixed secretions and sperm-(SV) and protein patterns from washed sperm and walls of the seminal vesicle (SVW) and from the paragonadal fat body (fb); CBB-G, gels stained for proteins with Coomassie brilliant blue-G; PAS, gel stained for carbohydrates with per-iodic acid Schiff's reagents. See text for explanation of electrophoretic band labelling and numbering systems.

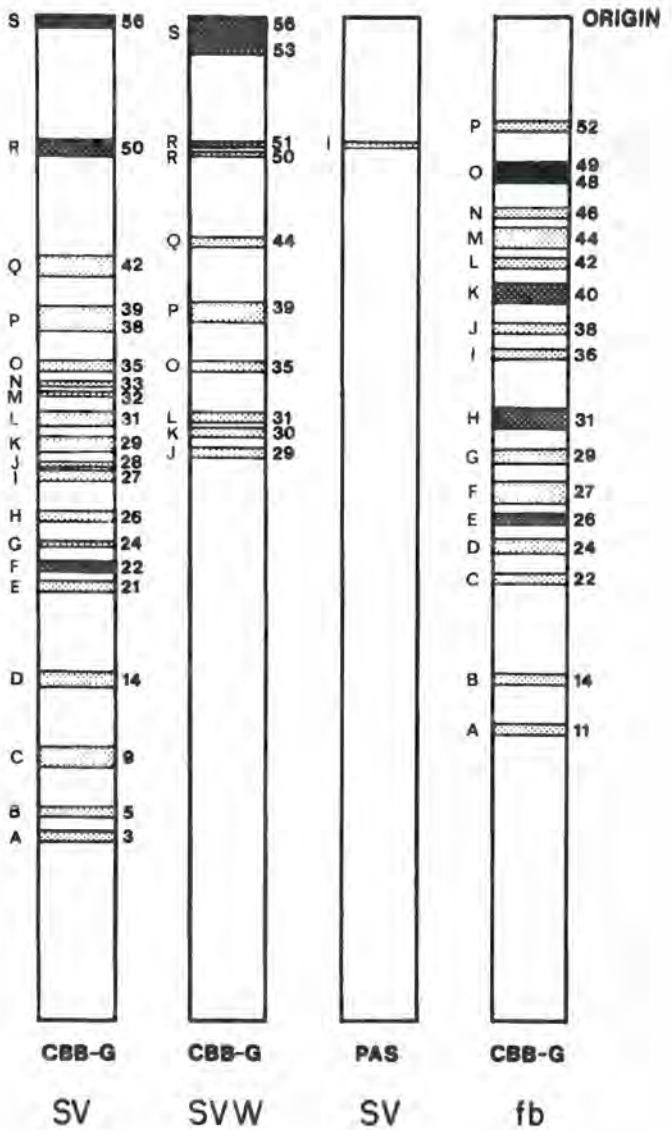

ORIGIN
S 56
R 50
Q 42
P 39
38
O 35
N 33
M 32
L 31
K 29
J 28
I 27
H 26
G 24
F 22
E 21
D 14
C 9
B 5
A 3
S 56
53
R 51
50
Q 44
P 39
O 35
L 31
K 30
J 29
I
P 52
O 49
48
N 46
M 44
L 42
K 40
J 38
I 36
H 31
G 29
F 27
E 26
D 24
C 22
B 14
A 11
CBB-G
CBB-G
PAS
CBB-G
SV
SVW
SV
fb

FIGURE 6

the exception of band fb-C (Figure 6). A single PAS-positive band, appearing near the origin, did not correspond to any of the protein bands in the same region.

3.1.12 Haemolymph

The haemolymph yielded 18 protein fractions of which 7 appeared in the slow protein migration zone (Figure 7). The remaining bands appeared in the medium zone. There were no bands in the fast moving zone. The 7 slow migrating proteins were present in high concentration as indicated by the high intensity of staining. This made their identification difficult, but the remaining bands were well separated. Three PAS-positive bands were obtained. Periodic acid-Schiff-positive band HE-I,II and III, appeared in the region of protein bands He-I,M and R respectively, indicating that these bands may be glycoproteins. Two bands (He-a and b, Figure 7) appeared with Sudan black B. Band He-a appeared inbetween protein bands He-N and He-O. Protein band He-Q corresponded to Sudan black B positive band He-b, indicating that this may be a lipoprotein.

FIGURE 7

Electrophoretic patterns of proteins, glycoproteins and lipoproteins from the haemolymph (He); CBB-G, gel stained for proteins with Coomassie brilliant blue-G; PAS, gel stained for carbohydrates with per-iodic acid Schiff's reagents; SBB, gel stained with Sudan black B for free and protein or carbohydrate bound lipids. See text for explanation of electrophoretic band labelling and numbering systems.

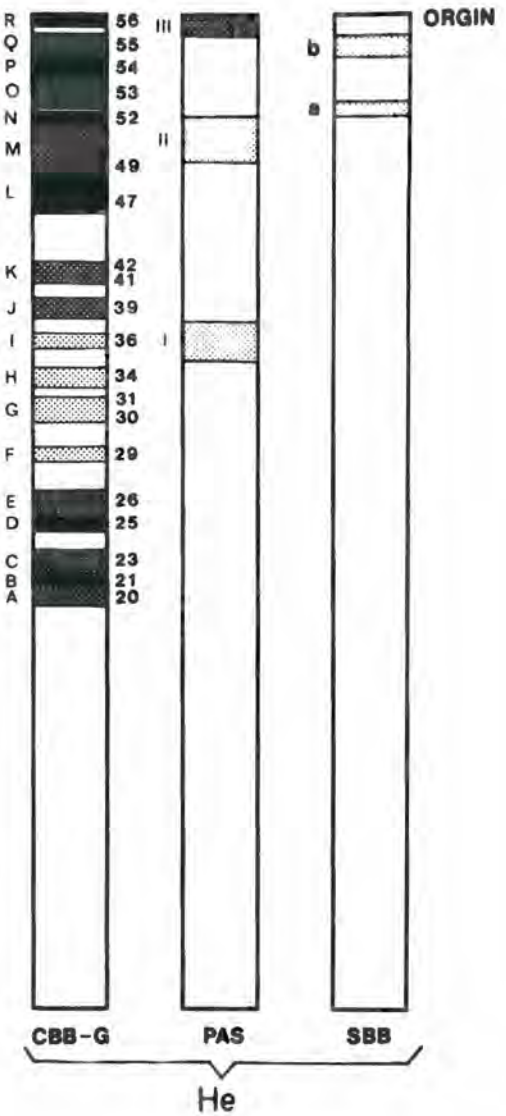

R
Q
P
O
N
M
L
K
J
I
H
G
F
E
D
C
B
A
56
55
54
53
52
49
47
42
41
39
36
34
31
30
29
26
25
23
21
20
III
II
I
ORGIN
b
a
CBB-G
PAS
SBB
He

FIGURE 7

3.1.13 General comparison

None of the glands have identical protein composition though there are a number of protein bands common to some glands. The major similarities between protein fractions existed for bands 2-G and 3-J (Figure 3), 4-J, 5-J and 6-F (Figure 4). Similarly, bands 3-L (Figure 3), 4-L (Figure 4), 11-K and 12-K (Figure 5) and SV-L (Figure 6) appeared to have the same electrophoretic mobility. When an intensely stained protein band from secretion of a gland corresponds to two or more bands from protein pattern of secretions of other gland(s), it does not necessarily imply a similarity in these protein bands. An example of such a situation is that in the region of bands 5-K and 6-G (Figure 4) are also located bands 3-K and 3-L (Figure 3) and Ho-O and Ho-P (Figure 5) protein bands. Similarly, bands 4-K, L and M (Figure 4) and SV-L, M and N (Figure 6) correspond to bands 5-K and 6-G (Figure 4).

The other major protein band common to secretions of gland 1,11,12 and homogenous glands were band 1-O (Figure 3), 11-O, 12-N and Ho-U (Figure 5). This fraction occurs in maximum concentration in gland 11 (indicated by the intensity of staining and width of the band), and

consists of at least two protein components, although only a single component may be present in gland 12. Band 2-L (Figure 3) lies in the same region as band 1-O (Figure 3), 11-O and 12-N (Figure 5). A number of protein bands from other gland secretions separated in the region of the intensely stained, major protein fraction (11-O, Figure 5) from secretions of gland 11. In the region of band 11-O (Figure 5) were bands 2-N, 2-O and 3-P (Figure 3), 4-R, 5-N and 6-I (Figure 4). It is interesting to note that one protein fraction appeared to be common to all gland secretions, fat body and the haemolymph, namely band 1-P, 2-P and 3-Q (Figure 3), 4-T, 5-P and 6-S (Figure 4), Ho-X, 11-Q and 12-O (Figure 5), SV-R, fb-P (Figure 6) and He-N (Figure 7). This band was approximately 0.6 cm from the origin. While this might have been an artifact, it did not appear in the blank gels which were stained for proteins.

In addition to the common major protein bands, a number of protein bands (narrow and faintly stained) were also common in some of the gland secretions. Protein band designated as 1-C, 2-A (Figure 3), 6-A (Figure 4), 11-B (Figure 5) and SV-C (Figure 6) appear to be similar in their electrophoretic mobility. Similarly, bands 1-E, 2-C,3-E (Figure 3),Ho-G and 12-E (Figure 5) appear in one migration

zone. In order to avoid the monotony of describing the common minor bands, protein patterns of various gland secretions, fat body and haemolmph have been shown in Tables 1-3. These Tables have been constructed based on fast (Table 1), medium (Table 2) and slow (Table 3) protein migration zones. Each protein band has been represented by a dot. Wide and intensely stained bands in the electropherograms (Figures 3-7) have been represented by vertical shaded areas in the Tables. Protein fractions have been numbered (represented on the vertical axis of all Tables) so that bands with a common number and common to some of the samples represent a similarity in their electrophoretic mobility. The same numbering system has also been used for the various electropherogram presentations (Figures 3-7).

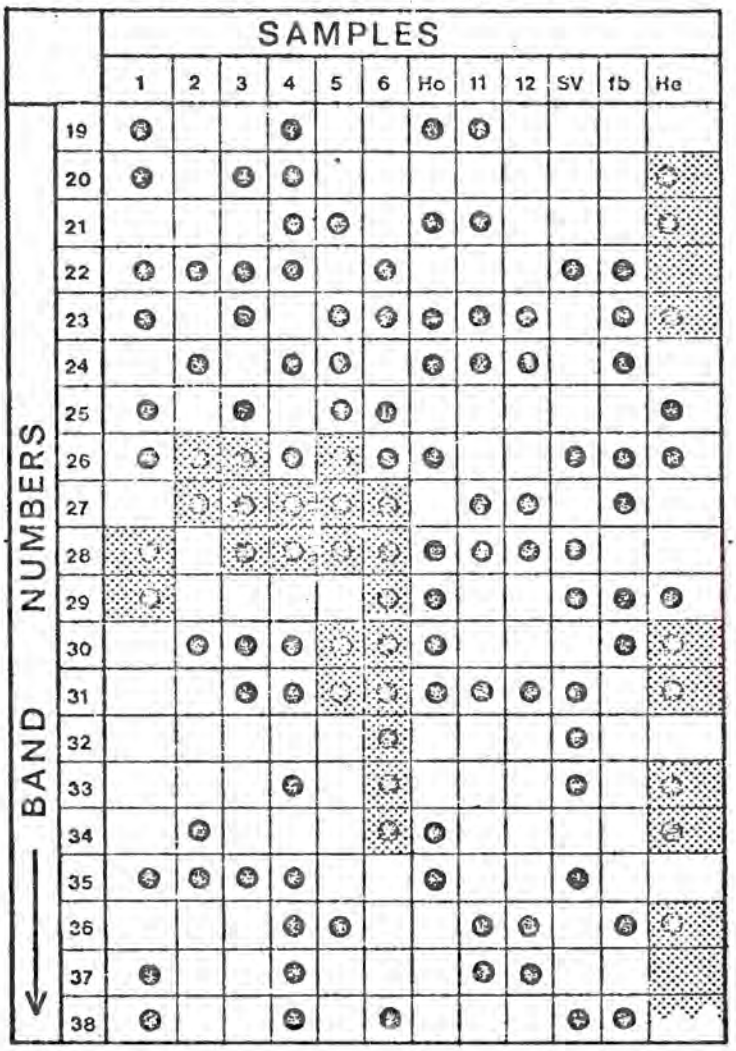
In addition to similarities in protein fractions of the secretions of various glands, some of the haemolymph proteins also had electrophoretic mobilities similar to some of the protein fractions in gland secretions. Haemolymph protein band He-K (Figure 7) was in the same migration zone as protein bands 1-O (Figure 3), Ho-U, 11-O and 12-N (Figure 5). Similarly protein band He-F (Figure 7) had a similar electrophoretic mobility as protein bands 1-L (Figure 3), 6-F (Figure 4), Ho-N (Figure 5 and fb-G

Table 1. Comparison of protein bands from various accessory reproductive glands, the paragonadal fat body and the haemolymph in the fast moving zone of electropherograms (Refer to Figures 3-7)

BAND NUMBERS ↓	SAMPLES											
	1	2	3	4	5	6	Ho	11	12	SV	fb	He
1	●											
2												
3										●		
4	●						●					
5			●						●	●		
6					●							
7			●					●				
8				●	●							
9	●	●				●	●	●				
10			●	●			●					
11				●	●		●		●		●	
12								●	●			
13		●										
14							●			●	●	
15	●		●	●			●		●			
16					●							
17	●	●	●				●		●			
18		●										

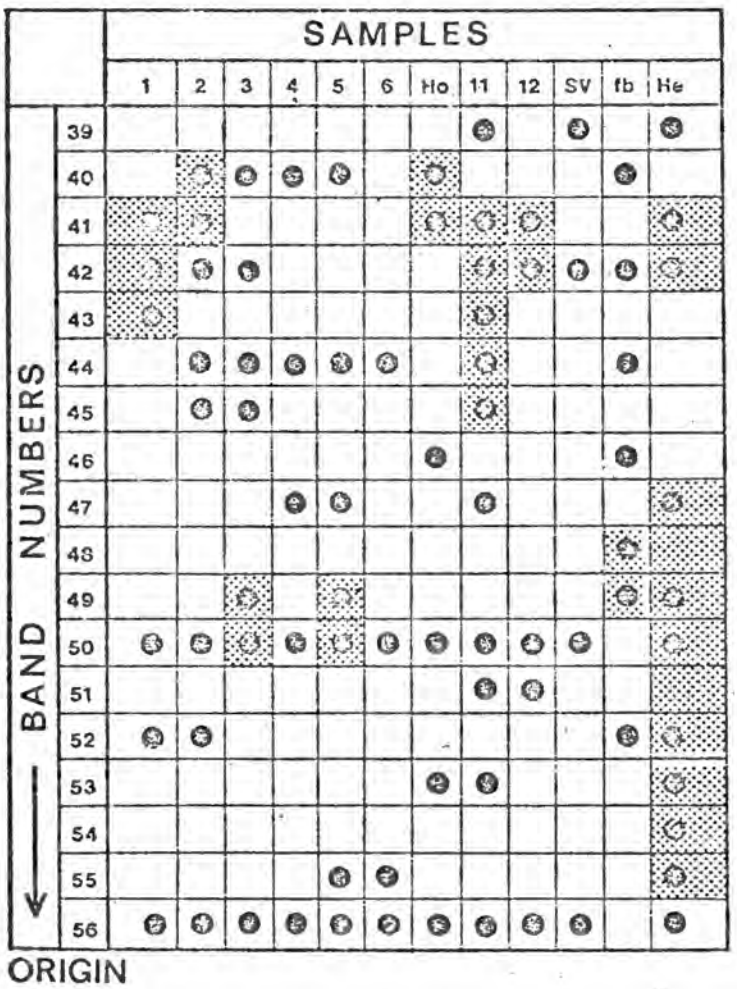
Notes : Each dot represents a protein band

Table 2 : Comparison of protein bands from various accessory reproductive glands, the paragonadal fat body and the haemolymph in the medium moving zone of electropherograms (Refer to Figures 3-7)



Notes : Each dot represents a protein band. Shaded blocks represent either a single band or multiple bands but due to their staining intensity appear as single bands

Table 3. Comparison of protein bands from various accessory reproductive glands, the paragonadal fat body and the haemolymph in the slow moving zone of electro pherograms (Refer to Figures 3-7)



Notes: Each dot represents a protein band. Shaded blocks represent either a single band or multiple bands but due to their staining intensity appear as single bands

(Figure 6). The other similarities in protein fractions of secretions from the glands, fat body and haemolymph have been presented in Tables 1-3.

Though a number of the protein bands appear to be common to many gland secretions, some protein fractions are characteristic of a single gland secretion. Protein bands 1-A (Figure 3) is present only in gland 1 while SV-A (Figure 6) is peculiar of the seminal vesicle. While bands 5-A and 5-D (Figure 4) are present only in gland 5 secretions, band 2-B and 2-D (Figure 3) are present only in secretions of gland 2.

The PAS-positive fractions 1-I (Figure 3), 11-I and 12-I (Figure 5) corresponding to the protein bands 1-O (Figure 3), 11-O and 12-N (Figure 5) respectively, may be similar glycoprotein complexes, since protein bands 1-O, 11-O and 12-N have a similar electrophoretic mobility. Glands 3,4 and 5 also seem to have similar glycoprotein complexes, bands 3-I and 3-J (Figure 3) in gland 3, 4-II and 4-J in gland 4, 5-I and 5-J (Figure 4) in gland 5 electrophoretic analysis.

Normal saline extracts of gland secretions, fat body and seminal vesicle did not reveal lipid or lipoprotein bands with Sudan black B. That the staining technique worked was confirmed by the appearance of lipoprotein bands in the haemolymph (Figure 7). Secretions of glands 3 and 5 have been shown to consit of acidic lipoprotein complexes (Odhiambo, 1969a). It was assumed that lipids or lipoproteins were present in levels that could not be detected by electrophoresis.

3.2 Immuno-electrophoresis

Some abbreviations have been used in this Chapter to represent guinea-pig anti-serum to whole gland complex, individual gland or the haemolymph. The abbreviations have been formulated in the manner shown in the two examples below :

Guinea-pig anti-serum to whole gland complex (excluding seminal vesicle and fat body) - abbreviated to Gp-a-G.Cl

Guinea-pig anti-serum to gland 1 - abbreviated to Gp-a-Gl.1

Results from cross-reacting individual gland secretions against Gp-a-G.Cl have been presented first. Pure secretions from

various glands were electrophoresed in 1% Noble agar on microscope slides and the centre troughs filled with 50 µl Gp-a-G.Cl. Secretions of all glands except glands 2,6, seminal vesicle and the paragonadal fat body reacted with Gp-a-G.Cl to give single antigen-antibody precipitates (Figure 8). Glands 1,11 and 12 secretions reacted with Gp-a-G.Cl to form a precipate in the same region indicating that these gland secretions have an antigen common to all of them. However, gland 12 reacted to give a faint precipitate reflecting on the low concentration of this antigen. Secretions of the two opaque white glands, gland 3 and 5, gave a common antigen-antibody reaction. A diffused, faint precipitate appeared by cross-reacting gland 4 secretion with Gp-a-G.Cl (Figure 9). This band was in the same region as the precipitate obtained from secretions of gland 3 and 5 (Figure 8). In fact, when combined secretions from glands 3 plus 4 and 4 plus 5 were electrophoresed and reacted with Gp-a-G.Cl a single band was precipitated (Figure 9). This indicated the presence of an antigen common to secretions of glands 3,4 and 5. No precipitin was obtained on cross-reacting gland 6 secretion with Gp-a-G.Cl. Similarly, neither the seminal vesicle nor the paragonadal fat body gave a precipitin with Gp-a-G.Cl.

Antigenscommon to some gland secretions were confirmed by electrophoresing various combinations of secretions of glands

FIGURE 8

Photographic record of the immunoelectrophoresis of secretions from accessory reproductive gland (Gl) 1,2,3,4,5,6,11,12 and the homogenous glands (Ho Gls) from 19 day old adult male S. gregaria. In all cases the antigens were precipitated by antibodies prepared against accessory gland complex (minus the seminal vesicle and the paragonadal fat body) extracts from 19 day old adult male S. gregaria.

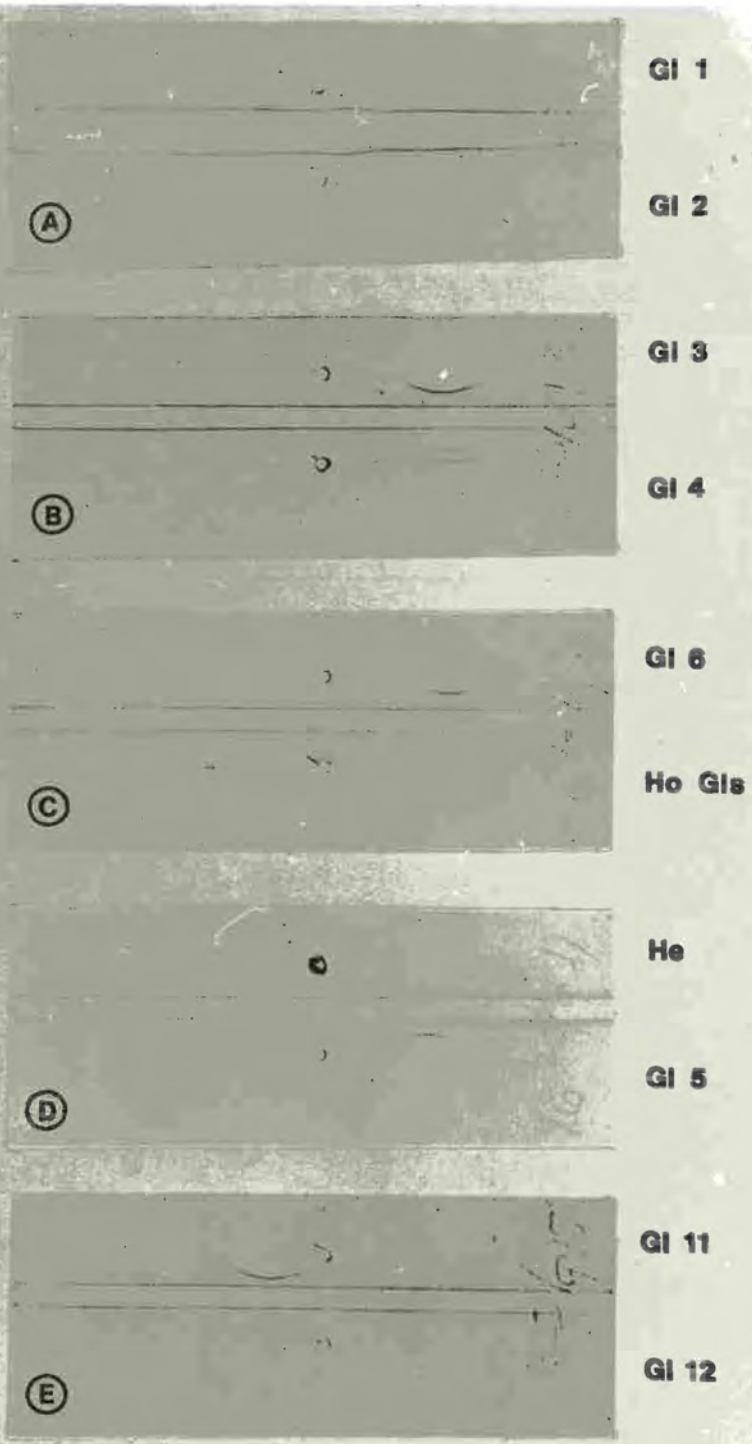


FIGURE 8

FIGURE 9

Photographic record of the immunoelectrophoresis of secretions from various individual accessory reproductive glands (Gl) and combinations of glands (Gls) from 19 day old adult male S. gregaria. In all cases the antigens were precipitated by antibodies prepared against whole accessory gland complex (minus the seminal vesicle and the fat body) extracts from 19 day old adult male S. gregaria.

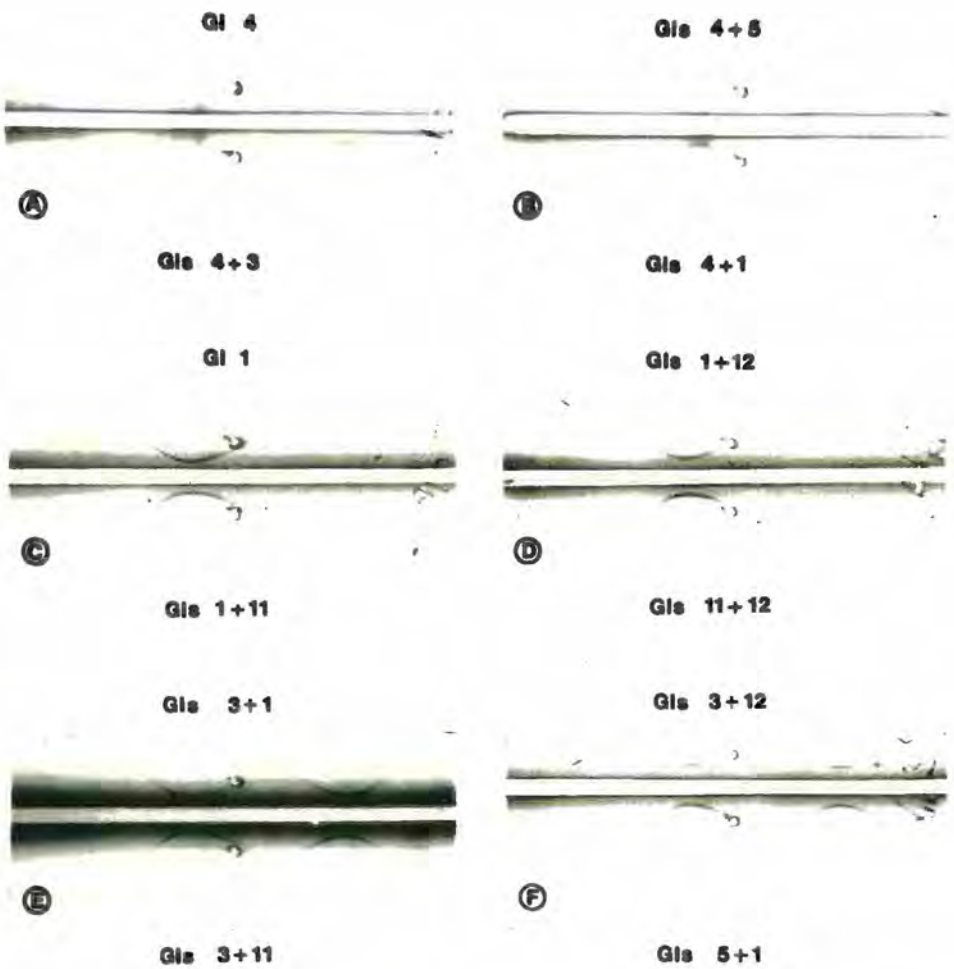

Gl 4
Gls 4+5
A
B
Gls 4+3
Gls 4+1
Gl 1
Gls 1+12
C
D
Gls 1+11
Gls 11+12
Gls 3+1
Gls 3+12
E
F
Gls 3+11
Gls 5+1

FIGURE 9

and cross-reacting with Gp-a-G.Cl (Figure 9). These reactions indicated while one antigen was common to glands 1,11 and 12 another antigen was common to secretions of glands 3,4 and 5.

The results outlined above confirmed the antigenic nature of the ARG secretions when injected into the guinea-pigs. However, due to the single precipitation reactions of the various gland secretions as against the multiple protein patterns of these secretions (Chapter 3.1), it seems that either the other protein fractions are not found in adequate quantities to start an immune response in the guinea-pig or that these proteins are not antigenic.

Larger quantities of individual glands were dissected and antibodies to these individual glands were raised from guinea-pigs. The various anti-sera, thus obtained, were used (i) to identify the number of antigens in each of the various gland secretions and (ii) to identify antigens common to various glands and the haemolymph.

Sera from guinea-pigs injected with glands 3,5 and 6 did not give precipitation reactions even against their respective gland secretions. These results were obtained, however, from the first bleeding after which the guinea-pigs had started to

die. It is possible that during the time before bleeding the guinea-pigs, due to a slow immune response, enough anti-body production had not occurred, hence no precipitation reaction. Similarly, gland 2 secretion did not react with Gp-a-Gl.2 to give a precipitating arc.

Figure 10 shows antigenic similarities and differences in the various gland secretions. A precipitin reaction common to glands 1,11 and 12 appeared when secretions of these glands were electrophoresed and reacted with Gp-a-Gl.1 and Gp-a-Gl.11 on one slide (Figure 10). However, there were differences in the sharpness and staining intensity of these arcs. The precipitate resulting from reaction of gland 11 secretions and Gp-a-Gl.11 was diffused with a sharp band lying in the diffused area (Figure 10). While a diffused band appeared on reacting electrophoresed secretions of gland 12 and Gp-a-Gl.11, a sharp precipitate band appeared from gland 12 secretions and Gp-a-Gl.1 reaction.

Two faint precipitin reaction arcs resulted from cross-reacting gland 4 secretion with Gp-a-Gl.4 (Figure 10). Gland 2 secretion did not reveal any precipitin line with either Gp-a-Gl.1 or Gp-a-Gl.4. Similarly, combined secretions from homogenous glands did not result into a band with Gp-a-Gl.1. A single precipitate was obtained when Gp-a-Gl.6, from the first bleeding of

FIGURE 10

Photographic record of the immunoelectrophoresis of secretions from accessory reproductive glands (Gl) 1,2,3,4,11,12 and the homogenous glands (Ho Gls) from 19 day old adult male S. gregaria. Antigens were precipitated by the following antibodies;

a. Guinea-pig anti- gland 6

b Guinea-pig anti- gland 11

c Guinea-pig anti- gland 12

d Guinea-pig anti- gland 1.

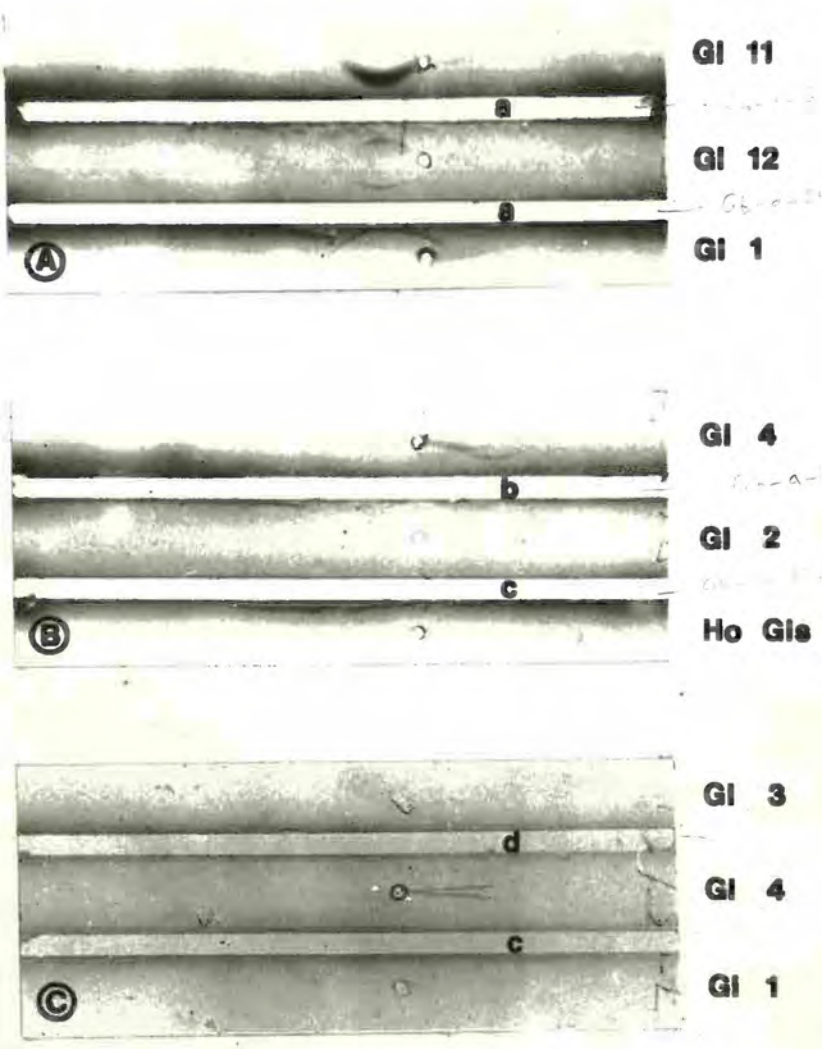


FIGURE 10

guinea-pigs was reacted with secretions of gland 4 (Figure 10). However, on the same slide gland 3 secretion did not give any precipitation with Gp-a-G1.6.

Two sharp precipitin arcs were obtained on electrophoresis of haemolymph followed by cross-reaction with Gp-a-haemolymph. This Gp-a-haemolymph was then subsequently used to identify antigens in haemolymph common to individual gland secretions. Single similar precipitation reactions appeared from secretions of gland 1,11 and 12 when reacted with Gp-a-haemolymph (Figure 11). These precipitin arcs correspond to those obtained by gland 1 against Gp-a-G1.1, Gp-a-G1.11 or Gp-a-G1.12. None of the other gland secretions resulted into precipitating bands with Gp-a-haemolymph.

3.3 Free Amino Acid Analysis

Data presented in this Chapter on the free amino acid composition of the various ARG secretions, paragonadal fat body and the haemolymph is a result of a combination of two-dimensional (2D) paper chromatography and high-voltage electrophoresis (HVE). HVE was performed at pHs' 1.9,3.6 and 6.5. Two dimensional chromatography revealed spots which sometimes were difficult to identify; such spots, on the other hand, could be identified using HVE.

FIGURE 11

Photographic record of the immunoelectrophoresis of the haemolymph (He) and secretions from accessory reproductive glands (Gl) 1,2,4,11,12 and the homogenous glands (Ho Gls) from 19 day old adult male S. gregaria. Immunoelectrophoresis of the normal guinea-pig serum (N-Gp-S) is also presented. In all cases antigens were precipitated by antibodies prepared against the haemolymph from 19 day old adult male S. gregaria.

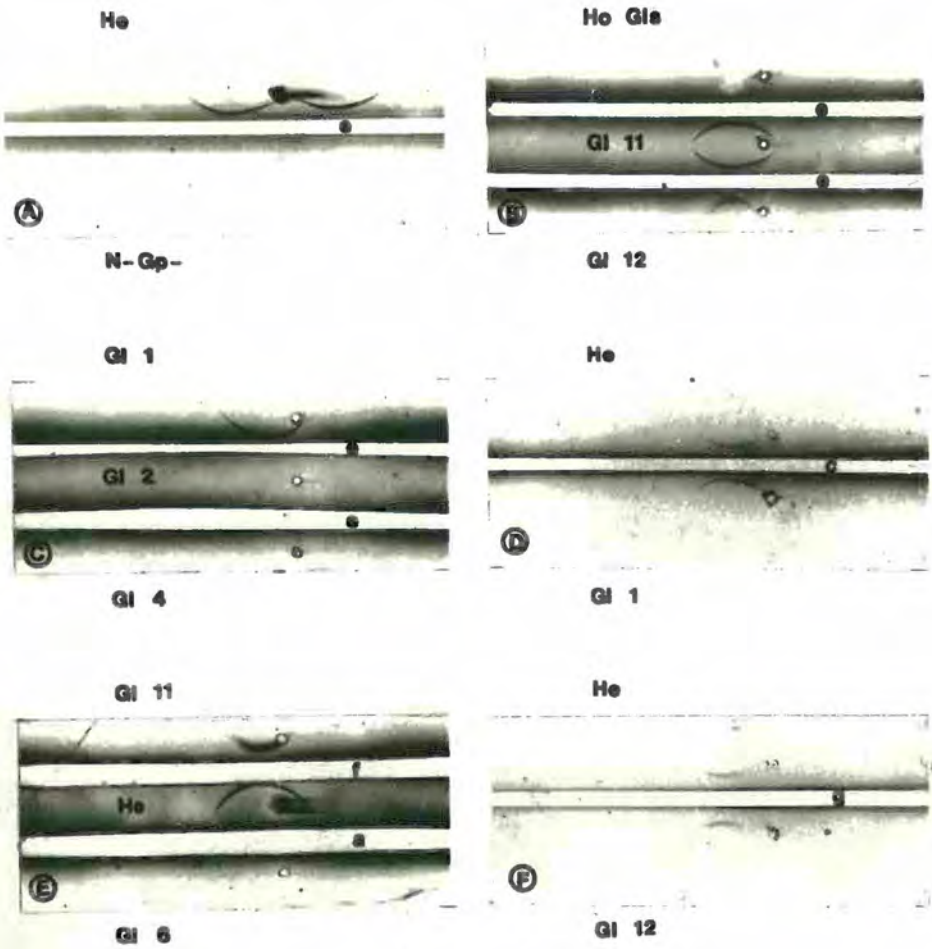

He
Ho Gle
Gl 11
A
B
N-Gp-
Gl 12
Gl 1
He
Gl 2
C
D
Gl 4
Gl 1
Gl 11
He
He
E
F
Gl 6
Gl 12

FIGURE 11

A total of 18 free amino acids could be identified from 80% methanolic extracts of individual gland secretions, fat body washings and haemolymph : arginine, aspartic, α-alanine, citruline, cysteine, glutamic, glycine, histidine, hydroxy-proline, leucine/iso-leucine, lysine, methionine, phenylalanine, proline, serine, valine, threonine and tyrosine. However, while aspartic and citruline were not present in the haemolymph hydroxy-proline and threonine were present only in the haemolymph. Inspite of the use of a combination of 2D-chromatography and HVE, an unidentified spot appeared in the methanolic extracts of the secretions of glands 1,2,3,4,5,6 and 11. This unidentified spot moved a short distance from the point of sample application on the chromatogram. It is speculated that this unidentified spot may be a polypetide. Distribution of the amino acids in the gland secretions, fat body and haemolymph is presented in Table 4.

As revealed in Table 4, the amino acids of significant quantitative importance were α-alanine, glycine, methionine, phenylalanine, serine and aspartic. α-Alanine was present in maximum concentration in secretions of glands 3,5,6,1 and 2. However, methanolic extracts of all the other gland secretions, fat body and haemolymph had trace amounts of this amino acid. The relative amounts of amino acids were indicated by the intensity of the ninhyrin reaction and the size of the spot.

Table 4. Free amino acid distribution in accessory reproductive gland secretions, paragonadal fat body and haemolymph of 19 day old adult male S. gregaria

	AMINO ACIDS																
SAMPLES	α- Alanine	Arginine	Aspartic	Citrulline	Cysteine	Glutamic	Glycine	Histidine	Hydroxy-proline	Leucine/iso-leucine	Lysine	Methionine	Phenylalanine	Proline	Serine	Threonine	Tyrosine	Valine
Gland 1	••		•		o	o	••			•	o	•	•		•			
Gland 2	•	•	o		o	•	•			•	•	•	o		•			
Gland 3	•••		o	o	o	o	••			•	o	o	o		o			
Gland 4	•	o	o			o	o				o	o	o		o			
Gland 5	•••	•	•			•	•			o	o	•	•		o			
Gland 6	•••	o	o		o	o	•				o	o	•		o			
Homogenous glands	o	o	•			•									••			
Gland 11	•		o			o	•			o			o		•			o
Gland 12	o	o		o	o	o	••	o		•	o	•		o	o		o	
Seminal vesicle	o	o	o															
Paragonadal fat body	o												o					
Haemolymph	o	o			•	o	o	•••	o	o	•	o	o	o	o	o	o	o

Notes: Open and closed circles represent varying staining intensities with ninhydrin solutions;

••• — High

•• — Medium

• — Moderate

o — Faint

Glycine, like α-alanine, was also present in larger quantities in secretions of glands 1,3,12,2,5,6 and 11. Secretion of gland 4 and the haemolymph contained trace amounts of glycine (Table 4). Aspartic is another common amino acid except that it was not present in the paragonadal fat body and the haemolymph. Glutamic and serine were present in glands 1,2,3,4,5,6,11,12, homogenous glands and the haemolymph (Table 4). Serine was present in greatest concentration in the homogenous glands. Except for glands 1,3 and 11 and the fat body arginine was common to the rest of the samples. Methionine was common only to the first six glands, gland 12 and the haemolymph (Table 4). Phenylalanine, besides occurring in the secretions of the first 6 glands was also present in gland 11 secretion and the haemolymph.

Citruline, cysteine, proline, tyrosine and valine were present in trace amount and only in a few of the glands. While citruline was present only in gland 3 and 12 secretions, cysteine was present in secretions of glands 1,2,3,6 and 11 and in the haemolymph. Valine was found to occur in trace amounts in secretions of gland 1 and 11 and in the haemolymph. It is also interesting to note that proline, tyrosine and ethanolamine were present only in the secretions of gland 12 and the haemolymph. Not only was the secretion of gland 12 unique in having some of the amino acids and ethanolamine, but it also had the maximum

number of amino acids amongst samples of secretions of various glands. Methanolic extracts of gland 12 secretion revealed 13 amino acids beside ethanolamine (Figure 12). The presence of ethanolamine, in gland 12 secretions and the haemolymph was confirmed by HVE at pH 6.5.

3.4 Conclusions

Electrophoresis of the secretions of various ARGs of male S. gregaria have revealed multiple protein and in some cases multiple glycoprotein patterns. None of the individual glands and the homogenous glands secretions analysed have identical protein composition, though there are a number of protein bands common to some glands. Besides differences in the protein and glycoprotein patterns of the gland secretions, none of the gland secretions electrophoresed into a similar number of protein fractions. However, 21 protein bands were obtained from the secretions of glands 4 and 11. Secretions of the homogenous glands revealed a maximum of 26 protein bands, whereas only 10 of the 19 protein fractions were found to originate from the seminal vesicle secretion. These results, in general, agree with those obtained by Odhiambo (1969a). However, differences in the classification of glands 3 and 5 have been found. While Odhiambo (1969a, 1969b), basing his results on histochemical and ultra-

FIGURE 12

Diagrammatic representation of a two-dimensional paper chromatogram of free amino acids separated from 80% methanolic extracts of the secretions of accessory reproductive gland 12 and detected with 2% ninhydrin in acetone;

BUT, η-Butanol-Acetic acid-water (12:3:5 v/v/v)

PHE, Phenol-Water (4:1 v/v)

Running time, 15 hours in each direction

Key to Amino acids :

1 Cysteine

2 Glutamic

3 Serine

4 Glycine

5 Threonine

6 α-Alanine

7 Histidine/Lysine

8 Citruline

9 Proline

10 Tyrosine

11 Methionine

12 Leucine/iso-leucine

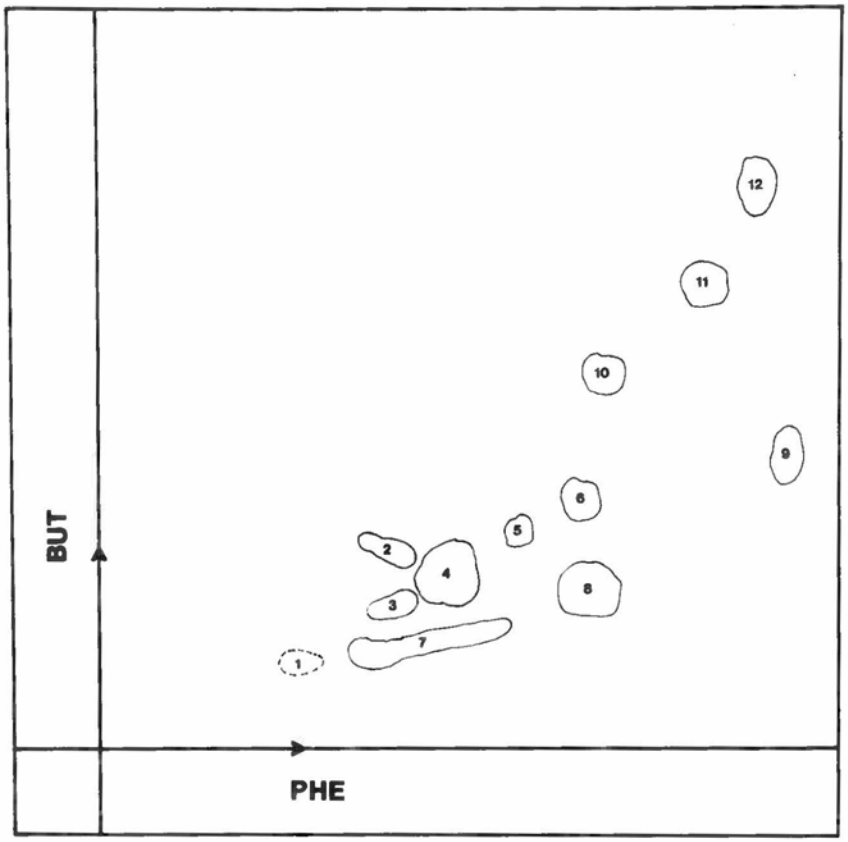


FIGURE 12

structural studies, found the secretions of glands 3 and 5 similar in nature, results in this study differ. Histochemical tests are generally qualitative and with a few exceptions they only broadly identify substances present. Electrophoresis, though also qualitative, gives some quantitative information. Based on this, secretions of glands 3 and 5 were found not only different in protein and glycoprotein patterns but also in the number of the protein and glycoprotein fractions. While gland 3 secretion revealed 19 protein and 3 glycoprotein bands, gland 5 secretions separated into 17 protein and 5 glycoprotein bands. Secretions of these two glands have been shown to contain acidic lipoproteins (Odhiambo, 1969a). However, with the techniques used in this study, it has not been possible to detect lipoproteins in any of the gland secretions. The fact that the staining techniques for lipoproteins worked was indicated by the appearance of 2 lipoprotein fractions in the haemolymph.

Because of the multiple protein patterns obtained from various gland secretions, it would not be surprising if there are protein fractions which are common to some of the gland secretions (Tables 1-3). On comparing the various protein electropherograms a number of protein fractions were found common to secretions of one gland or the other. However, even when the technique is performed with the greatest care, comparisons of electropherograms

are not always accurate, especially when each sample is electrophoresed on a separate gel. This problem has been overcome by immunoelectrophoresis on the secretions of individual samples. Various cross-reactions of the electrophoresed secretions and Gp-a-gland(s) or haemolymph sera have revealed the presence of antigens common to some of the glands. Unfortunately, the antisera produced contained antibodies to single antigens. This method is by no means exhaustive and these experiments need to be redone with greater concentration of ARG proteins. Nevertheless, while secretions of glands 1,11 and 12 and the haemolymph were found to have a common antigen, an antigen common to secretions of glands 3,4,5 and 6 was also present. Considering the results from disc electrophoresis and immunoelectrophoresis, it can be said that the antigen common to the secretions of glands 1,11 and 12 and the haemolymph is protein band 1-O (Figure 3), 11-O and 12-N (Figure 5) and He-K (Figure 7) respectively. The reason for proposing this is made stronger by the fact that these protein bands, at least in the case of secretions of gland 11 are present in large concentration and are probably responsible for the immune response in the guinea-pigs. However, it is surprising that the protein band 1-L (Figure 3) of gland 1 which appears to have a similar concentration as protein band 1-O did not give another immune reaction in the guinea-pigs injected with gland 1 homogenate. It might be possible that the protein

fraction 1-L (if 1-O is the antigenic protein) of gland 1 does not create an immune response in the guinea-pig. Using similar assumptions as outlined above and comparison of electropherograms, it would seem that the antigen common to secretions of glands 3,4,5, and 6 is the protein band 3-J (Figure 3), 4-J, 5-J and 6-F (Figure 4) of the respective glands.

By comparing various electrophorograms, a number of protein fractions were found common to some of the gland secretions, fat body and the haemolymph (Tables 1-3). An example of such proteins is protein bands 1-L (Figure 3), 6-F (Figure 4),Ho-N (Figure 5), fb-G (Figure 6) and He-F (Figure 7). Using immunochemical and radio-tracer techniques, Friedel and Gillott, (1976) demonstrated that in M. sanguinipes the fat body synthesizes certain proteins, which are released into the haemolymph, and are subsequently accumulated by the ARG. Proteins that are common to fat body, haemolymph and gland(s) secretion could represent a synthesis-release-and uptake pathway of proteins.

Free amino acid analysis of methanolic extracts of gland secretions, fat body and haemolymph revealed 18 amino acids besides ethanolamine in secretion of gland 12 and the haemolymph. The amino acids of quantitative importance (as indicated by the intensity of ninhydrin reaction and size of the spot) were

α-alanine, glycine, methionine, phenylalanine, serine and aspartic (Table 4). Of these, α-alanine was present in maximum concentration in secretions of glands 3,5,6,1 and 2 and in trace amounts in the rest of the samples.

CHAPTER IV

FREE SUGAR ANALYSES

4.1 Paper Chromatography

Free sugars from 80% methanolic extracts of the various gland secretions, paragonadal fat body and the haemolymph were detected on chromatograms using alkaline silver nitrate and aniline phthalate reagents.

The free sugar separation of methanolic extracts of the gland secretions and fat body is represented in Figure 13. Spots corresponding to trehalose and/or inositol, fructose and an unidentified substance in the region of amino sugars were present in all samples. The unidentified substance did not react with alkaline silver nitrate, but left a white spot. However, there was a light brown reaction area around the white spot. This seemed to indicate that an amino sugar was also present although partially masked by the unidentified spot. Spots corresponding to the sucrose standard were present in methanolic extracts of gland 11 and in seminal vesicle scretions, while trace amounts of sucrose were also present in the secretions of glands 3 and 4.

FIGURE 13

Diagrammatic representation of a descending paper chromatographic separation of free sugars separated from 80% methanolic extracts of the secretions from glands 1-6, 11, the homogenous glands (Ho), the seminal vesicle (SV) and the fat body (fb) and detected with alkaline silver nitrate:

Solvent system, n-Butanol-acetic acid-water (4:1:1 v/v/v/v)

Running time, 18 hours

Key to sugars :

Am Amino sugar (Glucuronic acid)

C Galactose

E Ribose

F Fructose

In Inositol

L Lactose

Mix Standard sugar mixture

S Sucrose

T Trehalose

W Unidentified "white" spot

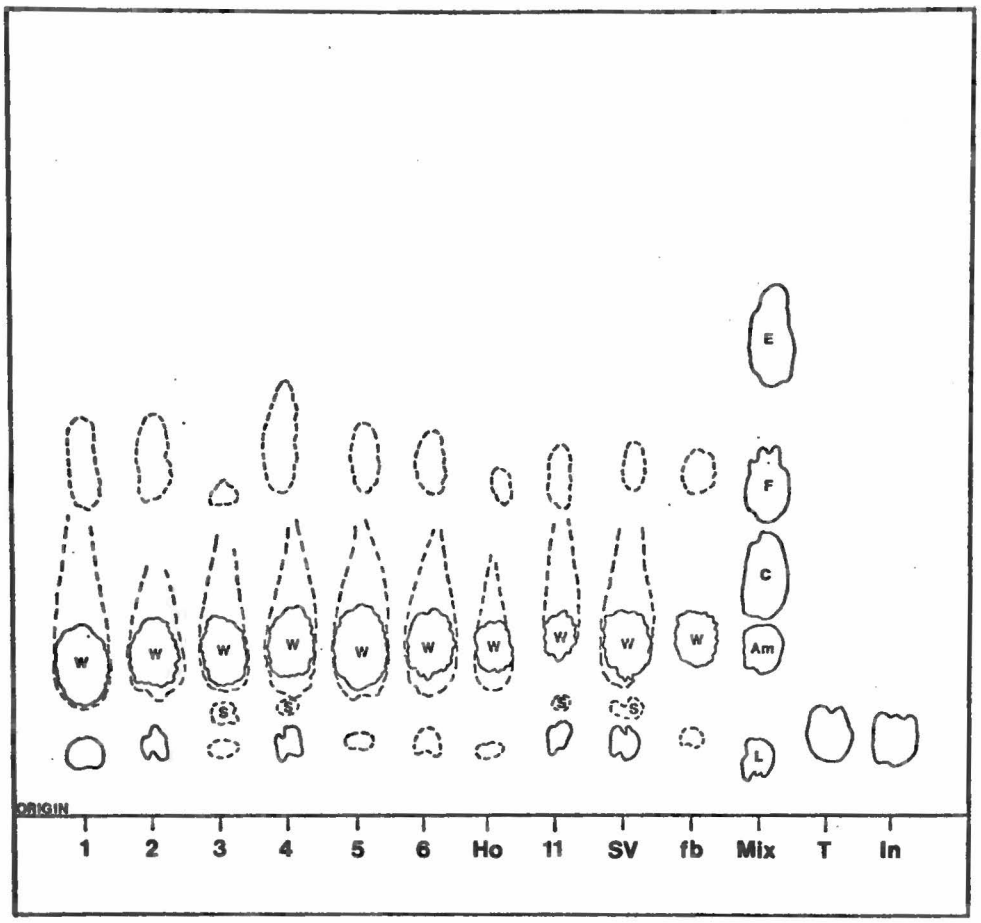


FIGURE 13

Spots corresponding to fructose, trehalose and/or inositol did not appear when the chromatograms were developed in aniline phthalate reagent (Figure 14). This reagent has a lower sensitivity than alkaline $AgNO_3$ and even the trehalose and inositol standard did not stain with this technique. Once again the unidentified substance appeared as an unreacted spot in the migration zone of amino sugars on chromatograms treated with aniline phthalate.

Methanolic extracts of the gland 12 secretion revealed free sugar spots corresponding to trehalose, an amino sugar and glucose on chromatograms developed in alkaline $AgNO_3$. Once again an unreacted white spot appeared in the migration region of amino sugars. Chromatography of the haemolymph yielded 6 spots. In addition to spots for glucose, galactosamine, sucrose, trehalose and/or inositol, two other unidentified spots appeared. One of the latter was near the site of sample application and it reacted with alkaline $AgNO_3$. An unreacted white spot containing the unknown substances was surrounded by a reaction spot which corresponded to trehalose/inositol.

When the chromatograms were developed in 0.2% ninhydrin in acetone "white unreacted spots" still appeared in the region of amino sugars just as they had appeared on chromatograms treated

FIGURE 14

Diagrammatic representation of a descending paper chromatographic separation of free sugars separated from 80% methanolic extracts of the secretions from glands 1-6, 11, the homogenous glands (Ho), the seminal vesicle (SV) and the fat body (fb) and detected with aniline phthalate reagent;

Solvent system, n-Butanol-acetic acid-water (4:1:1 v/v/v)

Running time, 18 hours

Key to sugars :

Am Amino sugar (Glucuronic acid)

Ar Arabinose

C Galactose

E Ribose

F Fructose

L Lactose

Mix Standard sugar mixture

S Sucrose

W Unidentified "white" spot

Xy Xylose

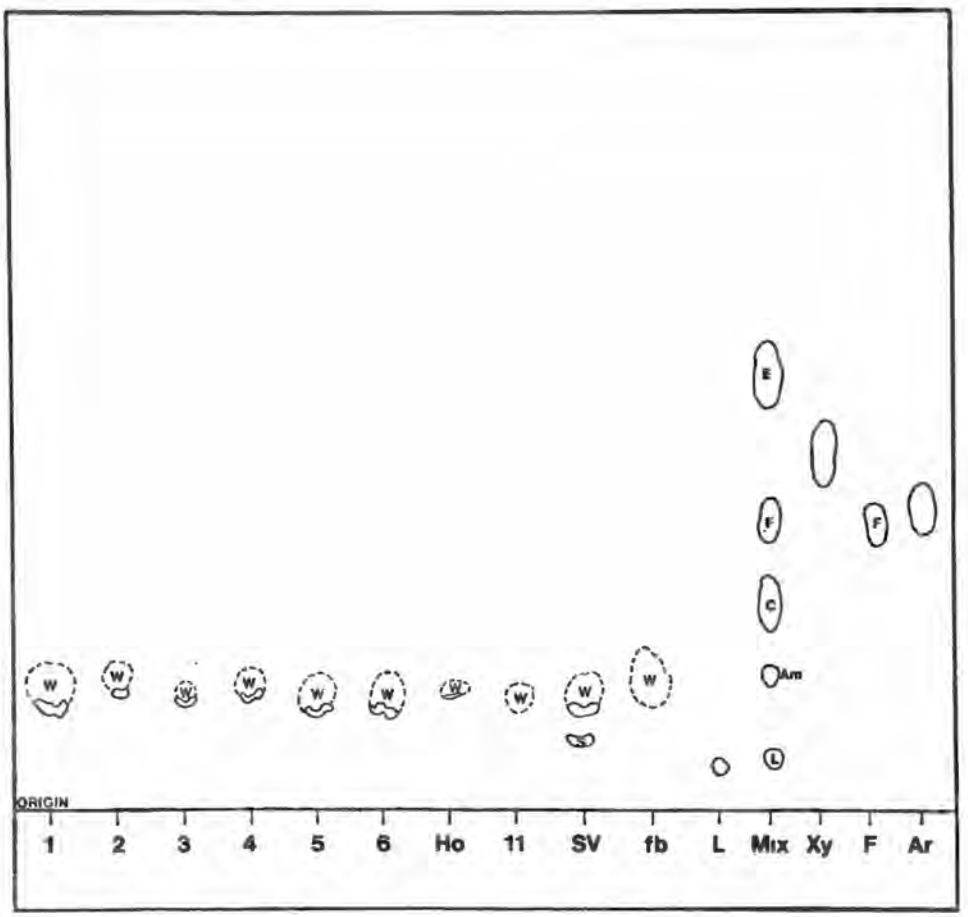


FIGURE 14

with alkaline $AgNO_3$ or aniline phthalate reagents. The "white spots" arising from the secretions of glands 3,5,6, the homogenous glands and the seminal vesicle gave only faint ninhydrin positive reactions. However, the pink colour resulting from the ninhydrin reaction was concentrated around the "white spots" indicating the presence of amino sugars.

4.2 Spot Tests with the Unidentified "White Spot"

The unknown substance from the "white spots" was eluted and concentrated as described in Chapter 2.4.2. The unknown was then subjected to analysis with several spot tests. The lack of reaction with phosphomolybdic reagent (Merck, 1967) indicated the absence of phosphates. The presence of an amino group was indicated by a faint positive reaction with ninhydrin. Several tests were performed for the presence of purines and/or pyrimidines in the unknown.

4.2.1 Eosine - Mercury Stain for Purines

The unknown compound was applied on an activated silica gel 'G' coated glass plate and found to be UV active (fluoresced under UV light). Adenosine standards and a methanolic extract of tick excreta, which contains guanine

(Balashov, 1959; Hamdy, 1972), were also UV active. When the spotted plate was sprayed with eosine-mercury stain the unknown did not have an initial fluorescence of the same relative intensity as the adenosine standard. After 24 hours, however, the remaining fluorescence of the unknown was more intense than that of the standard. The colour of the adenosine spot was uniformly red-violet, but only the border of the unknown spot was red-violet.

4.2.2 Silver Nitrate - Dichromate Stain for Purines

The adenosine standard spot reacted with the silver nitrate-dichromate stain to yield an initial red colour, but the extract of the tick excreta and the unknown gave no colour reaction. Overnight, the red colour from the adenosine turned yellow in the centre of the spot. On the other hand, the unknown compound had turned red while the tick excreta was white in the centre.

4.2.3 Wood's Reagent for Purines

Wood's reagent gave strong positive reactions (blue colour) with ATP and adenosine. Faint reactions were obtained with the unknown compound and with tick excreta. Uridine tri-phosphate and CTP gave faintly

positive reactions in the centre of the spotted areas but stained white on the periphery.

4.2.4 Mercury Nitrate - Ammonium Sulphide Reagent for Purines and Pyrimidines

Adenosine, UTP and ATP gave positive reactions (black spots) with mercury nitrate-ammonium sulphide reagent, but the unknown compound gave only a faint reaction.

4.2.5 Sakaguchi Test

The Sakaguchi test for arginine and guanidines resulted in a positive orange coloured reaction with arginine standard. The methanolic extract of tick excreta gave a faintly positive reaction, while the unknown gave a transient positive reaction.

4.3 Conclusions

Differences in the chemical nature of the ARGs of male S. gregaria are reflected in the distribution of free sugars in the secretions of the glands. These differences, however, are not as marked as those obtained from the protein analyses (Chapter 3).

Secretions from all the glands revealed the presence of compounds with Rf values similar to trehalose and/or inositol, an amino sugar and an unidentified compound. In the solvent system used (n-butanol - acetic acid - water, 4:1:1 v/v/v) 3 amino sugars standards (glucosamine, galactosamine and glucuronic acid) were not well separated, even when the migration time was prolonged to 39 hours. Moreover, the presence of the unknown compound in an unreacted area in the same region completely obscured the identification of the amino sugars. The evidence for the presence of an amino sugar lies in the appearance of a brown or reddish brown reaction area circling the unreacted white spot when the chromatograms are developed with alkaline $AgNO_3$ or aniline phthalate reagents. This evidence is supported by the faint pink reactions around the unreacted white spots which were obtained with the ninhydrin reaction. Furthermore, the amino sugar, galactosamine could be identified in extracts of the haemolymph.

Fructose was identified in the secretions of glands 1,2,3, 4,5,6,11, in the homogenous glands, the seminal vesicle and in the fat body. Sucrose was demonstrated in the methanolic extracts of the secretions of 4 glands (glands 3,4,11 and the seminal vesicle) and the haemolymph. Glucose was confined to gland 12 and the haemolymph.

Methanolic extracts of the secretions of the ARGs, fat body and the haemolymph contained a compound which could not be identified with the alkaline $AgNO_3$ or aniline phthalate reagents. Spot tests on concentrated eluants of the unidentified compound support the conclusion that it is a purine derivative. The transient positive reaction of the unknown with the Sakaguchi test gives further support since taurocyamine and glycocyamine (guanine derivative) give transient positive reactions with this test (cited by Dawson et al., 1972).

This is the first time that the secretions of the various ARGs of the desert locust have been analysed for free sugars. The presence of a purine like compound has never been demonstrated.

CHAPTER V

LIPID AND PHOSPHOLIPID COMPOSITION

5.1 Neutral Lipid Analysis

A typical thin-layer chromatographic separation of the neutral lipids from chloroform-methanol (2:1 v/v) extracts of the various gland secretions and the fat body is represented in Figure 15, and the results of all the separations are tabulated in Table 5. Six types of neutral lipids were identified in the various gland secretions. An unidentified spot was present on the chromatogram (Figure 15) for secretions of glands 1,2,5,6,11, the seminal vesicle and the fat body. This unidentified compound migrated with a Rf value of 0.65 and appeared just behind the spot for triglycerides.

The most striking feature of gland 4 secretions was the absence of all classes of neutral lipids (Figure 15; Table 5). Secretions of gland 6 contained 6 types of neutral lipids and an unidentified component. In ascending order these included a monoglyceride (α-monopalmitin), a sterol (cholesterol), a diglyceride (dipalmitin), a fatty acid (palmitic acid), the unidentified compound, a triglyceride (tripalmitin) and a sterol ester (cholesterol oleate). Gland 5 secretions were separated into spots corresponding

FIGURE 15

Diagrammatic representation of a thin-layer chromatographic separation of the neutral lipids from chloroform-methanol (2:1 v/v) extracts of secretions from glands 1-6, 11, the homogenous glands (Ho), the seminal vesicle (SV) and the paragonadal fat body (fb), detected with iodine vapour;

Solvent system, Petroleum ether (b.pt. 60-80^{o}C) - diethyl ether-acetic acid (60:40:1 v/v/v)

Running time, 25 minutes for 15 cms

Key to neutral lipids :

CE Cholesterol oleate (Sterol ester)

Ch Cholesterol (Sterol)

DG Dipalmitin (diglyceride)

Fa Palmitic acid (fatty acid)

MG α-Monopalmitin (monoglyceride)

TG Tripalmitin (triglyceride)

U Unidentified compound

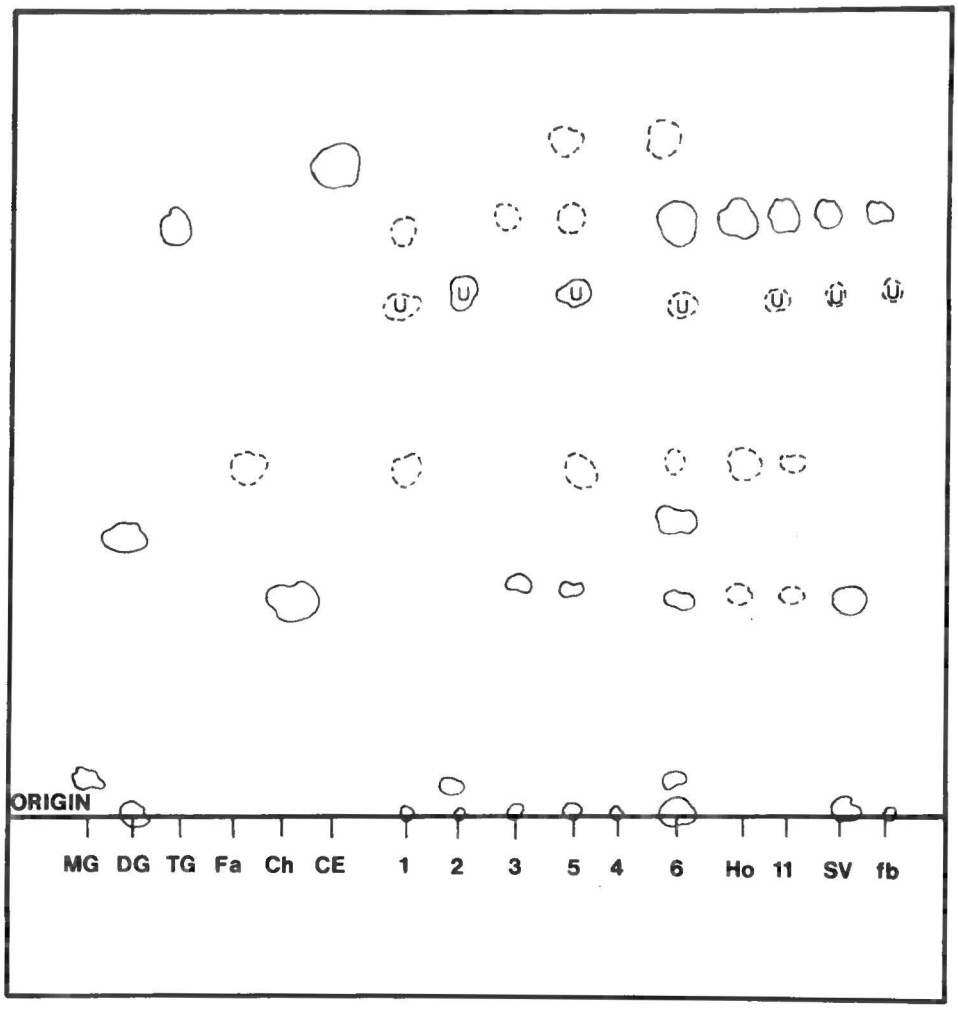


FIGURE 15

Table 5. Neutral lipid composition of the accessory reproductive gland secretions, fat body and the haemolymph of 19 day old adult male S. gregaria

	NEUTRAL LIPIDS						
	Monoglyceride	Sterol	Diglyceride	Fatty acid	Triglyceride	Sterol ester	Unidentified
SAMPLES							
Gland 1				O	O		O
Gland 2	●						●
Gland 3		●●			O		
Gland 4							
Gland 5		●●		O	O	O	●
Gland 6	●	●●	●●	O	●●	O	O
Homogenous glands		O		O	●●		
Gland 11		O		O	●●		O
Gland 12	●				●		
Seminal vesicle		●●			●●		O
Paragonadal fat body					●●●		
Haemolymph	●●	●●	●	O	●	●	

Notes: The closed and open circles represent varying intensities of reaction with iodine vapour;

●●● – Very strong

●● – Strong

● – Moderate

O – Weak

to a sterol, a fatty acid, the unidentified compound, triglyceride and a sterol ester, but secretions of gland 3 gave two spots, one corresponding to a sterol and the other to a triglyceride (Figure 15; Table 5). The secretions of glands 6,11,12, homogenous glands, seminal vesicle and the fat body were rich in triglycerides as indicated by the intensity of the iodine reaction. Haemolymph extracts contained all classes of neutral lipids, but the unidentified compound was absent.

5.2 Phospholipid Composition

The phospholipid composition of the various samples has been presented in Table 6. The secretions of ARGs contained trace amounts of phospholipids but sphingomyelin and lysolecithin were absent in all secretions. Figure 16 illustrates the separation of phospholipid compounds from individual gland secretions and the paragonadal fat body. A phospholipid corresponding to the phosphatidylethanolamine standard was present in all secretions except those of glands 1 and 2 and the paragonadal fat body. Spots with an Rf value similar to phosphatidylserine were present in secretions of glands 2,3 and 4. Secretions from all glands except 2 and 3 contained trace amounts of phosphatidylcholine. Phosphatidylinositol was demonstrated in the secretions of glands 2,5,6,12, the homogenous glands and in the fat body. A compound migrating behind

Table 6. Phospholipid composition of the accessory reproductive gland secretions, fat body and the haemolymph of 19 day old adult male S. gregaria

	PHOSPHOLIPIDS						
	Phosphatidyl – ethanolamine	Phosphatidylserine	Phosphatidylinositol	Phosphatidylcholine	Sphingomyelin	Lysolecithin	Unidentified
SAMPLES							
Gland 1				O			
Gland 2		O	●				
Gland 3	O	O					
Gland 4	O	O		O			O
Gland 5	O		O	●			
Gland 6	O		O	O			
Homogenous glands	O		O	O			
Gland 11	O			O			
Gland 12	O		O	O			
Seminal vesicle	O			O			
Paragonadal fat body			O	O			
Haemolymph	●			●			

Notes: The closed and open circles represent varying reaction intensities with iodine vapour;

● - Moderate

O - Weak

FIGURE 16

Diagrammatic representation of a thin-layer chromatographic separation of phospholipids from chloroform-methanol (2:1 v/v) extracts of the secretions from glands 1-6, 11, the homogenous glands (Ho), the seminal vesicle (SV) and the paragonadal fat body (fb), detected with iodine vapour;

Solvent system, Chloroform-methanol-acetic acid-water (60:10:3.5 v/v/v/v);

Running time, 45 minutes for 15 cms

Key to phospholipids :

A Phosphatidylethanolamine

B Phosphatidylserine

C Phosphatidylinositol

D Phosphatidylcholine

E Sphingomyelin

F Lysolecithin

U Unidentified compound

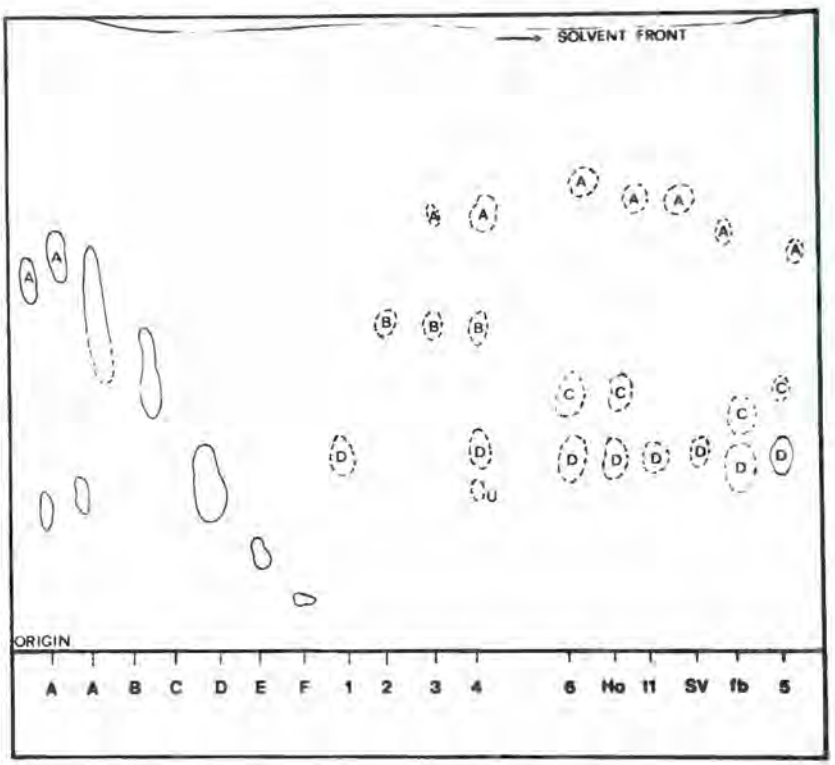


FIGURE 16

spots corresponding to lysolecithin was present in the extracts of gland 4 secretion. This compound could have been a breakdown product of one of the phospholipids.

5.3 Conclusions

As a class, neutral lipids and phospholipids were found to be common components in the secretions from most glands, although neutral lipids were not present in the secretion of gland 4. While definite differences were found in the neutral lipid composition of the various glands, close similarities were obtained in the secretions of glands 5,6,11, homogenous glands and the seminal vesicle. The phospholipid composition of the various ARG secretions was less variable. Secretions of glands 1,2,3,4,11 and the seminal vesicle revealed definite differences, but the secretions of glands 5,6,12 and the homogenous glands were strikingly similar in their phospholipid composition.

CHAPTER VI

ENZYME STUDIES

Alkaline phosphatases, malic dehydrogenases and non-specific esterases were present in high concentrations in the electrophoresed secretions of all glands. Lower concentrations of acid phosphatases, malic enzyme and α-glycerophosphate dehydrogenase were observed in some of the gland secretions, but isocitrate dehydrogenase was not detected in any of the samples. Xanthine dehydrogenase was detected as a single band in the paragonadal fat body.

6.1 Alkaline Phosphatase (AlPase)

A graphic representation of the AlPase distribution in the various samples used is presented in Figure 17. Secretions of the first six glands were rich in AlPase, although the relative concentration was lower in gland 2. Alkaline phosphatase concentrations in the homogenous glands, glands 11 and 12, the paragonadal fat body and the haemolymph were low. Alkaline phosphatase activity could not be detected in the functional seminal vesicle. The staining reaction with the secretions of first 6 glands was so intense that the reaction had to be stopped within one hour. The rest of the samples were left overnight in the reaction mixture

FIGURE 17

Diagrammatic representation of alkaline phosphatase zymograms of secretions from the accessory reproductive glands, washings of the paragonadal fat body (fb) and the haemolymph (He). X,Y and Z represent the three migration zones of alkaline phosphatase bands. Samples were applied in 20% sucrose (1:1 v/v) on top of the spacer gel and electrophoresed for 40 minutes.

Gels 1-6 were stained for 1 hour with the method of Toledo and Magalhães (1973), while the remaining gels were stained overnight at room temperature

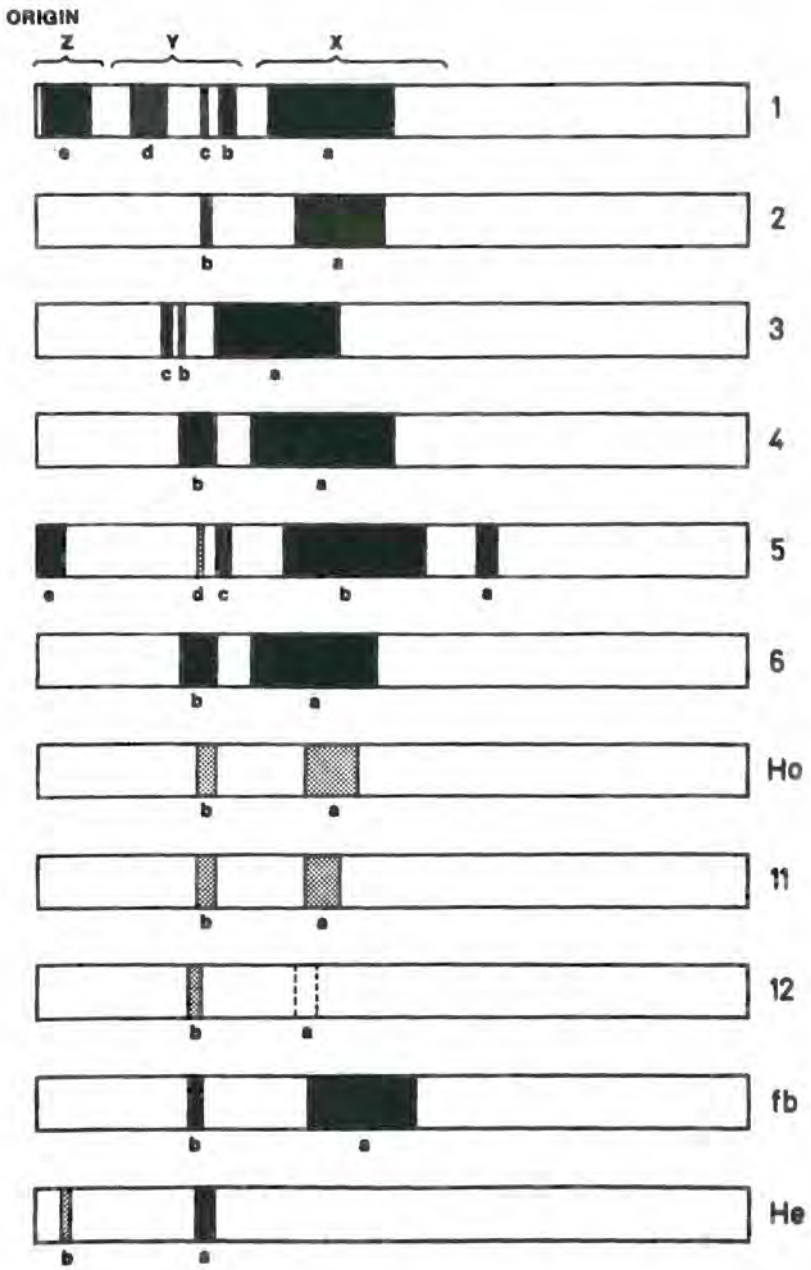

ORIGIN
Z
Y
X
1
e
d
c b
a
2
b
a
3
c b
a
4
b
a
5
e
d c
b
a
6
b
a
Ho
b
a
11
b
a
12
b
a
fb
b
a
He
b
a

FIGURE 17

at 35°C until faint AlPase bands appeared.

Alkaline phosphatase activity was confined to 3 slow mobility zones (X), (Y) and (Z) (Figure 17) on the gels. The secretions of the first 6 glands produced intensely stained bands in zone (X) of the zymograms (Figure 17). A faintly stained band was also produced in zone (X) by secretions of the homogenous glands, glands 11 and 12 and the fat body. Single bands in zone ($\overset{Y}{*}$) were produced by the secretions of glands 2,4,6,11,12, homogenous glands, fat body and the haemolymph. Secretions of glands 1,3 and 5 produced 2 AlPase bands in zone (Y) (Figure 17). An AlPase band was present in zone ($\overset{Z}{*}$) of the zymograms for secretions from glands 1 and 5 and from the haemolymph. An additional AlPase band appeared between zones (Y) and (Z) in the zymograms from the secretions of gland 1.

6.2 Malic Dehydrogenase (MDH)

Two distinct zones of MDH activity were found on gels stained for MDH. Secretions of glands 1-6 produced single MDH bands in zone (U) (Figure 18) but with varying staining intensities and electrophoretic mobilities. Single MDH bands were obtained in zones (X) and (W) from the secretion of gland 3. An additional band between zones (U) and (W) stained intensely and

FIGURE 18

Diagrammatic representation of malic dehydrogenase zymograms of secretions from the accessory reproductive glands, washings of the paragonadal fat body (fb), and the haemolymph (He). Samples were applied in 20% sucrose (1:1 v/v) on top of the spacer gel and electrophoresed for 40 minutes. Gels were stained with the method of Toledo and Magalhães (1973)

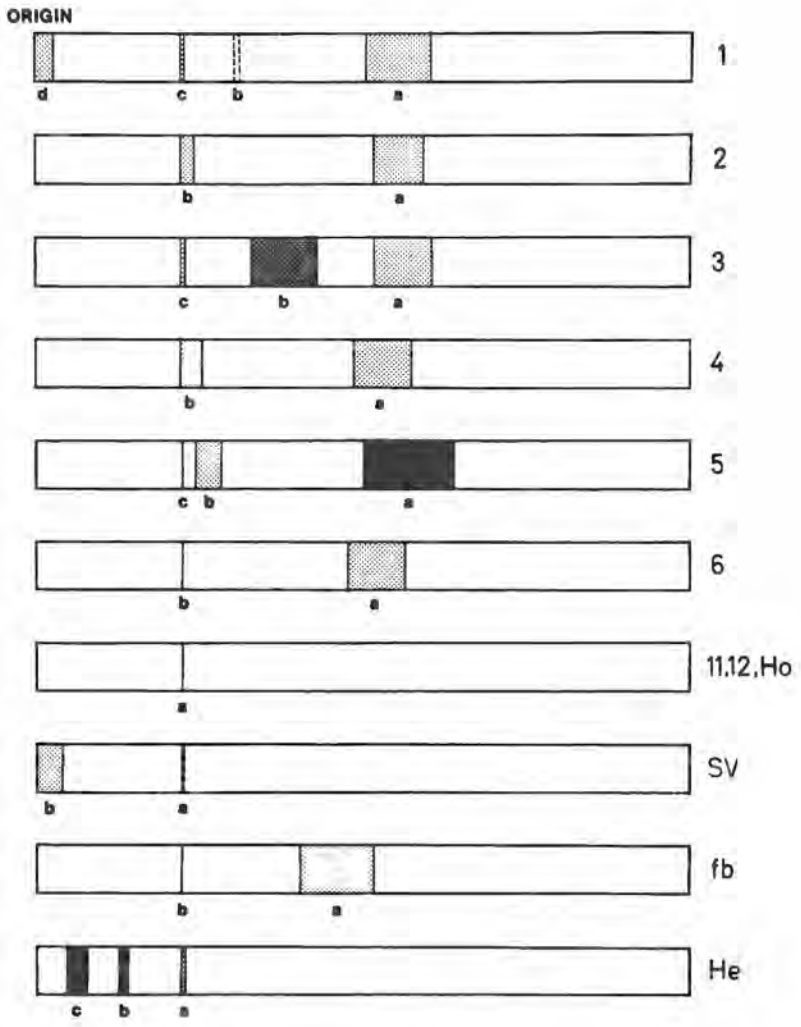


FIGURE 18

was not present in the zymograms of the other glands. A single MDH band in zone (W) (Figure 18) was common to the secretions of glands 1-4, 6-16, the fat body and the haemolymph. The zymogram of gland 5 was distinct due to the presence of two bands in zone (W). Malic dehydrogenase bands were obtained near the origin of the separation gels in secretions from gland 1 and from the seminal vesicle. In addition, from the haemolymph extracts 3 MDH bands were obtained in the slow mobility zone. Malic dehydrogenase activity could not be detected in the secretions of glands 11 and 12 or in the homogenous glands.

6,3 Acid Phosphatases (AcPase)

The secretions of glands 1,3,4,5 and 6 produced single faint bands of AcPase (Figure 19) with similar electrophoretic mobilities. The highest AcPase activity, as indicated by the staining intensity, was observed in the secretions of gland 4. Acid phosphatase activity was not detected in the secretions from glands 2,11,12, the homogenous glands, the seminal vesicle and the fat body. Three AcPase bands were detected in haemolymph samples. One of these bands, He-b (Figure 19) corresponded to the single AcPase bands from glands 1,3,4,5 and 6 secretions.

FIGURE 19

Diagrammatic representation of acid phosphatase zymograms of secretions from accessory reproductive glands 1,3,4,5 and 6 and the haemolymph (He). Samples were applied in 20% sucrose (1:1 v/v) on top of the gel and electrophoresed for 40 minutes. Gels were stained with the method of Terranova and Leopold (1973)

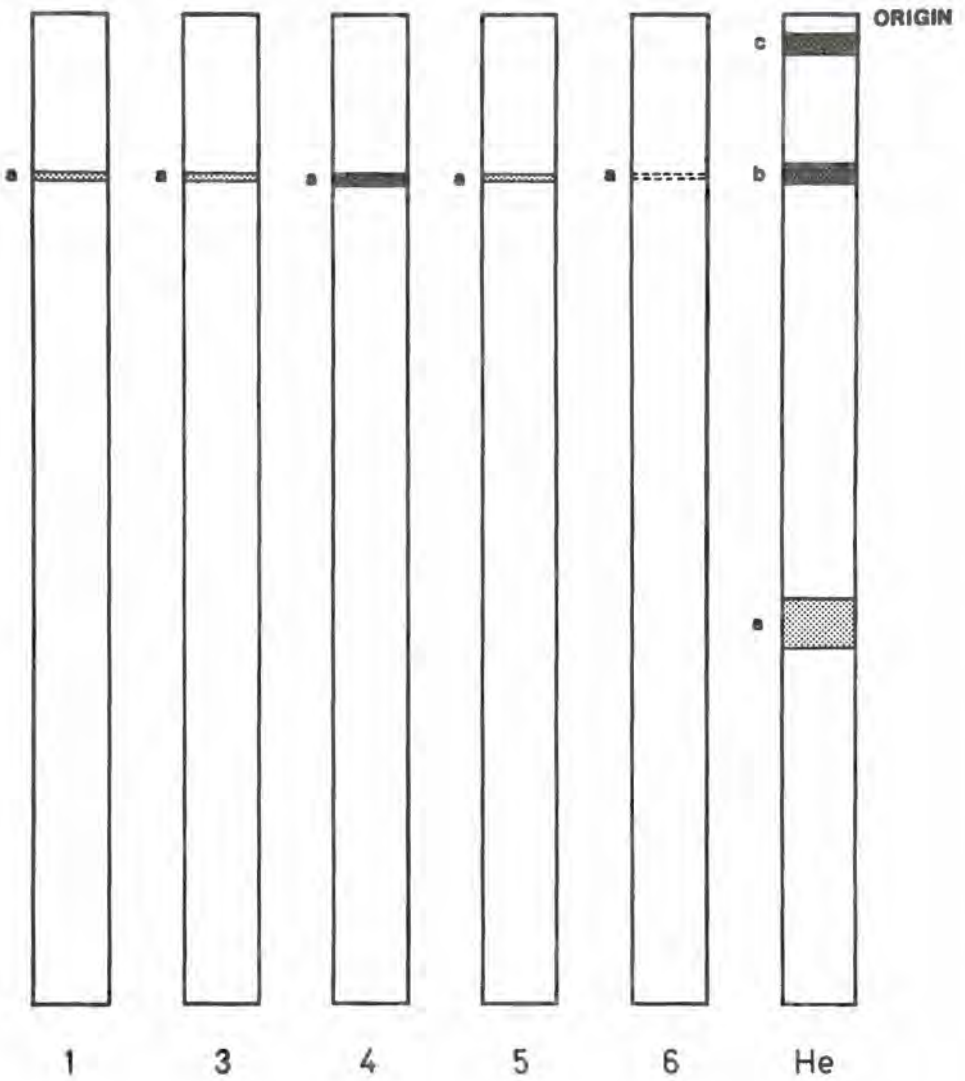


FIGURE 19

6.4 α-Glycerophosphate Dehydrogenase (α-GPDH)

α-Glycerophosphate dehydrogenase activity was demonstrated in the secretions of glands 1,2,4,5,11, the homogenous glands, the seminal vesicle, the fat body and the haemolymph (Figure 20). Secretions from glands 3,6 and 12 did not exhibit any α-GPDH activity. As shown in Figure 20, bands 1-a, 2-b, 4-a, 5-a and SV-a have similar mobilities. Band fb-a from the fat body and band 2-a from gland 2 had slightly higher, but different, electrophoretic mobilities. α-Glycerophosphate dehydrogenase bands were obtained at the origin of the separation gel for the secretions of glands 1,2,4,5, the homgenous glands and the fat body. However, these bands stained with varying intensities for the different gland preparations. Four α-GPDH bands were obtained from the haemolymph. The haemolymph band He-b appeared to correspond to band 1-b, but stained more intensely.

6.5 Malic Enzyme (ME), Xanthine Dehydrogenase (XDH) and Isocitrate Dehydrogenase (IDH)

Malic enzyme activity could not be detected in any of the gland secretions, but 2 ME bands appeared in the haemolymph. Both of these bands were near the origin of the separation (Figure 21).

FIGURE 20

Diagrammatic representation of α-glycerophosphate dehydrogenase zymograms of the secretions from accessory reproductive glands 1,2,4,5,11, the homogenous glands (Ho), the seminal vesicle (SV), and the washings from the paragonadal fat body (fb) and the haemolymph (He). Samples were applied in 20% sucrose (1:1 v/v) on top of the spacer gel and electrophoresed for 40 minutes. Gels were stained with the method of Toledo and Magalhães (1973)

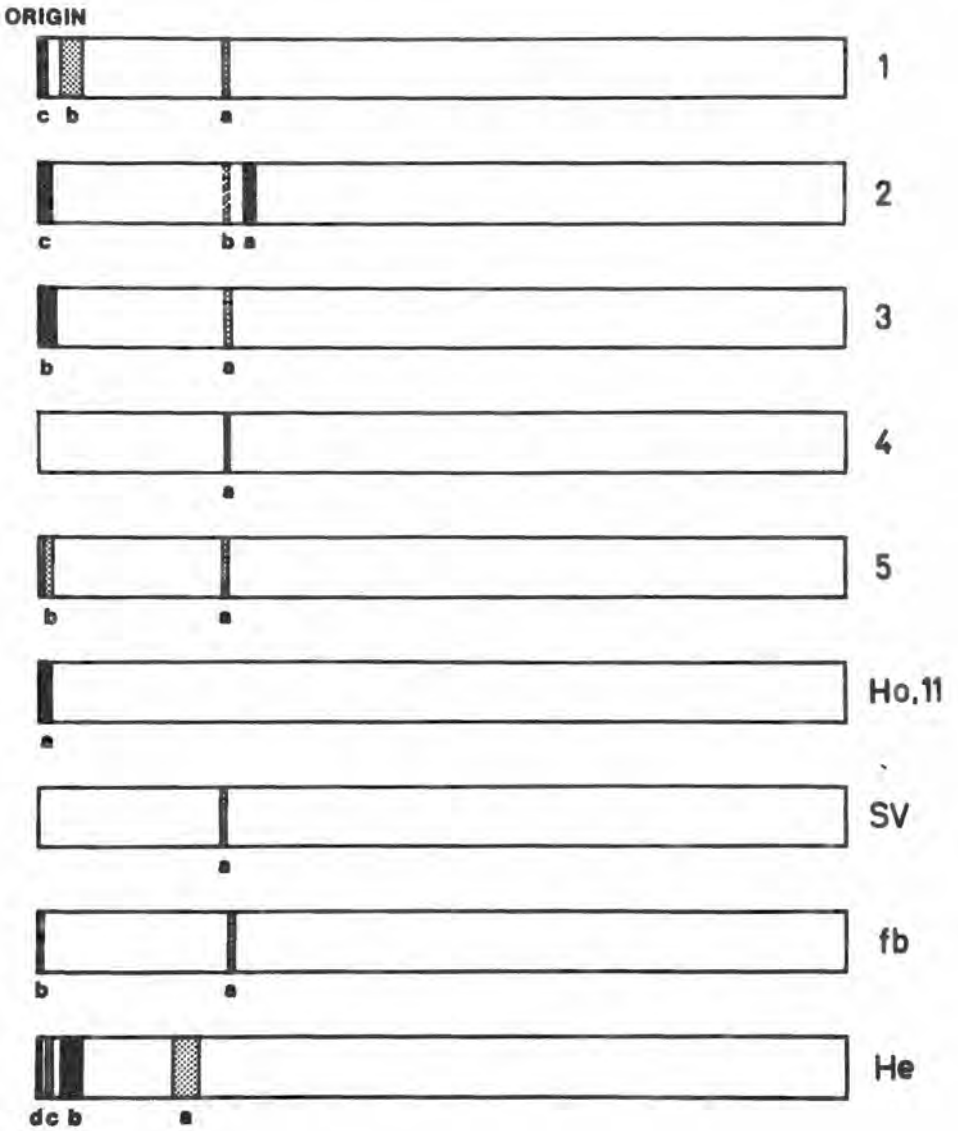


FIGURE 20

FIGURE 21

Malic enzyme (ME) and Xanthine dehydrogenase (XDH) zymograms from the samples of the haemolymph (He) and washings of the paragonadal fat body (fb) respectively. Samples were applied in 20% sucrose (1:1 v/v) on top of the spacer gel and electrohoresed for 40 minutes. Gels were stained with the method of Toledo and Magalhães (1973)

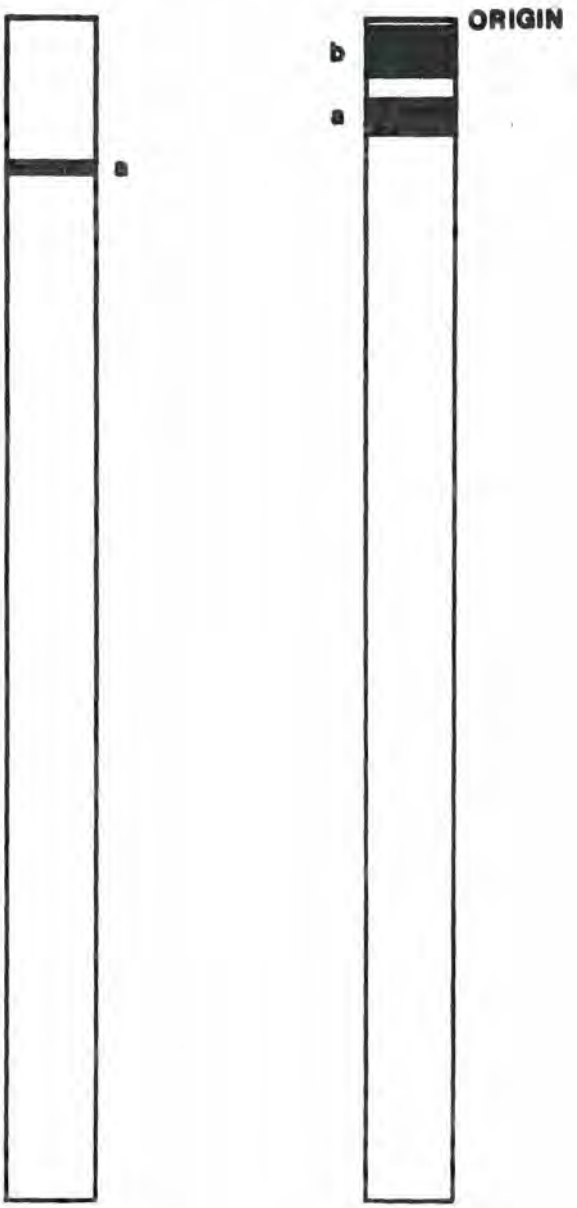


FIGURE 21

Xanthine dehydrogenase could not be demonstrated in the gland secretions or in the haemolymph. However, a single band was obtained from the fat body washings (Figure 21).

Iso-citrate dehydrogenase could not be detected in any of the preparations.

6.6 Non-specific Esterases

Four distinct zones of esterase activity were demonstrated in the zymograms of the various gland secretions, the fat body and the haemolymph. These zones are designated as (Q), (R), (S) and (T) in Figure 22.

Gland 5 secretions showed the maximum number of esterase bands. All zymograms had a single esterase band in zone (Q) (Figure 22), but none of the bands had similar electrophoretic mobilities. A single band was demonstrated in zone (R) for the secretions of glands 2,4,6,11,12 and the homogenous glands (Figure 22), while multiple bands were obtained in zone (R) for secretions from glands 1, 4 and 5. No bands were present in zone (R) for the secretions of gland 3 and the seminal vesicle.

One or two esterase bands were present in zone (S) of the

FIGURE 22

Diagrammatic representation of non-specific esterase zymograms of secretions from accessory reproductive glands 1,2,3,4,5,6,11,12, the homogenous glands (Ho), the seminal vesicle (SV),washings of the paragonadal fat body (fb) and the haemolymph (He). Q,R,S and T represent the 4 migration zones of non-specific esterase bands. Samples were polymerized in the large pore sample gels and electrophoresed for 35 minutes. Gels were stained with the method of Whitemore and Gilbert (1974)

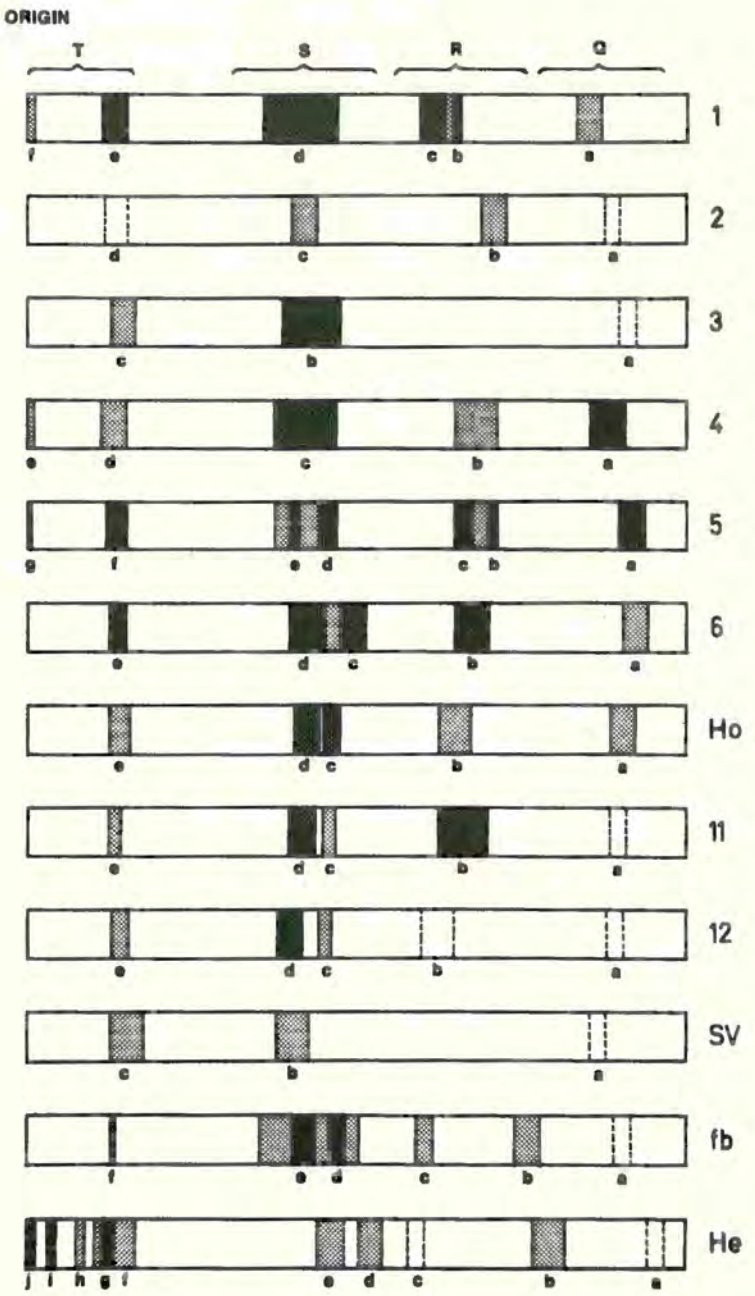


FIGURE 22

zymograms from all preparations. These bands stained intensely in all cases. Zone (T) contained a single esterase band in all the samples.

The paragonadal fat body and the haemolymph produced distinctly different zymogram patterns. Six esterase bands were present in the paragonadal fat body, but 10 bands were obtained from the haemolymph. Bands fb-b, He-b and fb-c, He-c appeared between zones (Q) and (R) and between zones (R) and (S), respectively. Two bands appeared in zone (S) of both zymograms. A narrow band appeared in zone (T) of the fat body preparation, while 5 esterase bands appeared in the same zone of the haemolymph zymogram. Band He-g appeared to correspond to the single bands in zone (T) of the zymograms of most of the gland secretions.

6.7 Comparison of Zymograms and Protein Electropherograms

The gland secretions and preparation of the fat body and haemolymph were electrophoresed under the same condition as those used for obtaining enzyme preparations but were stained with CBB-G. The protein patterns, thus obtained (Figure 23) were compared with the various zymogram patterns (Figures 17-21).

6.7.1 Similarities Between Protein and Alkaline Phosphatase Patterns

The similarities between the electrophoretic mobilities of AlPase and protein bands have been summarised in Table 7. For all the samples, the AlPase bands (Figure 17) corresponded to one or more protein bands (Figure 23). Bands 1-a, c and e (Figure 17) in the AlPase zymogram of gland 1 secretion appeared to correspond respectively to protein bands 1-13, 14 and 17 (Figure 23 of the same gland). Occasionally a single AlPase band was located in the region of 2 to 3 protein bands. For example, AlPase band 2-a appeared in the region of protein bands 2-12 and 13. Similarly AlPase band 3-a appeared in the region of protein bands 3-10, 11 and 12.

6.7.2 Similarities Between Protein and MDH Patterns

Malic dehydrogenase bands 1-a and 1-c from the zymograms of glands 1 secretion (Figure 18) corresponded to protein bands 1-12 and 1-15, respectively (Figure 23). Similarly MDH bands 2-a and 2-b from gland 2 secretions and bands 4-a and 4-b from gland 4 secretions

Table 7. Comparison of alkaline phosphatase and protein patterns

GLAND TYPE	AlPase BANDS*	PROTEIN BANDS**
1	a	13
	c	14
	e	17
2	a	12,13
3	a	10,11,12
	c	13
4	a	16,17
	b	18
5	b	11,12
6	a	14
	b	15
Homogenous glands	b	11
11	a	7
	b	8
12	b	8
Paragonadal fat body	a	6
	b	7
Haemolymph	b	11

Notes : * Refer to FIGURE 17

** Refer to FIGURE 23

corresponded to the protein bands 2-10 and 2-16, and 4-15 and 4-18, respectively (Figure 23). Malic dehydrogenase bands 3-b and 5-a corresponded to protein bands 3-11 and 5-10, 11 respectively. Malic dehydrogenase bands fb-a and fb-b from the fat body appeared to correspond to protein bands fb-6 and fb-7, respectively.

6.7.3 Similarities Between Protein and AcPase Patterns

The electrophoretic mobilities of AcPase bands from glands 4,5 and 6 (Figure 19) were similar to protein bands 4-18, 5-14 and 6-15 (Figure 23). The bands with AcPase activity in the haemolymph, designated as He-a, b and c (Figure 19), were located in the same region as protein fractions He-6, 10 and 11, respectively (Figure 23).

6.7.4 Similarities Between Protein and α-GPDH Patterns

α-Glycerophosphate dehydrogenase bands 1-a, 2-b and SV-a (Figure 20) corresponded to protein bands 1-15, 2-16 and SV-12, respectively (Figure 23). Two of the α-GPDH bands in the haemolymph, He-a and b, were located in the region of protein bands H-10 and 11, respectively.

FIGURE 23

Diagrammatic representation of the electrophoretic patterns of proteins from secretions of accessory reproductive glands 1,2,3,4,5,6,11,12, the homogenous glands (Ho), and the seminal vesicle (SV), washings of the paragondadal fat body (fb) and the haemolymph (He). Only those protein bands with electrophoretic mobilities in the range of the fastest and slowest moving isozyme bands have been shown. Samples were applied in 20% sucrose (1:1 v/v) on top of the gel and electrophoresed for 40 minutes. Gels were stained with Coomassie brilliant blue-G.

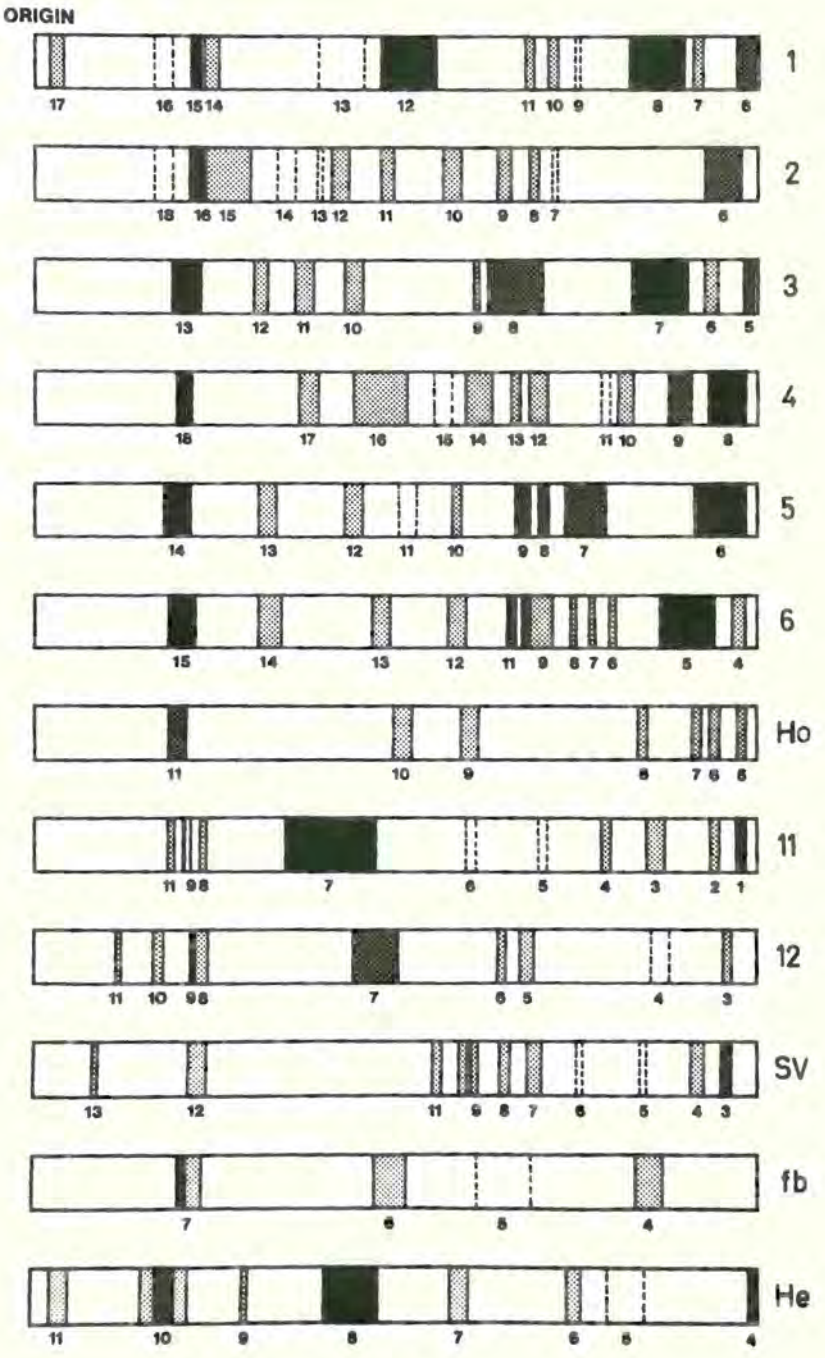

ORIGIN
1
17 16 15 14 13 12 11 10 9 8 7 6
2
18 16 15 14 13 12 11 10 9 8 7 6
3
13 12 11 10 9 8 7 6 5
4
18 17 16 15 14 13 12 11 10 9 8
5
14 13 12 11 10 9 8 7 6
6
15 14 13 12 11 9 8 7 6 5 4
Ho
11 10 9 8 7 6 5
11
11 9 8 7 6 5 4 3 2 1
12
11 10 9 8 7 6 5 4 3
SV
13 12 11 9 8 7 6 5 4 3
fb
7 6 5 4
He
11 10 9 8 7 6 5 4

FIGURE 23

6.7.5 Similarities Between Protein and ME Patterns

Malic enzyme activity was detected only in the haemolymph. Malic enzyme band He-b corresponded to protein band He-11 (Figure 23).

6.7.6 Similarities Between Protein and Non-specific Esterase Patterns

Similarities between the non-specific esterase and protein electrophoretic patterns of the various gland secretions, fat body and the haemolymph have been presented in Table 8. Non-specific esterase zymograms (Figure 22) were compared with the protein electropherograms (Chapter 3, Figures 3-7) since the sample application technique and electrophoresis times were similar in both cases. The secretions of various glands (1,4,5,6 and the homogenous glands), fat body and the haemolymph revealed esterase bands which often corresponded to more than one protein band. For example, the secretions of gland 1 contained non-specific esterase bands 1-c, d and f (Figure 22) which were located in the region of protein bands 1-K, M and N and R, respectively (Figure 3). However, in the zymograms for secretions from glands 2,3 and the seminal vesicle, only

TABLE 8 Comparison of non-specific esterase and protein patterns

GLAND TYPE	ESTERASE BAND*	PROTEIN BAND**
1	c	K
	d	M,N
	f	R
2	c	J
3	b	M
4	a	B,C
	b,c	G
	d	M,N,O,P
5	b	F
	c	H
	e	M
6	a	A
	b	C
	c	G
Homogenous glands	a	c
	b	K
	c	Q
	d	R
11	b	H,G
	d	R
12	d	M
Seminal vesicle	c	R

Notes: *Refer to FIGURE 22

**Refer to FIGURE 3-6

bands 2-c, 3-b and SV-c appeared in the same region as protein bands 2-J, 3-M and SV-R, respectively. There were also non-specific esterase bands which did not correspond to any of the protein bands. The single band in zone (T) of non-specific esterase zymograms from ARGs and the fat body and band He-h (Figure 22) did not correspond to any of the protein bands in electropherograms of the respective samples (Chapter 3; Figures 3-7).

6.8 Conclusions

The secretions of the various ARGs were tested for the presence of 8 enzymes. Three of these enzymes, ME, XDH and IDH, could not be detected in any of the ARG secretions. Semi-quantitative comparisons, based on the staining intensities and placement of bands for AlPase from the electrophoresed secretions, indicated that the first 6 pairs of glands could be distinguished from glands 11 and 12, the homogenous glands and the seminal vesicle. However, these first 6 pairs of glands also differed among themselves. Acid phosphatase was absent from the secretions of gland 2, while secretions of glands 1,3 and 5 contained 2 AlPase bands in zone (Y) (Figure 17). Only the secretions of gland pairs 1 and 5 exhibited AlPase activity near the origin of the separation gel. On the basis of MDH patterns (Figure 18) the secretions of the first 6

glands were quite distinct. Staining of the electrophoresed secretions for α-GPDH also revealed differences among the glands. Secretions of glands 3,6 and 12 showed no detectable α-GPDH activity. Non-specific esterase patterns also revealed differences in the glands. Gland 5 secretions contained the maximum number of non-specific esterase bands. These combined differences in enzyme patterns confirmed the earlier findings (Chapters 3,4 and 5) which showed that the secretions of the various ARGs differed in their chemical composition.

CHAPTER VII

SEQUENCE OF EVENTS DURING SPERMATOPHORE FORMATION

7.1 Events During Sperm Transfer and Description of Spermatophores

During copulation, male desert locusts of S. gregaria produce several relatively simple spermatophores within which the sperm and seminal fluids are transferred to the female. Male migratory locusts, on the other hand transfer a single elaborate spermatophore. When collected from the female ovipositors, the evacuated spermatophore is a transparent structure, consisting of a short thick walled tube and a thin walled bulb (Figure 24). Each spermatophore is approximately 1.5 mm in length and as many as 14 such spermatophores have been recovered after a 4 hour mating period (Pickford and Padgham, 1973). In this study, a maximum of six spermatophores were recovered from the ovipositors of a mated female after a single prolonged mating.

During sperm transfer, the spermatophore tube penetrates a short distance into the spermathecal aperture of the female while the spermatophore bulb remains within the males aedeagus. Sperm and seminal fluid are then "pumped" into the spermathecal duct of the copulating female. After the sperm and seminal fluid have

FIGURE 24

Phase contrast photomicrograph of an evacuated spermatophore collected from the ovipositors of a mated female;

Sp bl, spermatophore bulb; Spm, sperm; Spt, Spermatophore tube

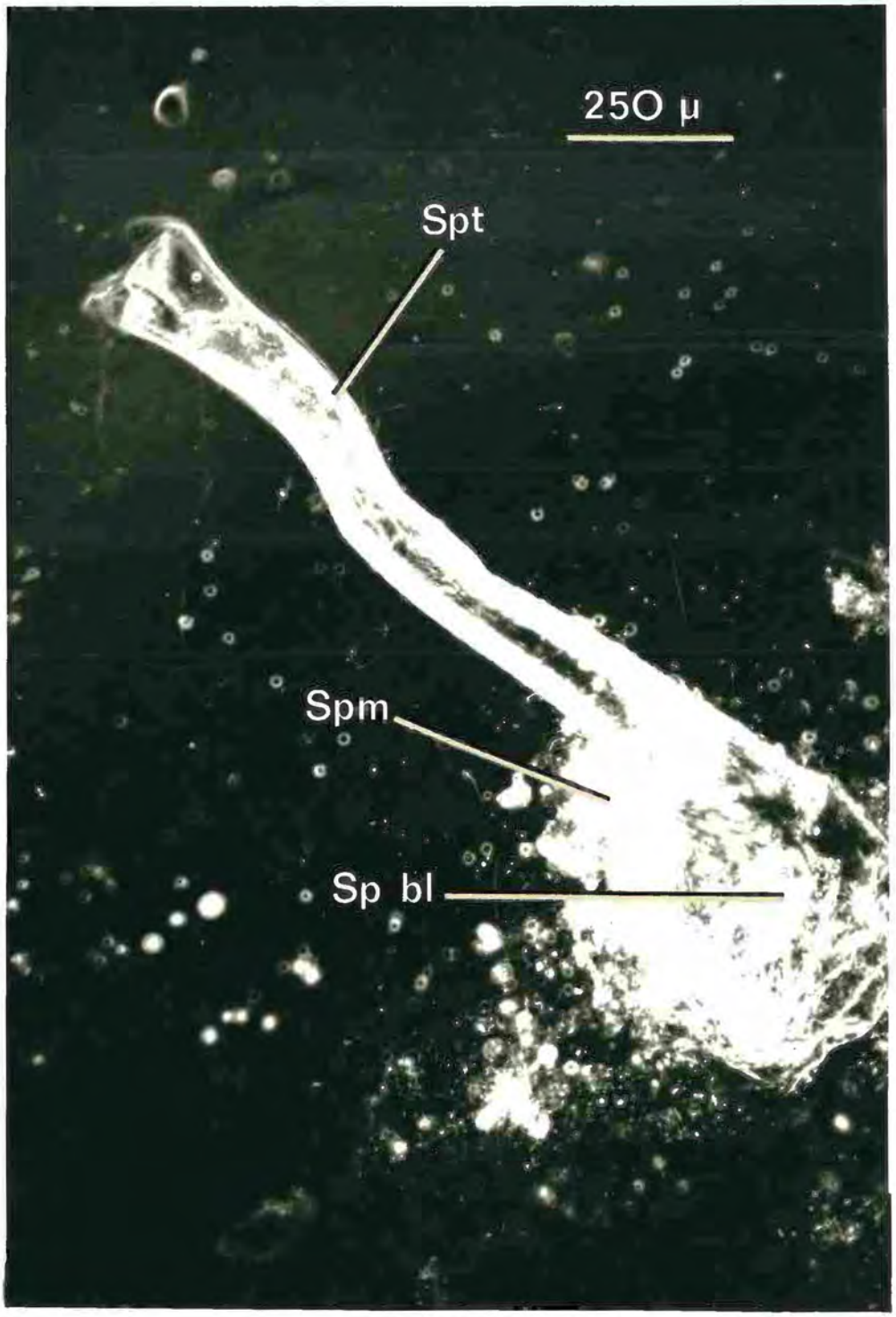


FIGURE 24

been transferred, the evacuated spermatophore and any excess sperm become lodged in the ovipositors of the copulating female. Subsequently the male produces another spermatophore and the processes of evacuation and expulsion are repeated.

In order to study the time required for spermatophore formation and sperm transfer, copulating pairs were interrupted at different periods ranging from 10 to 120 minutes after the onset of copulation.

7.1.1 0-30 Minutes

Spermatophore formation and sperm transfer to the spermathecal duct of the female were observed as early as 10 minutes after the beginning of copulation. By the end of 30 minutes an evacuated and expelled spermatophore was occasionally observed between the female ovipositors. Inactive sperm could be collected from between the ovipositors and/or around the aedeagus on these occasions. When the reproductive system of females were dissected after 30 minutes <u>in copula</u>, sperm were found to have migrated as far as preapical diverticulum of the spermatheca.

7.1.2 30-120 Minutes

A minimum of one and a maximum of two evacuated spermatophores were observed between the ovipositors by end of 60 minutes. Additionally, a third spermatophore was normally being formed in the aedeagus and that spermatophore would be transferred to the female before the end of the second hour in copula.

When unevacuated spermatophores were collected from the aedeagus and observed under a phase-contrast microscope it was observed that the openings of the spermatophore tubes were still sealed (Figure 25). An oily secretion and granular secretions resembling those from gland 11 or 12 (Odhiambo, 1969a) were observed inside the tubes of these spermatophores.

7.2 Electrophoresis of Spermatophores

Spermatophore supernatents or washings were electrophoresed as described in Chapter 2.8 and the gels were stained for proteins and glycoproteins with CBB-G and the PAS reagent. Lipoproteins were not present at detectable levels.

FIGURE 25

Phase contrast photomicrograph of the tip of the tube of an unevacuated spermatophore which was collected from the aedeagus of a copulating male. The secretions (Sc) at the tip of the spermatophore tube (Spt) resemble those of the accessory reproductive glands 11 and/or 12

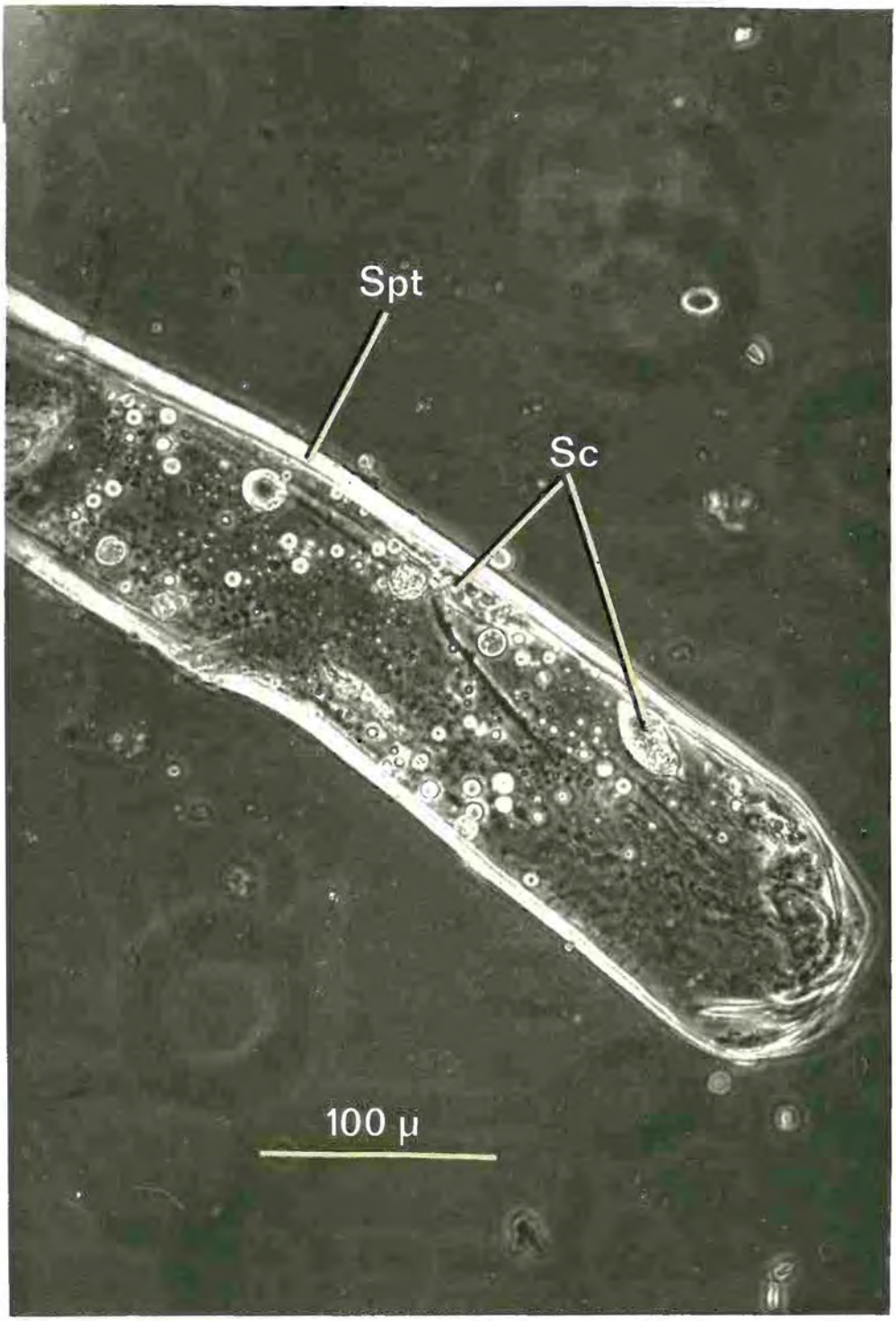


FIGURE 25

The protein and glycoprotein patterns of evacuated spermatophores collected from female ovipositors have been presented in figure 26. Fourteen protein fractions were detected. The protein bands designated as ♀ Sp-F, ♀ Sp-G, ♀ Sp-H and ♀ Sp-I were the major fractions as indicated by the width of the bands and by their staining intensity. The major protein fractions from evacuated spermatophores resembled the major protein fractions from the secretions of gland 5 (Figure 4). Four positive bands were also obtained (Figure 26) from evacuated spermatophores. The migration patterns of PAS-positive bands ♀ Sp-I, ♀ Sp-II, ♀ Sp-III and ♀ Sp-IV were similar to those of the protein bands ♀ Sp-F, ♀ Sp-I, ♀ Sp-K and ♀ Sp-M. These bands may be composed of glycoprotein complexes.

The electrophoresis of unevacuated spermatophores collected from the male aedeagus generated a protein which was different from that of evacuated spermatophores. Twenty protein fractions were obtained from the washings of unevacuated spermatophores (Figure 26). Seven of these were in the fast moving zone (electrophoretic zones were described in chapter 3.1). The major bands, ♂ Sp-C and ♂ Sp-L, were two of the six fractions in the medium zone. Eight protein fractions were present in the slow moving zone and band ♂ Sp-M was the major fraction in that region of the gel. Bands ♂ Sp-N, ♂ Sp-O, ♂ Sp-P and ♂ Sp-R also stained

FIGURE 26

Electrophoretic separation of proteins and glycoproteins from supernatents of homogenized evacuated spermatophores (♀Sp) and washings of unevacuated spermatophores (♂Sp); CBB-G, gels stained for proteins with Coomassie brilliant blue-G; PAS, gels stained for carbohydrates with per-iodic acid - Schiff's reagents

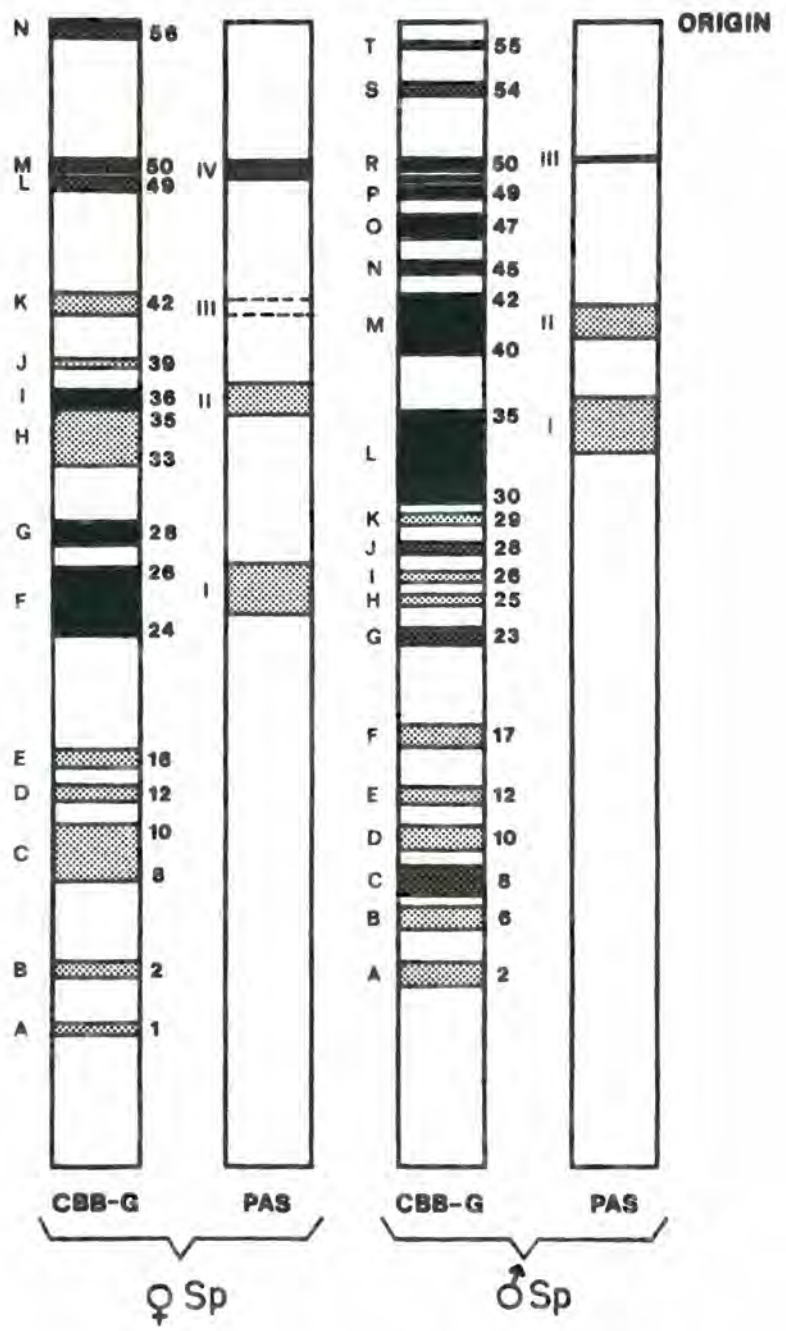

ORIGIN
N
56
M
L
50
49
K
42
J
39
I
36
H
35
33
G
28
F
26
24
E
16
D
12
C
10
8
B
2
A
1
IV
III
II
I
T
55
S
54
R
50
P
49
O
47
N
45
M
42
40
L
35
30
K
29
J
28
I
26
H
25
G
23
F
17
E
12
D
10
C
8
B
6
A
2
III
II
I
CBB-G
PAS
CBB-G
PAS
♀ Sp
♂ Sp

FIGURE 26

intensely with CBB-G. Three PAS-positive bands were obtained from unevacuated spermatophores. Of these, the PAS-positive bands ♂ Sp-I and II were located in the region of protein bands ♂ Sp-L and M, respectively (Figure 26), indicating the glycoprotein nature of these fractions.

7.3 Immunoelectrophoresis

No precipitin arc were obtained when the supernatent of evacuated spermatophores were reacted individually with guinea-pig anti-sera to glands 1,4,11,12 and the homogenous glands or with anti-sera to the whole gland complex (all glands minus seminal vesicle and fat body). Positive reactions were obtained when the electrophoresed washings of unevacuated spermatophores were reacted with the guinea-pig anti-serum to the homogenous glands and to the anti-sera to the whole gland complex (Figure 27). Two close precipitin bands were obtained with the anti-serum to the homogenous glands, one of which was similar to the band obtained from the reaction with anti-serum to the gland complex. Reactions with guinea-pig anti-sera to glands 3,5,6 and 16 could not be performed due to the difficulties mentioned in chapter 3.2.

FIGURE 27

Photographic record of the immunoelectrophoresis of washings of unevacuated spermatophores (♂Sp) and normal guinea-pig serum (N-Gp-S). The antigens were precipitated by the following antibodies :

c Guinea-pig anti-gland 12

d Guinea-pig anti-gland 1

e Guinea-pig anti-ARG mixture serum

f Guinea-pig anti-homogenous glands

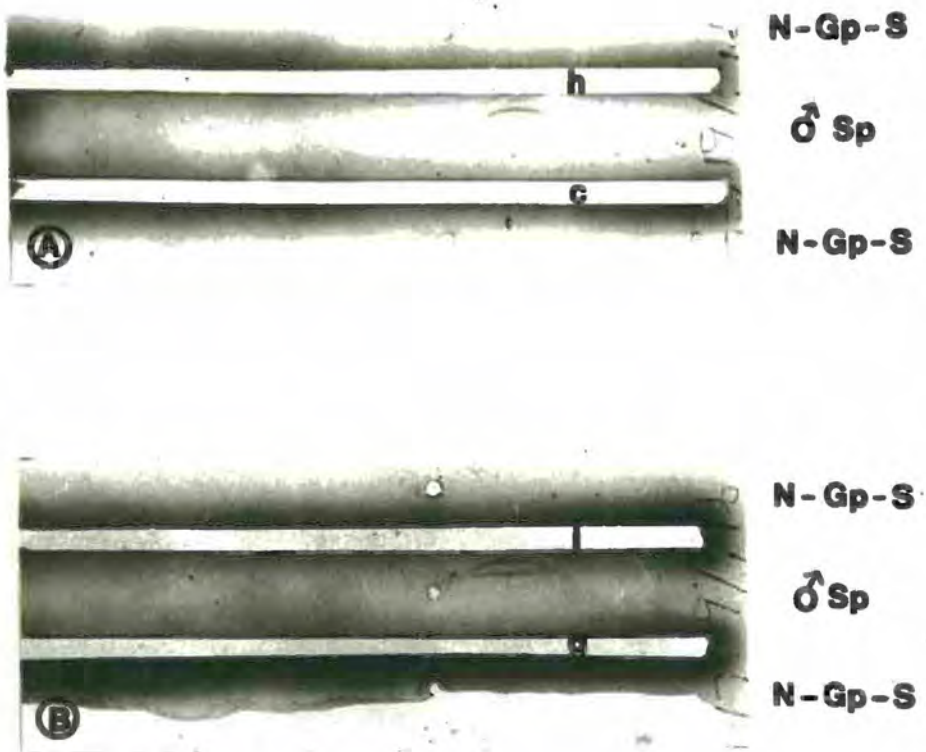


FIGURE 27

7.4 Conclusions

Fourteen protein bands appeared in the electrophoresed supernatents of the evacuated spermatophores which were obtained from females. Four of these bands represented major protein components, two of which (♀ Sp-F and I Figure 26) appeared to be glycoproteins. Twenty protein fractions were stained from the washings of unevacuated spermatophores obtained from males and 7 of these bands represented major components. Two PAS-positive bands (♂ Sp-L and M) appeared to represent glycoprotein complexes.

The electrophoretic patterns of the supernatents from homogenized evacuated spermatophores and the washings from unevacuated spermatophores were quite distinct. They may represent two largely different groups of proteins. The supernatent of the evacuated spermatophores could consist mainly of the polymerized products of the accessory glands which are used for the construction of the spermatophore itself. On the other hand electrophoretic pattern from the washings of unevacuated spermatophores may represent the polymerization products which comprise the seminal fluid component.

The above interpretation is supported by the immunoelectrophoretic analysis. Only one common antigen appears to be present in

the washings of the unevacuated spermatophores and the ARG complex. There were no reactions between the anti-sera to individual ARGs or ARG complex and the supernatent of evacuated spermatophores. The antigenicity of protein secretions of the individual ARGs appears to be highly modified by the polymerization processes which must accompany spermatophore formation.

CHAPTER VIII

EFFECT OF SPECIFIC ANTIBODIES ON THE DEVELOPMENT AND FUNCTION OF ACCESSORY REPRODUCTIVE GLANDS

8.1 Normal Accessory Reproductive Gland Pattern During Adult Development

At ecdysis adult males of S. gregaria have a pink body colouration. During the subsequent days of adult development the body colouration changes progressively to yellow (Penner, 1967). The acquisition of a yellow body colour is a secondary sexual characteristic and is correlated with the onset of reproductive maturation. Under the rearing conditions used in this study some males could form spermatophores and inseminate females after 12 days of adult development. The growth and development of the testes and ARGs is complete by day 19 when males are able to inseminate females. At that time they have acquired a bright yellow body colouration.

In order to form a basis of comparision for the experimental and control series, it was necessary to determine the normal growth patterns of the ARG complex during this period of adult development. Measurements of length and width of the ARG complex of locust 3,7,10 and 19 days after adult ecdysis were

taken and mean values have been presented in Table 9. The measurements varied from 3.10±0.04 mm (length) and 1.9±0.04 mm (width) for 3 day old locusts to 5.6±0.10 mm (length) and 2.29±0.17 mm (width) for 19 day old locusts. Because the variance of the meassurements in successive age groups increased in proportion to the increasing means, the length and widths were transformed into log values. The log width was plotted against log length for different days (Figure 28) to obtain an allometric growth curve for the ARG complex. The resulting growth curve did not appear to be linear. During the early days of adult development (till about day 7) the width of the ARGs increased at a faster rate than the length. Between days 7 and 10 the situation changed when the length increased at a slightly higher rate than the width. From day 10 onwards the relative rates of growth of the length and width were nearly equivalent (Figure 28). In order to determine the effect of specific antibodies or normal guinea-pig serum (normal serum) on the growth of the ARGs a linear growth plot was required. An ARG growth index was derived from the product of the length and width measurements. An increase in the value of this product should reflect an increase in the volume of ARG complex. Because the variance of the ARG indices also increased with the magnitude of the group means, they were converted to their log values. A linear growth plot was obtained when the log ARG indices were plotted against their respective

TABLE 9 Comparison of means, standard errors and variances for measurements (in mm) of ARG growth during post-ecdysial adult development

ARG measurements on various days	Numbers	Means (± SE) mm	Variance
Day 3			
ARG length	23	3.104 ± 0.043	0.043
ARG width	23	1.191 ± 0.035	0.028
Day 7			
ARG length	20	3.360 ± 0.066	0.087
ARG width	20	1.543 ± 0.039	0.030
Day 10			
ARG length	21	4.057 ± 0.093	0.173
ARG width	21	1.495 ± 0.043	0.039
Day 19			
ARG length	35	5.614 ± 0.101	0.357
ARG width	35	2.291 ± 0.069	0.168

FIGURE 28

Logarithmic growth curve showing the changing relationships between increasing accessory reproductive gland (ARG) complex width to increasing length on a log-log scale during the adult development of male S. gregaria. Plotted values represent the means ± standard errors of log transformed data. Figures in brackets represent the number of observations on respective days of development

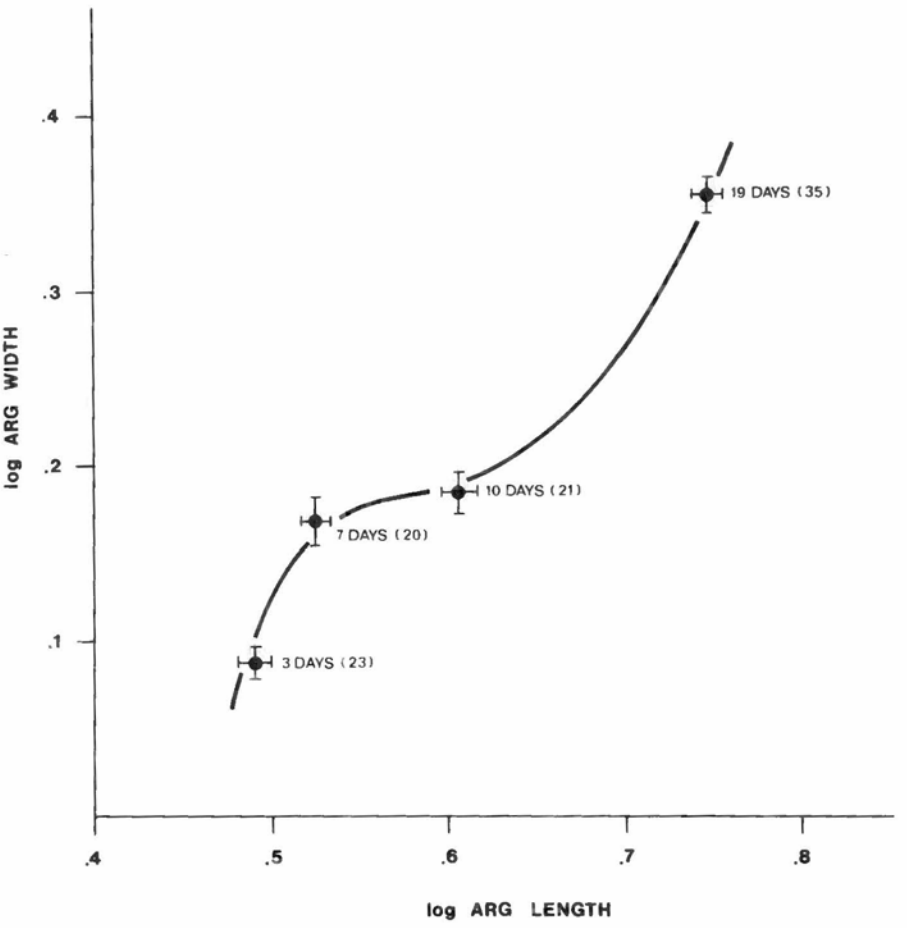

.4
.3
.2
.1
log ARG WIDTH
19 DAYS (35)
10 DAYS (21)
7 DAYS (20)
3 DAYS (23)
.4
.5
.6
.7
.8
log ARG LENGTH

FIGURE 28

days of development (Figure 29).

8.2 Effect of Various Treatments on ARG Growth

The various injection treatments of male locusts with guinea-pig-anti-ARG mixture serum (anti-serum) and normal serum have been described in the materials and methods Chapter 2.9. In each experimental and control series, the locusts were dissected and assayed on the nineteenth day of adult development when the length and width measurements of the ARG complex were taken. The log values for the ARG growth indices were calculated for each individual as described in the previous section. The group means and their standard errors for the log ARG growth indices in the controls and experimental series have been presented in Table 10. The significance of the various treatments effects was tested using a single classification analysis of variance with planned comparisons between the group values for the log ARG growth indices (Table 11). The injection of normal serum and anti-serum, in all experimental series, had a pronounced inhibitory effect on ARG growth which was highly significant ($P<.001$). Similarly, the inhibitory effects of the anti-serum were greater than those of the normal serum, in all series, and that difference was highly significant ($P<.001$). Although the mean log ARG growth index of males injected with normal serum on days 3,7 and 10 was lower than

FIGURE 29

Semi-logarithmic plot, illustrating the normal pattern of increase in the mean log-transformed values of the accessory reproductive gland (ARG) growth index (length x width) with days after adult emergence of male S. gregaria. The arrow indicates the day of adult development on which spermatophore production is first observed.

Inhibition of ARG growth on day 19, following the injection of mixed anti-sera raised in guinea-pigs to the antigens of the ARGs, is also illustrated and the equivalent day of normal development (EqND) corresponding to the respective level of inhibition is extrapolated from the plot of normal growth. If the ARG growth index continued to increase in the same log-function beyond day 19, then the maximum value of the ARG growth index (Max GI, open circle) calculated from the length and width reported by Odhiambo (1969a) would be reached between days 22 and 23 of adult development.

Series I treatments consisted of the injection of 0.1 ml of mixed anti-serum (I as) or normal serum (I ns) on days 3,7 and 10 of adult development. Series II treatments consisted of the injection of 0.1 ml of mixed anti-serum (II as) or normal serum (II ns) on days 7 and 10 after adult emergence. Series III treatments consisted of the injection of 0.1 ml of mixed anti-serum (III as) on day 10 after adult emergence

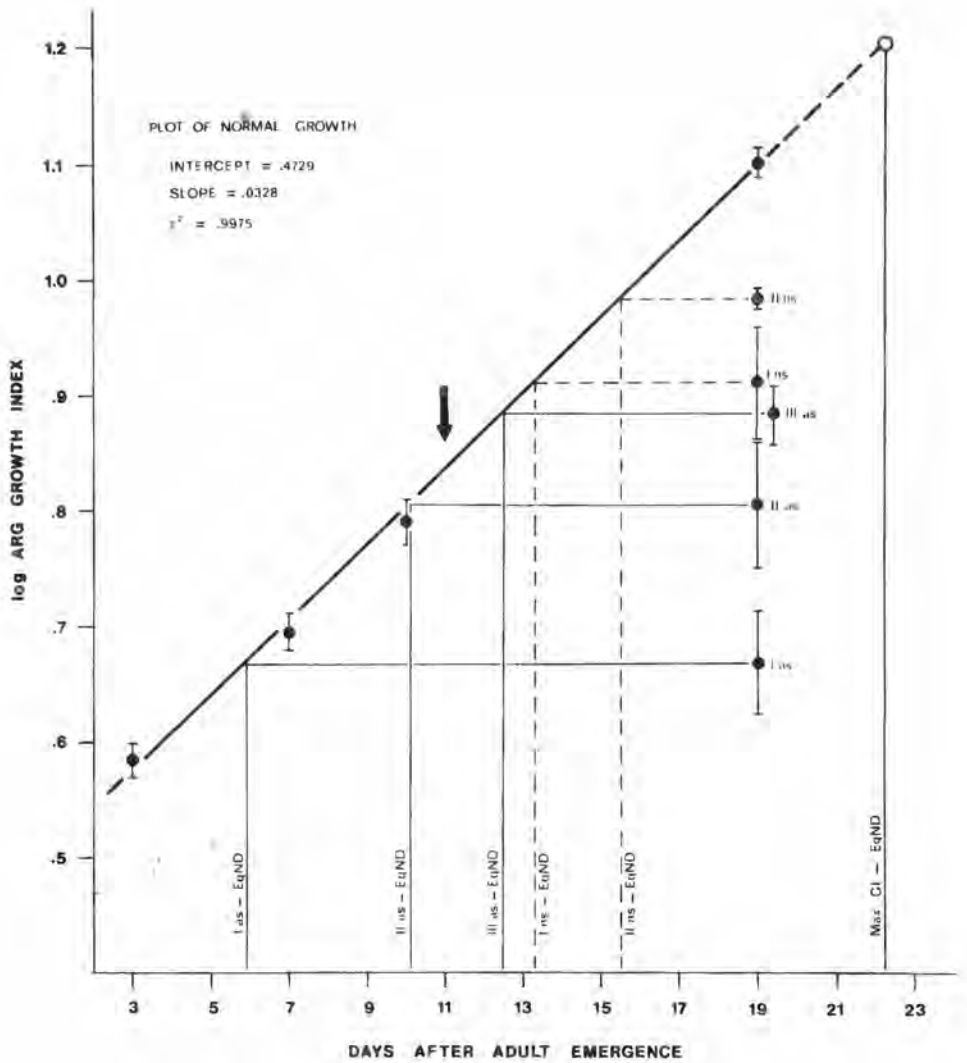

PLOT OF NORMAL GROWTH
INTERCEPT = .4729
SLOPE = .0328
r² = .9975
log ARG GROWTH INDEX
1.2
1.1
1.0
.9
.8
.7
.6
.5
3
5
7
9
11
13
15
17
19
21
23
DAYS AFTER ADULT EMERGENCE
Max CI – EqND

FIGURE 29

Table 10 Group means and standard errors for log ARG growth indices in the control and experimental series

Groups	Numbers	Mean ± SE log growth index
Control Series		
3 days old	23	0.5837 ± 0.0149
7 days old	20	0.6946 ± 0.0160
10 days old	21	0.7904 ± 0.0198
19 days old	35	1.1036 ± 0.0156
Experimental Series I, injected on days 3,7 and 10 with :		
Anti-serum	21	0.6670 ± 0.0459
Normal serum	18	0.9120 ± 0.0472
Experimental Series II, injected on days 7 and 10 with :		
Anti- serum	20	0.8054 ± 0.0552
Normal serum	8	0.9830 ± 0.0091
Experimental Series III, injected on day 10 with :		
Anti-serum	11	0.8836 ± 0.0260

TABLE 11 Effects of normal serum and anti-serum injections on log ARG growth indices - single classification analysis of variance with treatment sum of squares decomposed into planned comparisons

Source of variation	SS	df	MS	Fs	P(<)
Treatments	2.8214	5	0.5643	18.546	.001
Control Vs Injections	1.9196	1	1.9196	63.090	.001
Normal serum Vs anti-serum	0.4835	1	0.4835	15.892	.001
Among normal serum	0.0260	1	0.0260	0.855	NS
Among anti-serum	0.3923	2	0.9162	6.447	.005
Within	3.2556	107	0.0304		
Total	6.0770	112			

that for males injected on days 7 and 10, the difference among these inhibitory effects was not significant. On the other hand, the inhibitory effects of the injection of anti-serum on day 10, on days 7 and 10, and on days 3,7 and 10, were progressively more pronounced and differences among these treatments were significant at the .005 level.

When locusts were injected on days 3,7 and 10 with anti-serum (experimental series I) the ARGs were not filled with secretions by day 19 and could not be easily identified using the landmarks described by Odhiambo (1969a). The paragonadal fat body was either white or pale yellow as compared to the bright yellow of 19 day old untreated males. The glands of the normal untreated males were full of accessory secretions and could easily be identified (Figure 30). An extreme effect of the injection of anti-serum in experimental series I is demonstrated in Figure 31. The ARG complex resembled that of a 3 day old male. When compared to the normal development of males in the control series by a t-test the mean log ARG growth indices of the series I males injected with anti-serum were not significantly different from those of 7 day old normal males. However, they were significantly lower ($P<.05$) than those of normal males in the 10 day old group.

Injection of normal serum on days 3,7 and 10 (Series I)

FIGURE 30

Colour photomacrograph showing the accessory reproductive glands (ARGs) of normal untreated 19 day old adult male S. gregaria. The paragonadal fat body is yellow in colour and the first six glands, which are filled with secretions, are easily distinguishable.

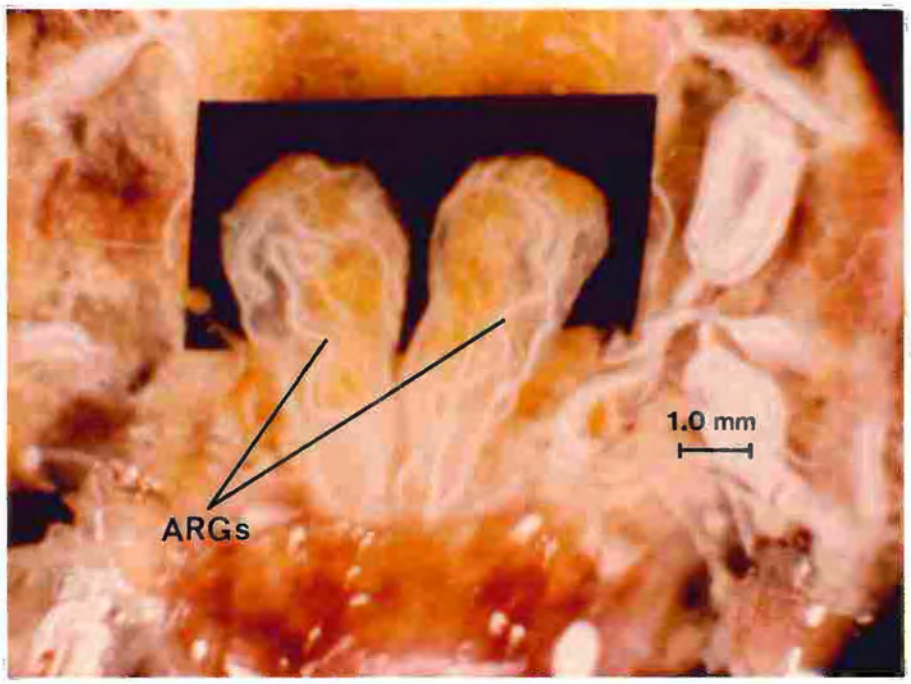


FIGURE 30

FIGURE 31

Colour photomacrograph showing the extreme inhibitory effect of guinea-pig anti ARG-serum on the growth of accessory reproductive glands of males injected on days 3,7 and 10, and dissected on day 19 of adult development. The paragonadal and perivisceral fat bodies are white and the individual ARGs, which are not filled with secretions are not distinguishable

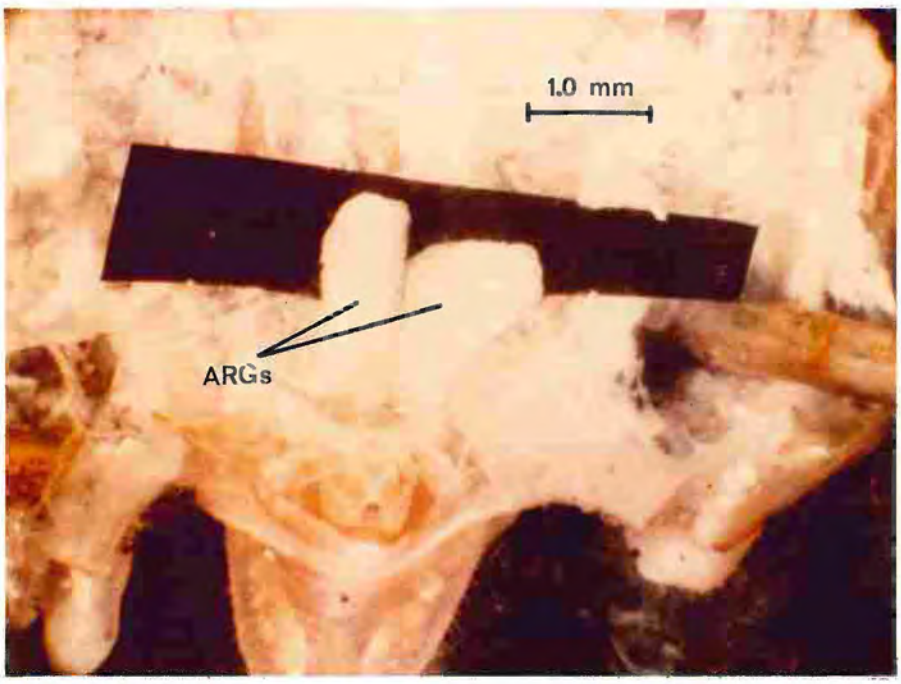


FIGURE 31

of adult development also inhibited the growth of ARGs. The mean log ARG growth index of series I serum injected males was greater than that of the 10 day old control groups, but was significantly lower (P<.001) than that of the 19 day old controls (Table 12). At the same time, the mean log ARG growth index of anti-serum injected males in series I was significantly lower (P<.001) than that of the 19 day old controls (Table 12). At the same time the mean log ARG growth index of anti-serum injected males in series I was significantly lower (P<.001) than that of normal serum injected series I males. When these males were placed with virgin females for mating, only the normal serum injected series I males inseminated females. The series I anti-serum injected males remained passive and did not attempt to copulate with females. The body colouration of anti-serum and normal serum injected males (series I) were different (Figure 32). While the normal serum injected males had attained the yellow body colouration, the anti-serum injected males were brownish and the femur colour was dark brown (Figure 32).

When the mean treatment log ARG growth indices were extrapolated on the plot of normal ARG growth for days 3,7,10 and 19 (Figure 29) the mean values for the anti-serum injected males corresponded to day 6 of normal development. The normal serum injected males had mean growth indices extrapolated to those of

TABLE 12 Effects of normal serum and anti-serum injections on log ARG growth indices - Tests for differences between group means

Comparisons	$t_{statistic}$	df	P(<)
Control Series			
3 day to 7 day	5.073	41	.001
7 day to 10 day	3.763	40	.001
10 day to 19 day	12.274	54	.001
Experimental Series			
I anti-serum to I normal serum	3.611	37	.001
I anti-serum to III anti-serum	3.168	31	.01
I anti-serum to II anti-serum	1.887	40	NS
II anti-serum to II normal serum	1.956	26	.05
II anti-serum to III anti-serum	0.845	30	NS
III anti-serum to II normal serum	2.983	17	.01
Between Experimental and Control Series			
I anti-serum to 10 day	2.419	41	.05
I anti-serum to 7 day	0.545	40	NS
II anti-serum to 19 day	5.478	54	.001
II anti-serum to 10 day	0.257	40	NS
III anti-serum to 19 day	6.826	45	.001
I normal serum to 19 day	4.668	52	.001
II normal serum to 19 day	3.567	42	.001

FIGURE 32

Colour photograph comparing the effects of guinea-pig anti ARG-serum (A) and normal guinea-pig serum (B) on the body colouration of males injected on days 3,7 and 10, and observed on day 19 after adult emergence. The colours painted on pronotums of the males were used to differentiate between experimental groups maintained in one cage

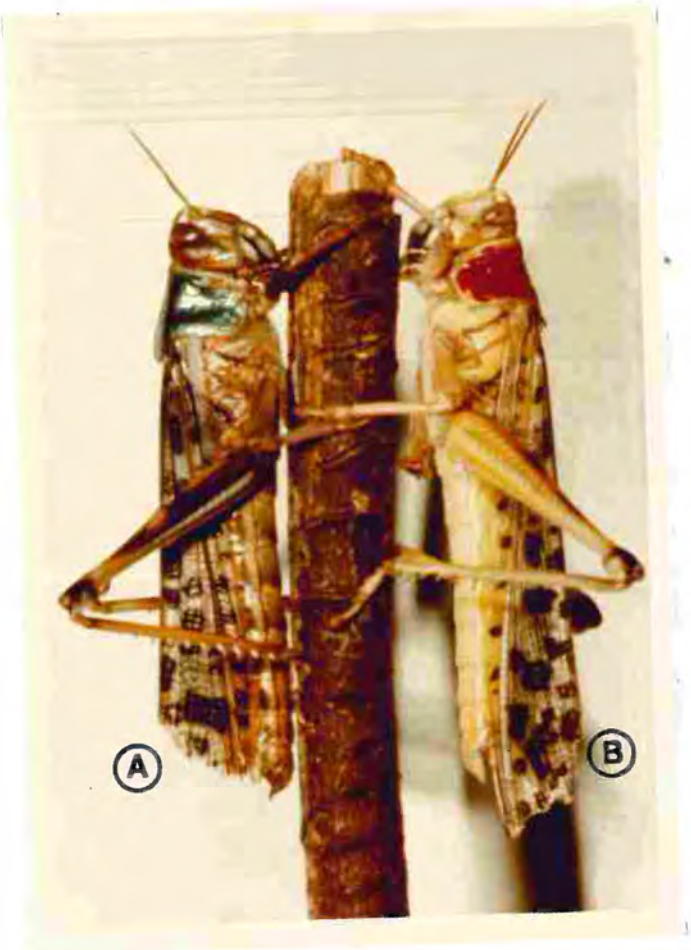


FIGURE 32

13 day old normal males.

Locusts which received anti-serum injections on days 7 and 10 (series II) attained a pale yellow body colouration by day 19. Although their ARGs contained some secretion and individual gland types could be identified they were not as developed as those of 19 day old untreated males. The colour of the paragonadal fat body varied from pale yellow to yellow. The mean log ARG growth index of series II anti-serum injected males was significantly lower ($P<.001$) than that of 19 day old untreated males (Table 12). However, their mean index corresponded closely to that of 10 day old untreated males (Figure 29) and was not significantly different from the mean for that group (Table 12).

The normal serum injected males in series II had a mean log ARG growth index which was significantly lower ($P<.001$) than that of 19 day old untreated males (Tables 12) and significantly higher ($P<.05$) than that of the series II anti-serum injections. Like the series I normal serum injected males, serum injected males from series II were able to transfer normal appearing spermatophores to receptive females. The anti-serum injected series II males, like those of series I, remained passive towards virgin females and did not attempt to copulate with them.

In series III, male locusts received only one injection of anti-serum on day 10 of adult development. The males attained a yellow body colouration and their ARGs were filled with accessory secretions. Individual glands could easily be identified using the landmarks described by Odhiambo (1969a). Males from this series readily copulated with receptive virgin females and transferred normal appearing spermatophores. Still, the mean log ARG growth index of the males in this series was significantly lower ($P<.001$) than that of the 19 day old normal untreated males (Table 12). By extrapolation (Figure 29) their growth was equivalent to that of 12 day old untreated males.

8.3 Normal Guinea-pig Serum Proteins

Electrophoresis of normal guinea-pig serum revealed 19 protein bands (Figure 33) of which bands A and B were the pre-albumin and albumin fractions, respectively. Comparison of protein patterns from ARG secretions (Chapter 3; Figures 3-7) and the normal guinea-pig serum protein pattern revealed a number of common protein bands. Protein bands 1-L (Figure 3), 4-J, 5-J and 6-F (Figure 4) were present in the same migration zone as normal guinea-pig serum protein band D. Similarly, band F of the serum had a similar electrophoretic mobility to bands 4-L (Figure 4), 11-K, 12-K (Figure 5), fb-H (Figure 6) and He-G

FIGURE 33

Electrophoretic patterns of proteins separated from normal guinea-pig serum (N-Gp-S); CBB-G, gel stained for proteins with Coomassie brilliant blue-G

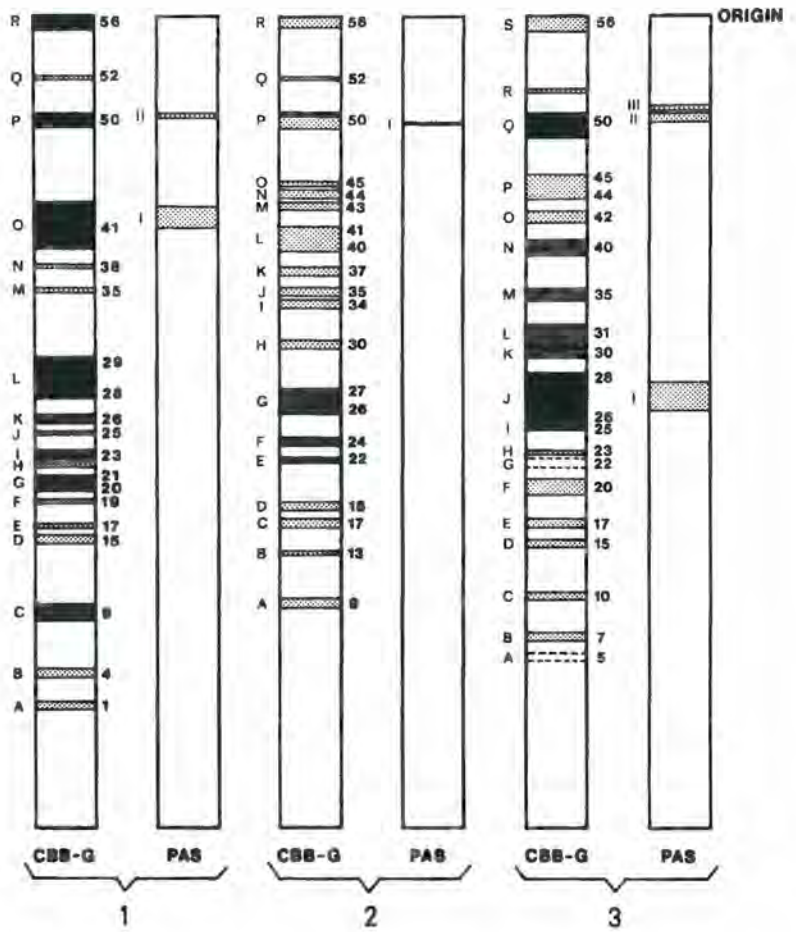


FIGURE 3

Table 13. Comparison of normal guinea-pig serum proteins with proteins from ARGs, fat body and the haemolymph of 19 day old adult male S. gregaria

SAMPLES	NORMAL GUINEA-PIG SERUM PROTEINS																	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Gland 1				L					N						Q				R
Gland 2							I								Q				R
Gland 3												P							
Gland 4				J		L			P										U
Gland 5				J															
Gland 6				F					H										L
Homogenous glands					O			S											Z
Gland 11						K													T
Gland 12						K													P
Seminal vesicle																			
Paragonadal fat body		A				H			J				O						
Haemolymph						G	H									P			R

Protein bands from Figures 3 - 7

Note : For normal guinea-pig serum protein patterns refer to Figure 33

(Figure 7). Other fractions in the normal guinea-pig serum may also have been common to some of the ARG secretion proteins (Table 13).

8.4 Conclusions

The growth of the ARGs in sexually maturing males can be inhibited by the injection of a mixture of antibodies against the ARG secretion proteins. Inhibition was maximized when the injection of antibodies began early (day 3) in adult development. The inhibition of ARG growth was correlated with the incomplete development of secondary sexual characters (yellow body colouration) and paragonadal fat body and the suppression of normal mating behaviour. The effects of anti-serum injections were less pronounced when the injections began on day 7 or day 10 of adult development. Infact, the injection of antibodies on day 10 did not delay the acquisition of yellow body colour or prevent spermatophore production by males which had matured to 19 days of age. Under the rearing conditions used in this study, male locusts could inseminate females by about day 12. Therefore, the ARG secretion complement may be nearly complete by day 10 of adult development although the glands continue to grow in size (Figure 29). In order to disrupt the reproductive capacity of males injected late in adult development, quantities of antibodies

larger than those used in this study might be needed.

One of the haemolymph proteins has been shown to be common to the secretions of glands 1,11 and 12 by immunochemical techniques (Chapter 3.2). Other haemolymph proteins common to ARG secretions and fat body have also been demonstrated by disc electrophoresis (Chapter 3.1). It is likely that the pronounced effect of the anti-serum on ARG growth was due to the precipitation of these proteins while they were still in the haemolymph and before they could be transferred to the glands. Other antibodies in anti-serum were to ARG secretion antigens which did not appear in the haemolymph. The inhibitory action of these antibodies might occur within the gland tissues themselves.

The inhibition of ARG growth also occurred when sexually immature males were injected with normal guinea-pig serum. While the growth of the ARGs was slightly inhibited the secondary sexual characters developed normally. This inhibition of growth may have been due to a reduction in the food intake of serum injected and/or the loss of haemolymph following injection.

CHAPTER IX

CONCLUSIONS AND DISCUSSIONS

The original aims of this study were two-fold : (1) to investigate the detailed chemical nature of various ARG secretions of male S. gregaria by electrophoretic, immunochemical and chromatographic techniques; and (2) to use these same techniques to elucidate the functions of various ARGs during spermatophore formation and secretion of the seminal fluids. As a result of the initial experimental results, a third objective emerged, namely to determine if antibodies raised against ARG secretions could be used to disrupt the normal development and/or functions of ARGs in sexually maturing or mature males. In this chapter, the conclusions and discussions have been focussed on these three objectives.

9.1 Characterization of ARG Secretions

Previous investigations demonstrated that 9 types of ARGs can be characterized in male S. gregaria on the basis of histological, histochemical and ultrastructural criteria (Odhiambo, 1969a, 1969b). In this study, however, the different electrophoretic techniques have demonstrated distinct protein and glycoprotein patterns for the secretions from the various ARGs.

For example, Odhiambo (1969a, 1969b) grouped glands 3 and 5 as a single type since they alone produced secretions which contained acidic lipoproteins. Several of the major protein bands from the secretions of these glands showed similar electrophoretic mobilities (Figures 3 and 4), but the over all electrophoretic patterns were different. Moreover, the secretions of gland 5 had 5 glycoprotein bands while gland 3 had only 2 such bands. The combined protein patterns for the homogenous glands, including gland pairs 7,8,9,10,13,14 and 15 were more complex than those from single pair of glands. Such complexity might be expected from the pooled secretions of several different types of glands. Together with the biochemical observations reported in the previous chapters, these data imply that each pair of accessory reproductive glands may have one or more unique roles in the secretions of accessory seminal fluids and/or spermatophore formation. This would not necessarily prelude several pairs of glands from sharing other common functions.

Multiple protein and glycoprotein patterns, such as those found in the secretions of the ARGs of male S. gregaria have been reported from several other insect genera, including Musca (Terranova et al., 1972), Drosophila (von Wyl, 1976) and Glossina (Dhadialla, 1973). These findings are intriguing since histological and ultrastructural studies have shown that the secretory

cells which form the accessory glands of M. domestica (Leopold, 1970; Riemann, 1973) and those of each gland type in S. gregaria (Odhiambo, 1969a, 1969b) were similar. Odhiambo (1969b) considered the multi-lobed accessory glands of S. gregaria to be an example of the "vertical" compartmentalization of the epithelial tissue. Consequently, the gland cells of S. gregaria are not only specialised to form distinct gland types, but histologically and ultrastructurally similar cells are also specialized to produce different types of proteins and glycoproteins.

The common nature of some of the protein bands in the various gland secretions has been confirmed by immuno-electrophoresis. These analyses revealed that one antigen was common to glands 1,11 and 12 and the haemolymph, while a second antigen occurred in the secretions of glands 3,4,5 and 6. On comparison of the electropherograms it seemed probable that the major bands 1-O (Figure 3), 11-O and 12-N (Figure 5) and He-K (Figure 7) were immunochemically identical. Similarly, protein bands 3-J (Figure 3), 4-J, 5-J and 6-F (Figure 4) probably represent a single antigen.

The molecular weights of various insect ARG secretory proteins when previously determined, have been shown to be relatively low as in Musca (Nelson et al., 1969; Terranova et al.,

1972), Drosophila (Baumann, 1974; von Wyl, 1976) and Hyalophora and Anthraea (Shepherd, 1974b and 1975). A comparison of the electrophoretic patterns obtained from male S. gregaria ARG secretions (Chapter 3) with the electrophoretic patterns for normal guinea-pig serum (Chapter 8), normal human serum and insulin (Dhadialla, unpublished observations) provides an estimate of their isoelectric point (pI) and a rough approximation of molecular weights. In general, proteins from the locust gland secretion, fat body and haemolymph have a lower electrophoretic mobility than insulin with its moleculer weight of 5,700 (Lehninger, 1970). Only a few of the faster moving locust protein bands had mobilities between those of insulin and albumin (pI of 4.9; Sober, 1970 : molecular weight of 68,000; Lehninger, 1970) Most of the locust protein bands were present between those for albumin and γG-immunologlobulins (pI of 5.8 - 7.3; molecular weight range of 151,000 - 161,000; Sober, 1970). By deduction it would appear that most of the locust protein fractions had molecular weights between 68 ,000 and 161,000, while a few of the molecular weights might have been as low as 5,700 . The paragonial secretions of D. melanogaster have a molecular weight range of 12,000 - 122,000 daltons (von Wyl, 1976). Similarly the molecular weights of the two matrone subunits which have been isolated from the male accesscry secretions of A. aegypti were 30,000 and 60,000 daltons respectively. Thus,these estimates of the mole-

cular weights for S. gregaria ARG protein fractions appear to be quite consistent with these observations on other insects.

Analysis of free amino acids in the methanolic extracts of various ARG secretions, fat body and haemolymph of S. gregaria revealed a total of 18 amino acids, of which α-alanine, glycine, methionine, phenylalanine, serine and aspartic acid were quantitatively important. Histochemical and spot tests have demonstrated amino acids in the secretions of L. nuttalli (Gerber et al., 1971b), P. americana (Vijayalekshmi and Adiyodi, 1973) and C. ischnocheles (Legg, 1973). Amino acids have also been demonstrated in their free form (Leopold, 1970; Leopold et al., 1971; Terranova et al., 1972; Chen and Oechslin, 1976) and bound in proteins (Frenk and Happ, 1976) of the glands and secretions of several insects. It should be noted that the first 6 pairs of ARGs in S. gregaria, together with gland 12, have secretions which were particularly rich in free amino acids. Vijayalekshmi and Adiyodi (1973) proposed that free amino acids may maintain the osmotic pressure of ARG secretions in Periplaneta. The free amino acid levels of ARG secretions are high in S. gregaria but further experiments must be designed and carried out before their role is known with certainty.

The analyses for free sugars, like those for free amino acids failed to reveal major differences between the individual pairs of glands. Trehalose and/or inositol and an amino sugar(s) were present in the secretions of all glands, but fructose was restricted to glands 1,2,3,4,5,6,11, the homgenous glands and the seminal vesicle. Sucrose was present in glands 3,4,11 and the seminal vesicle, but glucose was detected only in the secretions of gland 12. Inositol has been identified as the prominent sugar reserve in the ARGs of P. americana (Vijayalekshmi and Adiyodi, 1973) and glucose was found in the seminal plasma of that insect. As with the results for proteins and amino acids, the similarity between the sugar contents of the ARG secretions of S. gregaria and other insects imply a uniformity in their functions.

An unidentified compound from the gland secretions and the fat body of S. gregaria, and a second closely related compound from the haemolymph, have as yet unexplained functions. The first compound migrated in the region of the sugars or amino sugars but did not react with alkaline silver nitrate or aniline phthalate reagent. Njogu and co-workers (1970) encountered a compound with similar properties while analysing the sugars of trypanosomes. They concluded that their compound was an amino sugar. Their arguments cannot be convincingly extended to S. gregaria compounds, however, since amino sugars would show a positive reaction with

alkaline silver nitrate. The various spot tests performed on the unknown compound from the accessory secretions indicate that it is a purine, probably a guanine derivative. These tests are further supported by preliminary infra-red spectroscopic investigations.

Purine compounds, especially uric acid, are often stored in insect tissues as a form of deposit excretion (Bursell, 1967; Maddrell, 1971). Male cockroaches, Blattella germanica and several other species, accumulate excess uric acid in their ARGs and it is "excreted" at the time of mating when the newly formed spermatophore is completely covered with uric acid (Roth and Dateo, 1964). The guanine-based unknown compounds from S. gregaria were detected in relatively small amounts and it does not appear that they are involved in deposit excretion. Quantities of guanylate cyclase have been reported to accumulate in the ARGs prior to mating and to appear in the ARG secretions of the male cricket, A. domestica (Fallon and Wyatt; cited by Filburn and Wyatt, 1976). These authors argued that the build up of c-GMP in the male ARGs prior to copulation was one evidence for its involvement in a hormonal mechanism controlling spermatophore formation. Further research should be undertaken to assess the possibility that the unknown compounds reported in this study are involved in similar hormonal mechanisms in S. gregaria.

Histochemical studies have revealed lipids, phospholipids and phospholipoproteins in the ARGs of L. nuttalli (Gerber et al., 1971b)and A. persicus (Tatchell, 1962). Odhiambo (1969a, 1969b) reported the presence of acidic-lipoproteins in the secretions of ARGs 3 and 5 of male S. gregaria. In this study, lipoproteins could not be detected when electrophoresed gels were stained with Sudan black B. It is possible that if lipoproteins were present, they were not at levels which could be detected by disc electrophoresis. Both lipids and phospholipids were found in chloroform-methanol (2:1 v/v) extracts of the various ARG secretions and in some cases individual glands showed characteristic patterns. Gland 6 secretions contained all classes of neutral lipids but the secretion of gland 4 was devoid of neutral lipids. The neutral lipid compositions of glands 5 and 6 were found to be similar but monoglycerides were absent from the gland 5 secretions. Spots corresponding to sterols were found in the secretions of all glands except 1,2,4 and 12 while sterol esters were present only in the secretions of glands 5 and 6. The lipid contents of S. gregaria ARG secretions were not unusual since sterols and/or their esters has also been demonstrated in the ARG secretions of P. americana (Vijayalekshmi and Adiyodi, 1973).

The ARG secretions of S. gregaria also contained trace amounts of phospholipids in the form of phophatidyl compounds of

ethanolamine, serine, inositol and choline. Phosphatidylethanolamine and phosphatidylcholine were common phospholipids in the ARG secretions of Schistocerca. Phosphatidylserine was present in the secretions of glands 2,3 and 4, while phosphatidylinositol was found in the secretions of glands 2,5,6,12 and the homogenous glands. Phosphatidylcholine was the only phosphatidyl compound in the secretions of gland 1. A predominance of phosphatidylcholine over phosphatidylethanolamine has been demonstrated in the ARG secretions of Periplaneta (Vijayalekshmi and Adiyodi, 1973).

Tests for the presence of enzymes in the ARG secretions and tissue-extracts of male S. gregaria were made for two purposes. The distribution of enzymes is often used in studies on tissue organization, development and metamorphosis (Wagner and Selander, 1974) and in the next section of this chapter the distribution of some enzymes will be related to probable gland function. But differences in the enzymes distribution can also be used to characterize the gland secretions. Three enzymes (IDH, XDH and malic enzyme) were not present at detectable levels in any of the accessory secretions. Alkaline phosphatase levels were high in secretions from the first 6 pairs of glands, but were low in secretions from glands 11,12 and the homogenous glands. Similarly, MDH occurred in larger amounts in the first 6 pairs of glands. Non-specific esterase activity was present in all secretions but

was at its highest level in gland 5 secretions. Acid phosphatases appeared as single bands in the electrophoresed secretions of glands 1,3,4,5 and 6. Individually the distribution of enzymes tends to separate the gland secretions into several types. When the data for all enzymes are combined, however, each secretion has its own distinctive combination.

The use of electrophoretic, chromatographic and immunochemical techniques have revealed significant differences in the biochemical composition of the various secretions of the ARGs, the washings from the paragonadal fat body and preparation of the haemolymph of S. gregaria males. In contrast to previous investigations (Odhiambo, 1969a, 1969b) the secretions of glands 3 and 5 were shown to be quite distinct in terms of their protein, glycoprotein and enzyme patterns and in their free amino acid, free sugar, neutral lipid and phospholipid composition. Odhiambo (1969a) also found that the secretions of glands 2 and the homogenous glands had a "fibrous mucopolysaccharide" character, while in this study only single PAS-positive bands were observed in the secretions of these glands. In this respect the secretions of glands 2 and the homogenous glands could not be distinguished from the other secretions. One possible explanation for these contradictory findings may be related to the age of the males used in each study. Odhiambo (1969a, 1969b) used "sexually mature"

males whose age could have varied from 12 to 23 or more days post ecdysis. Cycles in the increase of both lipid and protein content have been observed during the period of breeding activity in the pseudoscorpion, C. ischnocheles (Legg, 1973). Moreover, Odhiambo (1969a) characterized the secretions of gland 2 and the homogenous glands as mucopolysaccharides on the basis of PAS tests, even though the toluidine blue and alcian blue gave negative results. Periodic acid-Schiff's reagents are used as general tests for the presence of amino sugars, uric acid, sulphuric acid residues and glycoproteins, while toluidine blue and alcian blue are specific for mucopolysaccharides (Pearse 1960). Infact, Odhiambo's data like the observations reported in this study, indicate that the secretions of these glands contain glycoproteins.

The other differences between the observations of Odhiambo (1969a, 1969b) and those reported in this study are more easily explained. Anatomically, Odhiambo grouped the ARGs into 4 categories including the opalescent (1 pair), white (4 pairs), and hyaline glands (10 pairs), and the seminal vesicle. With histochemical and ultrastructural criteria, however, he was able to distinguish 9 gland types. The observations reported in this study were at the biochemical level and at this level of organization the secretions of each gland appear to be distinct for all glands other than the homogenous glands. Infact, the observations

on the pooled secretions of the homogenous glands imply that they too might consist of biochemically distinct types. This still needs to be confirmed.

9.2 Functions of Accessory Reproductive Glands Related to Spermatophore and Accessory Seminal Fluid Production

The accessory reproductive gland secretions of male arthropods have been shown to produce the materials involved in spermatophore production (Tatchell, 1962; Hartmann, 1970; Leahy, 1973; Chaudhury and Dhadialla, 1976; Frenk and Happ, 1976) which are largely proteinaceous in nature (Khalifa, 1949; Davey, 1960; Gregory, 1965). Disc gel electrophoresis of the supernatent of homogenized spermatophores collected from females after evacuation revealed 14 protein fractions, of which 4 were glycoproteins. Similarly, the electrophoretic patterns from the washings of unevacuated spermatophores collected from males contained 20 protein bands of which 2 were glycoproteins. Besides the differences in the number of bands, the differences in the electrophoretic patterns indicated that the constituent protein fractions were quite different. It is likely that the protein fractions from the evacuated spermatophores represented the solubilized structural components of the spermatophore itself. The fractions obtained from the unevacuated spermatophores, on the other hand, represented

the proteinaceous components of the accessory seminal fluids.

It had been hoped that immunoelectrophoretic techniques could be used to determine the ARG source of spermatophore proteins in S. gregaria. Frenk and Happ (1976) used this technique successfully to demonstrate the contribution of the bean shaped ARG of T. molitor to spermatophore formation. When electrophoresed samples of the evacuated spermatophores of S. gregaria were reacted with guinea-pig anti-serum to the ARG complex or to individual glands, there were no precipitin reactions. As noted previously in chapter VII, there are two possible explanations for these results. It is possible that the anti-sera, which were raised against a combination of ARG antigens (even in the case of anti-sera to individual glands) were so weak that the technique lacked the sensitivity needed to precipitate the spermatophore proteins. But it should be remembered that the spermatophore is probably formed as the final product of a series of polymerization reactions possibly involving the proteins, glycoproteins, lipids and/or phospholipids, within the mixed secretion of the ARGs. In that case the antigenicity of the structural components should be expected to be quite different from those in the individual gland secretions.

The immunoelectrophoretic technique did show that the washings of unevacuated spermatophores contained two antigens in common with the secretions of the homogenous glands. As argued in Chapter VIII, it appears likely that these antigens may represent some of the components of the accessory seminal secretions of S. gregaria. Pickford and Ewen (1969) demonstrated the involvement of the short hyaline glands of male Melanoplus in the production of a substance which enhanced oviposition in that species. Leahy (1973) showed that the implantation of the ARG complex of mature male S. gregaria into virgin females stimulated the onset of oviposition without mating. Since the short hyaline glands of Melanoplus correspond anatomically to the homogenous glands of S. gregaria, it is possible that the antigen common to the secretions of the homogenous glands and to the washings of unevacuated spermatophore plays a role in the enhancement of oviposition. Substantiation of the role of this common antigen could be obtained by isolating the factor with gel filteration techniques and injecting it into mature virgin females.

As was the case with S. gregaria, free amino acids, carbohydrates and phospholipids have been found in the ARG secretions of other arthropods. The first 6 pairs of ARGs and gland 12 produce high levels of amino acids in the desert locust. Vijayalekshmi and Adiyodi (1973) proposed that amino acids were

important in maintaining the osmotic pressure of the seminal fluids in Periplaneta. If amino acids perform a similar role in the secretions of the desert locust, then glands 1-6 and 12 are likely to be their major source. Some amino acids, for instance glycine, have a significant buffering capacity and their solutions are routinely used for that purpose. The high level of amino acids in the ARG secretions of the desert locust could be related to their role in buffering the pH of the individual and mixed seminal secretions during spermatophore formation and/or subsequently during sperm tansfer. Infact, the solidification of the spermatophore of Rhodnius depends on pH changes in the bulbous ejaculatorious.

Baumann (1974a) showed that glucose was present in the paragonial secretions of Drosophila and that it was an important component of the active factor which stimulated egg maturation and oviposition. In S. gregaria only the secretions of glands 12 contained that reducing sugar. Glands 3,4,11 and the seminal vesicle were important source of carbohydrates such as trehalose and/or inositol, sucrose, and glucose, and these could provide a significant substrate for the metabolic requirements of the spermatozoa during their transfer to the female. Glands 5,6,11 and the seminal vesicles are important sources of another class of potential substrates, namely the phospholipids. The levels of certain phospholipids, including phosphatidylcholine, phosphatidylethanolamine,

phosphatidylserine and others were shown to change dramatically during the pre and post ejaculatory processes of bovine spermatozoa (Poulos et al., 1973). Further research might determine whether phospholipids provide an important substrate for the the metabolism of S. gregaria spermatozoa during their final stage of development.

Among the enzymes, some of the functions attributed to alkaline phosphatase are of special interest. In human skin fibroblasts studied in vitro (Waters, 1973), synthesis of collagen fibrils was correlated with the activation of AlPase. In S. gregaria this particular enzyme was found in high concentration in the secretions of glands 1-6. Odhiambo (1969b) has described the formation of fibrous crystalline inclusions in the secretions of the first pair of glands of the desert locust. Recently secreted materials consisted of electron-dense granular masses embedded in a matrix of fine fibrous material. After a period of time in the lumen the secretions were transformed into "highly crystalline, glycoprotein granules", the growth of which was accomplished by the "addition of fibrous components to the edges of mature granules and their polymerization soon thereafter." It therefore appears possible that the high AlPase levels in the secretions of gland 1 (and in glands 2-6 as well) may be associated with the elaboration and polymerization of secretory products

some of which will form structural components of the spermatophore.

Acid phosphatase activity was found in the tissues of the spermathecal complex of Musca by Terranova and Leopold (1973). These authors suggested that tissue were involved in the elaboration of substances necessary for sperm maintenance and fertilization following storage. Although gland homogenates were not analysed for AcPase activity in this study, traces of AcPase were found in the secretions of glands 1,3,4,5 and 6. It is quite likely that more activity would be found in the glandular tissues themselves. In that case, the AcPase activity of these glands might be associated with the elaboration of necessary seminal secretions.

The results of these investigations provide no definite indication of a marked division of labour between the individual glands of the ARG complex. While some glands may secrete products which are principally used as structural components of the spermatophore, others may secrete the accessory components of the seminal fluids, and it appears likely that many glands have mixed functions. The fibrous nature of their secretions and the high levels of AlPase activity suggest that the first 6 pairs of glands produce structural proteins. These same glands, however, produce

large amounts of free amino acids, while glands 4,5 and 6 have high levels of phospholipids and glands 3 and 4 were high in carbohydrate production. The homogenous glands had an antigen in common with the washings of the unevacuated spermatophore and this antigen could represent an important component of the accessory seminal secretions of S. gregaria. Based on these results it is clear that histological, biochemical and immunochemical techniques might produce more meaningful data on the functions of individual ARGs in the desert locust when combined with surgical and radiotracer techniques.

9.3 Inhibition of ARG Growth with Specific Antibodies

The potential use of specific antibodies to investigate reproductive processes has been demonstrated in this study. When sexually immature males of S. gregaria were injected with anti-serum during various days of adult development their ARG growth was significantly retarded. Inhibition of ARG growth was also produced with normal guinea-pig serum in the injection controls. However, the inhibition of ARG growth produced by the anti-serum was greater than that produced in the injection controls and the differences between the group means were highly significant.

The normal growth of the ARGs during adult development in Schistocerca has been shown to be under the influence of corpus allatum hormone (Odhiambo, 1966c). Odhiambo (1966a, 1966b) observed that the most pronounced cytological and histological changes occurred in the corpus allatum between the third and fifth days of adult development. However, he also reported progressive changes in the size of the corpus allatum and in the size of the constituent cells which could be correlated with age through the first 10 days of adult life. The growth and development of the ARGs in S. gregaria probably consist of several processes which extend well past the temporal phase of corpus allatum enlargement. The maximum length and width of the ARGs of the male desert locust reach 8 by 2 mm, according to the report by Odhiambo (1969a) and these values yield a log growth index of 1.20. Assuming that the linear pattern of increase in the log ARG index would hold during growth beyond day 19, an extrapolation of the relationship in Figure 29 indicates that the ARGs would reach the size reported by Odhiambo between the 22nd and 23rd day of development.

From these known facts it seems probable that early ARG growth is under the control of the corpora allata and consists of the actual growth and differentiation of the ARG glandular epithelia. Subsequent phases of growth might also be under the control of the corpus allatum, but they would principally reflect the

accumulation of secretory products within the glandular epithelia as well as within the ARG lumina. It is clear that the simple linear plot of the log ARG growth index on days of development does not reflect the complexity of these growth relationships.

The mechanisms involved in the inhibition of ARG growth by injection of normal sera and ARG anti-sera may be as complex as those involved in the control of normal growth. The inhibition of ARG growth following the injection of normal guinea-pig serum appeared to be associated with reduced feeding on the part of the developing adult male. That reduction could have been caused by the actual injection process or by some metabolic feedback mechanism which had been activated by the elevation of locust haemolymph protein as a result of the serum injection. A similar mechanism could have contributed to the inhibition of ARG growth following injection of the anti-serum. The highly significant differences following injections of normal serum as compared to the injection of anti-serum implies that additional mechanisms were involved in the inhibition of growth in the latter instance.

Friedel and Gillot (1976) showed that radio-labelled ARG secrerions were incorporated unchanged into developing glands of Melanoplus following their injection into the haemolymph. The conclusions of that study were that many components of the ARG

secretions were synthesized in the fat body of Melanoplus and subsequently transferred to the ARGs via the haemolymph. When the anti-sera were injected into developing male S. gregaria it is probable that they reacted with circulating antigen and prevented its transport to and incorporation within the developing ARGs. Prior to the injection of the anti-serum the development of ARGs would have been normal and at the end of the injection period (day 10 in all cases) it is possible that the level of antigen again rose in the haemolymph, assuming its continued production by the fat body, and that growth of the ARGs continued until the day of assay. It is also possible that the retarted growth of the ARGs had been monitored by a feed-back mechanism so that the activity of the corpus allatum was maintained at the level of activity associated with pre-day-10 growth. In such a case the ARGs of the anti-serum injected males might have eventually grown to normal size and the males would have developed their secondary sexual characteristics as well as interest in mating.

A more permanent form of growth inhibition may also have been involved. If the antibodies which were injected into developing males were taken up by the tissues of the ARGs or of the fat body (which may be a major source of certain components of ARG secretions) then the inhibition of growth might have been largely irreversible. Just as Friedel and Gillot (1976) were able

to show that radio-labelled secretions could be incorporated unchanged in the developing ARGs of Melanoplus, so Wigglesworth (1943) showed that proteins in the diet of an insect can cross the midgut of an insect without appreciable change. Schlein and co-workers (1976a) used indirect fluorescent antibody technique to substantiate that the gut wall of Sarcophaga does not bar the passage of serum immunoglobulins raised to various tissues of that fly. These data suggest that antibodies to ARG secretory antigens could easily enter target tissues where their reactions could interupt normal growth. Future research on the inhibition of ARG growth should focus on the actual sites of antibody reaction in order to elucidate the actual mechanisms of inhibition.

The correlation between inhibition of ARG growth and the acquisition of secondary sexual characteristics, principally the development of yellow pigmentation in epidermal and paragonadal fat body tissues, may be due to primary and secondary interactions with injected antibodies. The yellow pigmentation of S. gregaria is due solely to the accumulation and deposition of carotenoids (Goodwin, 1952, cited by Rowell, 1971). These compounds could be by products of the metabolic processes which under lie the growth and differentiation of the reproductive system in the male. If inhibition of ARG growth were due to the inhibition of these metabolic events then retardation of pigment deposition would be a

direct consequence of inhibition. On the other hand if the hormonal reactions between the corpus allatum were influenced by the inhibition of ARG growth, then the absence of pigmentation might indicate that the appearance of this secondary sexual character was related to the endocrine state of the male locust. Absence of normal pigmentation would then be due to a secondary effect related to the males changed endocrine state as a result of the inhibition of ARG growth.

9.4 Use of Specific Antibodies in Future Research

Friedel and Gillot (1976) studied the synthetic pathways of ARG secretions in Melanoplus by a combination of radio-tracer and immunochemical techniques. They did not, however, prepare antibodies to individual glands, let alone to the individual antigens present in each gland. Our knowledge on the relationships between growth and function in the male accessory reproductive glands of S. gregaria is based on relatively gross studies (Odhiambo, 1966c and the results of this investigation). Rates of maturation and the timing of functional cycles in individual glands are unknown. Although the anti-sera used in this study contained few antibodies to ARG antigens, they had a pronounced influence on ARG growth. It appears likely that injection of specific antibodies to various ARG antigens might have produced a more selective form

of inhibition. Following the precipitation of specific antigens to individual glands, combined immunochemical, radio-tracer and biochemical techniques could be used to supplement observations based on the surgical removal of individual glands. In fact, the inhibitory influences of antibodies to single ARG antigens might represent a form of "immunochemical ablation." The use of antisera raised against specific antigens might go a long way in providing data on the mechanisms of ARG growth and inhibition, on the co-ordination of the cycles of secretory synthesis and release in individual glands and on the importance of the various gland components in the complex process of spermatophore formation and accessory seminal fluid secretion.

The development and application of sophisticated immunochemical techniques might provide some exciting information on the reproductive physiology of S. gregaria and other insects. The techniques may also provide a possible alternative to the chemical approach to insect control. When Schlein et al., (1976b) fed Stomoxys and Glossina species on rabbits which had been immunized against antigens from the tissues of the flies they obtained a high rate of mortality and observed lesions in the fly tissues against which the antibodies had been raised. If antibodies could be raised against one or more of the antigens which play critical roles in the maturation of the reproductive systems, then parasitic

arthropods which fed on immunized hosts might be effectively sterilized. The potential benefits of this form of parasite control should stimulate future research on the immunochemical inhibition of insect reproductive maturation.

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