



<https://theses.gla.ac.uk/>

Theses Digitisation:

<https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/>

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk/>
research-enlighten@glasgow.ac.uk

**EFFECTS OF AZADIRACHTIN ON PROTEIN SYNTHESIS
IN SPECIFIC TISSUES OF THE
DESERT LOCUST *SCHISTOCERCA GREGARIA***

by

SHAGUFTA IQBAL

M.Sc. Zoology

(University of Balochistan, Quetta, PAKISTAN)

**Thesis submitted for the Degree of
Doctor of Philosophy**

May 1999



**UNIVERSITY
of
GLASGOW**

Division of Biochemistry and Molecular Biology

Institute of Biomedical and Life Sciences

University of Glasgow

Glasgow G12 8QQ Scotland UK

ProQuest Number: 10391441

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10391441

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

THIS BOOK IS BOUND BY
M. DOWNIE & SON
UNIT H, PURDON ST
PARTICK
GLASGOW G11 6AF
TEL: 0141-339-0333

GLASGOW
UNIVERSITY
LIBRARY

116 10 (copy 2)

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

In the Name of Allah, the Beneficent, the Merciful

I DEDICATE THIS THESIS TO;

My parents **Mr and Mrs. M. Iqbal** and to my beloved daughter **Aida Ali.**

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to Dr. R.H.C. Strang for his constant advice, encouragement and ever-willing help extended to me to complete this work. It has been a great privilege to have worked with him. His co-operation and support is highly appreciated.

My thanks are also due to Dr. L.M. Fixter for his constructive criticism and guidance to improve my written work.

I am grateful to Professor G. Milligan for making the facilities of the department available.

I also express my deep appreciation to;

Dr. A. Campbell for her help in ELISA experiments;

Dr. J. Modha for his help in Western Blotting and Ouchterlony;

Mr. A. Scott for guidance in electrophysiological studies;

Mr. J. Green for technical assistance;

Dr. J. Kusel for moral support;

All the colleagues and friends who made my stay in Glasgow pleasant;

My friend Dr. Rukhsana for her tremendous support, my husband Sadaqat Ali for his consistent help and last not least my son Ziad Ahmed for his patience.

I acknowledge the financial support from the Commission of Commonwealth Universities and The Charles Wallace Pakistan Trust.

SUMMARY

Azadirachtin A, one of the most polar of the many terpenoids occurring in the seeds of the neem tree (*Azadirachta indica*) has long been known to be the most potent in terms of its toxic effects on insects. Although its exact primary mode of action remains unclear, much research has implied that it inhibits protein biosynthesis in various insect tissues. This then leads to many secondary effects, such as sterility, developmental failure and general loss of biological fitness. The aim of the work reported in this thesis was to choose two specific proteins, lysozyme and vitellogenin, which could be easily quantified, and synthesised mainly by the fat body. Previous work had suggested that the proteins synthesised by fat body were reduced by azadirachtin. It was hoped that the synthesis of both could be induced by appropriate chemical stimuli both *in vivo* and *in vitro*. The insect studied, the desert locust *Schistocerca gregaria*, is known to be sensitive to the effects of azadirachtin, and is sufficiently large to allow ease of tissue sampling.

Preliminary studies with the uptake of [³H]-leucine into the proteins of fat body confirmed that aza reduced incorporation into the acid-precipitable proteins of the tissue and of the haemolymph by respectively 42% and 25% over a short time course.

Injection of bacterial lipopolysaccharide (LPS) was found to double the amount of circulating lysozyme within 24 hours. Further results suggested that this originated in both the fat body and haemocytes. Subsequent studies concentrated on the fat body as it yielded more material.

An incidental observation from this work was that lysozyme induction and release may be a general result of any mechanical or chemical insult to the locusts.

Azadirachtin A (3 µg/g body weight) largely eliminated the increase in lysozyme activity, reducing it to resting levels. Studies with isolated fat body confirmed that the effect of both LPS and azadirachtin could be shown to be direct effects on the fat body itself.

In studying the production of vitellogenin, it was found necessary to estimate the protein by immunological means. Vitellin was purified from mature ovarian tissue, and was used to raise a polyclonal antiserum in rabbits. This antiserum was found to cross-react with vitellogenin, and was used as the basis for various immunological assays. An ELISA assay allowed quantitative assays of both vitellogenin in fat body and haemolymph, and of vitellin in ovaries. Over a period of a month after imaginal moult, the proportion of vitellogenin in the female haemolymph rose from undetectable below 8 days to a maximum of 70% of the total protein. Treatment of females at 15 and 19 days with azadirachtin reduced the amount of vitellogenin by 62% and that in ovaries by 73% by 48 hours after treatment. These effects *in vivo* could be largely reproduced *in vitro* with isolated fat-body, using methoprene, a Juvenile Hormone analogue, as the stimulus.

In conclusion, the results presented show that azadirachtin prevented at least part of the increased production, induced by appropriate factors, of two identified proteins. This is consistent with previous biological observations of the action of the terpenoid.

CONTENTS

	Page No
Dedication	2
Acknowledgement	3
Declaration	4
Summary	5
Contents	6
List of illustrations	17
List of tables	23
List of abbreviations	24
CHAPTER 1 INTRODUCTION	
1.1 Agriculture and insects	26
1.2 Protective measures	27
1.2.1 Synthetic pesticides	27
1.2.1.1 History of synthetic pesticides	27
1.2.1.2 Synthetic pesticides and human health	34
1.2.1.3 Synthetic pesticides and environmental safety	37
1.2.1.4 Synthetic pesticides and insect resistance	37
1.2.2 Alternative measures	38
1.2.3 Natural pesticides	41
1.2.3.1 History of natural pesticides	41
1.2.3.2 Recent research and natural pesticides	42
1.3 Traditional application	42
1.3.1 Neem in Pakistan	44
1.4 Neem tree (<i>Azadirachta indica</i>)	45

1.4.1 Taxonomic history	48
1.4.2 Neem: origin and distribution	48
1.4.3 Biology or morphology of neem tree	49
1.4.4 Commercial formulation of neem	54
1.4.5 Neem terpenoid chemistry and bioactivity	58
1.5 Azadirachtin	65
1.5.1 Isolation of azadirachtin	65
1.5.2 Chemical properties of azadirachtin	68
1.6 Effects of azadirachtin on insects	71
1.6.1 External effect	75
1.6.1.1 Antifeedant effects	75
1.6.2 Internal effects	80
1.6.2.1 Insect Growth Regulatory (IGR) effect	81
1.6.2.2 Effect on reproduction	85
1.6.2.3 Effect on mortality	87
1.6.2.4 Effect on 'fitness'	88
1.6.2.5 Endocrine effects	90
1.7 Targets of azadirachtin	91
1.8 Aims of the project	93

CHAPTER 2 EFFECTS OF AZADIRACHTIN ON THE RATE OF INCORPORATION OF [³H]-LEUCINE INTO THE SPECIFIC TISSUES OF THE FEMALE ADULT LOCUST *SCHISTOCERCA GREGARIA*

2.1 Introduction	96
2.1.1 Protein metabolism of fat body	97
2.1.2 Proteins of haemolymph	97
2.1.3 Azadirachtin and haemolymph proteins	98

2.1.4	Cycloheximide and protein metabolism	98
2.1.5	Aims	99
2.2	Materials and methods	100
2.2.1	Insects	100
2.2.2	Insect saline	100
2.2.3	Treatment of insects	100
2.2.3.1	Azadirachtin treatment	100
2.2.3.2	Incorporation of tritiated leucine	101
2.2.3.3	Cycloheximide treatment	101
2.2.4	Uptake of radiolabelled leucine by the haemolymph and fat body proteins	102
2.2.5	Tissue sampling	102
2.2.5.1	Simultaneous collection of haemolymph and fat body	102
2.2.6	Determination of protein amounts and radioactivity	103
2.2.6.1	Extraction of proteins from haemolymph	103
2.2.6.2	Extraction of proteins from fat body	103
2.2.6.3	Estimation of protein	104
2.2.7	Determination of radioactivity into haemolymph and fat body proteins	105
2.3	Results	106
2.3.1	Time course of incorporation of [³ H]-leucine into the haemolymph and fat body proteins of locust <i>S. gregaria</i>	106
2.3.2	Effect of azadirachtin and cycloheximide on incorporation of the [³ H]-leucine on the haemolymph proteins	106
2.3.3	Effect of azadirachtin and cycloheximide on incorporation of the [³ H]-leucine on the fat body proteins	
2.4	Discussion	106

CHAPTER 3 SUPPRESSION OF LYSOZYME PRODUCTION BY AZADIRACHTIN IN SPECIFIC TISSUES OF THE DESERT LOCUST *SCHISTOCERCA GREGARIA*

3.1 Introduction	116
3.1.1 Insect immunity	116
3.1.1.1 Sources/Types of immune response	117
3.1.1.1.1 Humoral response	117
3.1.1.1.2 Cellular response	120
3.1.2 Variation in response	123
3.1.2.1 Haemolymph immune response in insects after infection	124
3.1.2.2 Induction of antibacterial haemolymph proteins	124
3.1.3 Azadirachtin and antibacterial proteins in insects	125
3.1.4. Lysozyme	126
3.1.4.1 Action of lysozyme	127
3.1.4.2 Function of lysozyme	127
3.1.4.3 Occurrence and distribution of lysozyme in insect tissues	127
3.2 Materials and methods	130
3.2.1 Insects	130
3.2.2 Insect saline	130
3.2.3 <i>In vivo</i> experiments	130
3.2.3.1 Antibacterial response of the locust	130
3.2.3.1.1 Induction of response	131
3.2.3.2 Tissue sampling	131
3.2.3.2.1 Haemolymph collection	131
3.2.3.2.2 Collection of haemolymph haemocytes and fat body	132
3.2.3.2.3 Preparation of fat body samples for lysozyme assay	132
3.2.3.3 Growth inhibition of <i>E. coli</i> (α DH5)	133

3.2.3.4 Assay of lysozyme activity	133
3.2.3.4.1 Agar gel lysoplate assay	133
3.2.3.4.2 Spectrophotometric method	134
3.2.3.4.3 Unit of lysozyme activity	137
3.2.3.5 Estimation of protein	137
3.2.3.6 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)	137
3.2.3.6.1 Coomassie blue staining of proteins after SDS-PAGE	138
3.2.3.7 Azadirachtin	138
3.2.3.7.1 Administration of azadirachtin to locust	138
3.2.3.8 Cycloheximide treatment	140
3.2.3.8.1 Administration of cycloheximide to locust	140
3.2.4 <i>In vitro</i> experiments	141
3.2.4.1 Incubation medium	141
3.2.4.2 Fat body preparations for <i>in vitro</i> incubation	141
3.2.4.3 Chemicals	141
3.2.4.3.1 Lipopolysaccharide	141
3.2.4.3.2 Cycloheximide	142
3.2.4.3.3 Ampicillin	142
3.2.4.3.4 Effect of azadirachtin on lysozyme production in fat body	142
3.2.4.4 Incubation of fat body	142
3.2.4.4.1 Lysozyme induction in fat body by LPS	142
3.2.4.4.2 Effect of azadirachtin on the I.PS-induced lysozyme in fat body	142

3.2.4.4.3 Effect of cycloheximide on the LPS-induced lysozyme in fat body	145
3.2.4.5 Assay for lysozyme in fat body	145
3.2.4.5.1 Spectrophotometric method	145
3.3 Results	147
3.3.1 Growth inhibition of a Gram-negative bacterium	147
3.3.2 <i>In vivo</i> experiments	147
3.3.2.1 Agar gel lysoplate assay for lysozyme activity using <i>Micrococcus lysodieticus</i> as a substrate	147
3.3.2.1.1 Agar gel lysoplate assay of <i>E. coli</i> -induced lysozyme activity in locust haemolymph	147
3.3.2.1.2 Agar gel lysoplate assay for haemolymph lysozyme activity of LPS-treated locust haemolymph	154
3.3.2.2 Changes in lysozyme activity in response to bacterial LPS injection	154
3.3.2.2.1 Humoral lysozyme-like activity estimation by spectrophotometry	157
3.3.2.2.2 Lysozyme activity changes in fat body after 24 hours of LPS injection	160
3.3.2.2.3 Lysozyme activity changes in haemocytes and plasma of locusts after 24 hours of LPS injection	160
3.3.2.3 Effect of azadirachtin on lysozyme activity	165

3.3.2.3.1 Effect of azadirachtin on lysozyme distribution in haemolymph	165
3.3.2.3.2 Effect of azadirachtin on lysozyme-like activity in fat body	165
3.3.2.3.3 Effect of azadirachtin on LPS-induced lysozyme activity in haemolymph plasma and haemocytes	165
3.3.2.3.4 Effect of azadirachtin on the LPS-induced lysozyme activity in fat body	172
3.3.2.4 Medium of azadirachtin administration	172
3.3.2.4.1 Effect of ethanol as a solvent for azadirachtin on lysozyme-like activity in plasma and haemocytes of <i>S. gregaria</i>	175
3.3.2.4.2 Effect of ethanol as a solvent for azadirachtin on lysozyme-like activity in fat body of <i>S. gregaria</i>	180
3.3.2.4.3 Effect of azadirachtin solubilised in cyclodextrins on lysozyme-like activity in plasma and haemocytes of <i>S. gregaria</i>	180
3.3.2.4.4 Azadirachtin solubilised in cyclodextrins and effect on lysozyme activity in fat body	185
3.3.2.5 Selection of appropriate drug delivery vehicle	185
3.3.2.6 Effect of LPS and azadirachtin on haemolymph protein concentration	188
3.3.2.7 Gel electrophoresis of haemolymph and fat body	188

	188
3.3.3 <i>In vitro</i> experiments	188
3.3.3.1 <i>In vitro</i> LPS-induction of lysozyme-like activity in fat body of <i>S. gregaria</i>	195
3.3.3.2 Effect of azadirachtin on the LPS-induction of lysozyme activity in fat body <i>in vitro</i>	195
3.4 Discussion	

Chapter 4 EFFECT OF AZADIRAHTIN ON VITELLOGENESIS IN *SCHISTOCERCA GREGARIA*

4.1 introduction	211
4.1.1 Role of fat body in vitellogenesis	212
4.1.2 Uptake of vitellogenin by ovary	212
4.1.3 Vitellogenesis in locusts	213
4.1.3.1 Humoral regulation of vitellogenesis in locust	214
4.1.4 Vitellogenin and vitellin as biomarkers	218
4.1.5 Azadirachtin and insect reproduction	218
4.1.5.1 Azadirachtin and vitellogenesis	219
4.1.6 Aims	220
4.2 Materials and methods	220
4.2.1 Insects	220
4.2.2 Insect saline	220
4.2.3 <i>In vivo</i> experiments	220
4.2.3.1 Tissue collection	220
4.2.3.2 Purification of vitellin	223

4.2.3.3 Protein estimation	228
4.2.3.4 SDS-PAGE	228
4.2.3.5 Native PAGE	230
4.2.3.6 Antiserum production	230
4.2.3.7 Azadirachtin treatment	230
4.2.3.8 Immunological methods	231
4.2.3.8.1 Ouchterlony immunodiffusion	231
4.2.3.8.2 Enzyme-linked immunosorbant assay (ELISA) for vitellogenin and vitellin	231
4.2.3.8.3 Generation of the titre curve by ELISA	232
4.2.3.8.4 Estimation of haemolymph and fat body vitellogenin by ELISA	232
4.2.3.8.5 Vitellin of locust ovaries estimated by ELISA	233
4.2.3.8.6 Effect of zadirachtin on vitellogenin level in haemolymph of female locust	233
4.2.3.8.7 Effect of azadirachtin on fat body vitellogenin of locusts	233
4.2.3.8.8 Effect of azadirachtin on vitellin of locust	234
4.2.3.8.9 Western blotting	234
4.2.4. <i>In vitro</i> experiments	235
4.2.4.1 Incubation medium	235
4.2.4.2 Chemicals	235
4.2.4.2.1 Azadiarchtin	235
4.2.4.2.2 Methoprene	235
4.2.4.3 Fat body isolation	235

4.2.4.4 Incubation of fat body	236
4.2.4.5 ELISA for vitellogenin of incubated fat body of female locust <i>in vitro</i>	236
4.3 Results	236
4.3.1 Vitellin purification	238
4.3.1.1 PAGE of purified vitellin	238
4.3.2 SDS-PAGE for vitellin or vitellogenin in locust tissues	238
4.3.2.1 SDS-PAGE for ovarian vitellin	238
4.3.2.2 SDS-PAGE of fat body for vitellogenin	243
4.3.2.3.SDS-PAGE of haemolymph for vitellogenin	243
4.3.2.4 SDS-PAGE of fat body and ovarian samples of locust for vitellogenin or vitellin	243
4.3.3 Immunological techniques for vitellogenin or vitellin estimation	250
4.3.3.1 Ouchterlony immunodiffusion	250
4.3.3.2 Quantification by ELISA	250
4.3.3.2.1 Generation of standard titre curve using increasing amounts of antigen subjected to ELISA	255
4.3.3.2.2 Vitellogenin estimation in haemolymph of female adult locusts from different age groups by ELISA	255
4.3.3.2.3 Vitellogenin estimation in fat body of female adult locust by ELISA	255

4.3.3.2.4 Vitellin in the ovaries of mature locusts	261
4.3.3.3 Effect of azadirachtin on vitellogenin and vitellin	261
4.3.3.3.1 Effect of azadirachtin on locust haemolymph vitellogenin	261
4.3.3.3.2 Effect of azadirachtin on vitellogenin level of female adult locust fat body	267
4.3.3.3.3 Effect of azadirachtin on locust vitellin of ovarian vitellin	267
4.3.3.4 Western blotting the effects of azadirachtin on vitellogenin in haemolymph and fat body and ovarian vitellin	267
4.3.4 <i>In vitro</i> experiments	272
4.4 Discussion	272
CHAPTER 5 FINAL DISCUSSION	282
REFERENCES	287

List of illustrations

		Page No.
Chapter 1		
Fig 1.1	The structure of some of the earlier insecticides sometimes called the "first generation" insecticides.	28
Fig 1.2	The structure of some of the so-called "second generation" insecticides.	30
Fig 1.3	Numbers and types of insecticides/acaricides introduced between 1950 and 1990.	32
Fig 1.4	The structure of some insect hormones or compounds that interfere with the normal development of insects, sometimes named as "third generation" insecticides.	35
Fig 1.5	Insecticides and their acute mammalian toxicities.	39
Fig 1.6	Traditional uses of the Neem tree "A Village Dispensary".	46
Fig 1.7	Neem tree in the plains of Arafat, Saudi Arabia.	50
Fig 1.8	Ripe (yellow) and immature (green) fruits of <i>Azadirachta indica</i> .	52
Fig 1.9	Some of the commercial products of neem.	55
Fig 1.10	In Sudan in 1959, during a locust swarm all the vegetation destroyed except Neem tree.	59
Fig 1.11	Limonoids from neem seeds and their putative biogenetic precursor, tirucallol.	62
Fig 1.12	An outline of different procedures involved in isolation of azadirachtin from ground neem seed kernels.	66

Fig 1.13	Resolution by high performance liquid chromatogram (HPLC) of the major terpenoids in the neem seed kernels.	69
Fig 1.14	The effects of azadirachtin are classified into two, on the basis of its response in insects.	73
Chapter 2		
Fig 2.1	Analytical high performance liquid chromatogram (HPLC) of azadirachtin.	100
Fig 2.2	A device for simultaneous collection of fat body and haemolymph from the body of the locust.	104
Fig 2.3	The rate of incorporation of [³ H]-leucine into the haemolymph and fat body of the untreated locust, <i>S. gregaria</i> .	109
Fig 2.4	The effect of azadirachtin and cycloheximide on the rate of incorporation of [³ H]-leucine into the proteins of haemolymph and fat body of the female adult locust <i>S. gregaria</i> .	111
Chapter 3		
Fig 3.1	An outline of different types of immune response in insects.	118
Fig 3.2	Variation in the shape of the different types of haemocytes in <i>Rhodnius prolixus</i> .	121
Fig 3.3	Lysozyme hydrolysis the β 1-4 glycosidic bond between c ₁ of N-acetylmuramate (NAM) and c ₄ of N-acetylglucosamine (NAG).	128
Fig 3.4	A sketch of bacteriological agar gel lysoplate setting with 1.5% of <i>Micrococcus lysodieticus</i> .	135
Fig 3.5	Growth inhibition test for a Gram-negative bacterium in nutrient agar gel by haemolymph of locust.	148

Fig 3.6	Lysozyme activity indication by lysis of <i>Micrococcus lysodiekcticus</i> . in an agar gel lysoplate assay.	150
Fig 3.7	The lysis of <i>Micrococcus lysodiekcticus</i> in an agar gel by haemolymph of female <i>S. gregaria</i> . The locusts were injected by haet-killed <i>E. coli</i> 24 hours before.	152
Fig 3.8	The lysis of <i>Micrococcus lysodiekcticus</i> in an agar gel by haemolymph of female <i>S. gregaria</i> . The locusts were injected by LPS 24 hours before.	155
Fig 3.9	The activity of lysozyme in the haemolymph of mature <i>S. gregaria</i> after injection with bacterial lipopolysaccharide.	158
Fig 3.10	The activity of lysozyme in the fat body of mature <i>S. gregaria</i> after 24 hours of injection with bacterial lipopolysaccharide.	161
Fig 3.11	The activity of lysozyme in plasma and haemocytes of mature female locusts <i>S. gregaria</i> .	163
Fig 3.12	The effect of azadirachtin on the distribution of activity of lysozyme in the haemolymph of the desert locust <i>S. gregaria</i> .	166
Fig 3.13	The effect of azadirachtin on the activity of lysozyme in the fat body of the desert locust <i>S. gregaria</i> .	168
Fig 3.14	The effect of azadirachtin on the LPS-induced activity of lysozyme in the plasma and haemocytes of the desert locust <i>S. gregaria</i> .	170
Fig 3.15	The effect of azadirachtin on the LPS-induced lysozyme activity in the fat body of the desert locust <i>S. gregaria</i> .	173

		Page No
Fig 3.16	The effect of injection of 10% (v/v) ethanol on the level of activity of lysozyme in the haemolymph of the desert locust <i>S. gregaria</i> and response to azadirachtin.	176
Fig 3.17	The effect of azadirachtin solubilised in 1, 5 and 10% (v/v) of ethanol on the level of activity of lysozyme induced by LPS in the plasma and haemocytes.	178
Fig 3.18	The effect of azadirachtin solubilised in 1, 5 and 10% (v/v) of ethanol on the level of activity of lysozyme induced by LPS in the fat body of <i>S. gregaria</i> .	181
Fig 3.19	The effect of azadirachtin solubilised in cyclodextrins on the LPS-induced level of lysozyme activity in plasma and haemocytes.	183
Fig 3.20	The effect of azadirachtin solubilised in cyclodextrins on the LPS-induced lysozyme activity in the fat body of <i>S. gregaria</i> .	186
Fig 3.21	The effect of various treatments on the concentration of total protein in the haemolymph of <i>S. gregaria</i> .	189
Fig 3.22	Gel electrophoresis of haemolymph and fat body samples of locusts after 24 hours of LPS injection.	191
Fig 3.23	<i>In vitro</i> , LPS-induction of lysozyme activity by fat body of mature female <i>S. gregaria</i> , after 24 hours of incubation.	193

	Page No	
Fig 3.24	<i>In vitro</i> , effect of azadirachtin on LPS-induced lysozyme-like activity by fat body of mature <i>S. gregaria</i>	196
Fig 3.25	A hypothetical illustration of humoral induction of lysozyme synthesis by haemocytes and fat body of <i>S. gregaria</i> in response to bacterial lipopolysaccharide.	202
 Chapter 4		
Fig 4.1	Schematic illustration of the hormonal regulation of reproduction in female locusts.	216
Fig 4.2	The possible target tissues of <i>S. gregaria</i> , whose proteins might be affected by azadirachtin	221
Fig 4.3	Scheme of vitellin purification from mature oocytes of <i>S. gregaria</i> .	224
Fig 4.4	Purification of vitellin by ion-exchange chromatography.	226
Fig 4.5	Polyacrylamide gel electrophoresis (PAGE) of main peak fractions eluted by DEAE cellulose column.	239
Fig 4.6	SDS-PAGE of ovary of adult locust <i>S. gregaria</i> .	241
Fig 4.7	SDS-PAGE of fat body of adult locust <i>S. gregaria</i> .	244
Fig 4.8	SDS-PAGE of haemolymph of adult locust <i>S. gregaria</i> .	246
Fig 4.9	SDS-PAGE of fat body and ovarian samples of adult locust <i>S. gregaria</i> .	248
Fig 4.10	Ouchterlony immunodiffusion of vitellogenin and vitellin.	251
Fig 4.11	Characterisation of antiserum by enzyme-linked immunosorbant assay (ELISA).	253

		Page No
Fig 4.12	Generation of standard titre curve of vitellin by ELISA.	256
Fig 4.13	ELISA estimation of vitellogenin in haemolymph of female adult locust <i>S. gregaria</i> .	258
Fig 4.14	Effect of azadirachtin on vitellogenin content of haemolymph of 15 day old locusts estimated by ELISA.	263
Fig 4.15	Effect of azadirachtin on vitellogenin level of haemolymph of 19 day old locusts assayed by ELISA.	265
Fig 4.16	Immunodetection of the effect of azadirachtin on vitellogenesis in <i>S. gregaria</i> as revealed by SDS-PAGE blotting to nitrocellulose membrane and reaction with anti-vitellin sera.	270

	List of tables	Page No
Chapter 1		
Table 1.1	Natural compounds as commercial pesticides in world markets.	43
Table 1.2	Commercially produced neem-based pesticide products.	57
Table 1.3	Congeners of azadirachtin. Pure azadirachtins as isolated from 27 kg neem seeds.	64
Table 1.4	Number of neem-sensitive insect pest species arranged by order.	72
Table 1.5	The antifeedant effects of azadirachtin (AZAD) and 'Morgosan-O' (M-O) (0.3% AZAD) on a selected range of insect pest species in laboratory and green house trials.	76
Table 1.6	The insect growth regulatory (IGR) effect of azadirachtin on insects after injection, oral cannulation or topical application.	82
Chapter 3		
Table 3.1	Reference proteins of known Molecular weight.	139
Table 3.2	Step I: Induction of lysozyme by LPS in fat body <i>in vitro</i> .	143
Table 3.3	Step II: The effect of azadirachtin on the LPS induced induced lysozyme in fat body <i>in vitro</i> .	144
Table 3.4	Step III: The effect of azadirachtin and cycloheximide on the lysozyme induction in fat body by LPS <i>in vitro</i> .	146
Chapter 4		
Table 4.1	Reference proteins of known Mr.	229
Table 4.2	The effect of azadirachtin on the <i>in vitro</i> fat body vitellogenesis.	237
Table 4.3	Vitellogenesis in the fat body of female adult locusts of different age groups.	260
Table 4.4	Vitellin in the ovarian samples of locusts of different age.	262
Table 4.5	Effect of azadirachtin on vitellogenesis of fat body of female locust <i>S. gregaria</i> , after 48 hours of treatment.	268
Table 4.6	Effect of azadirachtin on the ovarian vitellin of female locust after 48 hours of treatment.	269
Table 4.7	The effect of azadirachtin on the <i>in vitro</i> fat body vitellogenesis of <i>S. gregaria</i> after 24 hours of incubation.	273

List of abbreviations

IGR	Insect growth regulatory
LPS	Bacterial lipopolysaccharide
PB	Phosphate buffer
PBS	Phosphate buffer saline
BSA	Bovine serum albumin
SD	Standard deviation
SDS	Sodium dodecylsulphate
HPLC	High performance liquid chromatography
JH	Juvenile hormone
TCA	Trichloroacetic acid
AZAD	Azadirachtin
PAGE	Polyacrylamide gel electrophoresis
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
TBS	Tris-buffer saline
HRP	Horse radish peroxide
ELISA	Enzyme-linked immunosorbant assay

Chapter 1

Introduction

1.1 Agriculture and insects

Settled agriculture, depending on relatively few crops, was initiated about 10,000 years ago and a huge part of the world's food is produced by monoculture crops today (Smith, 1998). According to a press release by the Assistant Director General of International Labour Organisation in 1997, a majority of the world's work force outside USA and Europe is involved in agriculture, (62% in Asia and 63% in Africa), (Taqi, 1996). Up to now 248,000 plant species have been identified, and more than 150 of them are under commercial cultivation. 90% of the world's food is supplied by 20 crops: just four of them - rice, maize, wheat and potato - supply more than half of the world's food consumption, (Zamora, 1996). The benefits of large scale monoculture has brought in its wake the problems of insect pests. Almost all plants are susceptible to insect attacks.

Agriculture has a strong impact on the economy of the world, so it is important to protect all the crops of any country from pests. Crops, grown either as food or as a cash crop, are liable to insect attack both when they are growing and also in stored form. For instance in China, during 1993-1995 when there was an outbreak of cotton bollworm, *Heliothis armigera* H. (Lepidoptera), this cash crop was reduced by 30% and total estimated losses from the pest damage were 100-120 million US dollars (Wu *et al.*, 1997).

The practice of storing food grains began about 4500 years ago as a safeguard against poor harvests and famines. Not only cash crops but stores of food grains are also vulnerable to attacks by insects. Crop failures due to adverse weather conditions and/ or pest attacks on growing or stored crops were the main cause of famines (Schmutterer, 1995). According to a survey report, one third of the world's food crop is destroyed annually by insect pests during growth, harvest and storage (Saxena *et al.*, 1989). 35% of all crops are lost during growth and 20% after harvest just because of

pests (Fox, 1997). Food losses due to insect pests in developing countries of Asia and Africa are higher. Arthropods, more specifically the insects, are the earth's most numerous, diverse and biologically successful group of animals. There is evidence that several species of insects attacked granaries and other food storage structures in ancient times, e.g in ancient Egypt, (Levinson and Levinson, 1985), and this is still the case today.

1.2. Protective measures

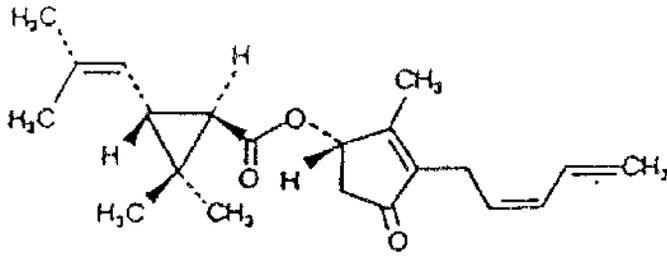
With the advancement of science and technology every effort is made to improve the quality of life for man on this planet. Pest control management to minimise the food losses is one of them. For thousands of years, until the middle of the 20th century, the only source of pesticides was natural plant extracts. The latter half of the this century, however, has been dominated by various synthetic chemicals, in which there is now a huge world-wide trade.

1.2.1. Synthetic pesticides

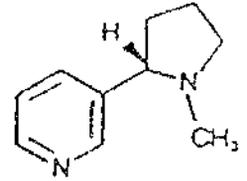
1.2.1.1. History of synthetic pesticides

The "first generation" of insecticides were plant metabolites, originating in folk-use, and introduced on an increasing scale about 100 years ago. They were natural insecticides such as pyrethrins, nicotine, rotenone or ryanodine, (Fig 1.1). The efficiency and selectivity of these products against insects was somewhat limited, but they were the best compounds available until about 1940. Around this time the synthetic insecticides, starting with DDT, were introduced as the "second generation" of insecticides (Forgash, 1984). They were very efficient, neuro-active compounds, e.g the chlorinated hydrocarbons, the organophosphates, the carbamates and the pyrethroids, (Fig 1.2). These developments had a profound impact on agriculture. These groups of toxic compounds dominated the insect market till 1975, as indicated in Fig 1.3.

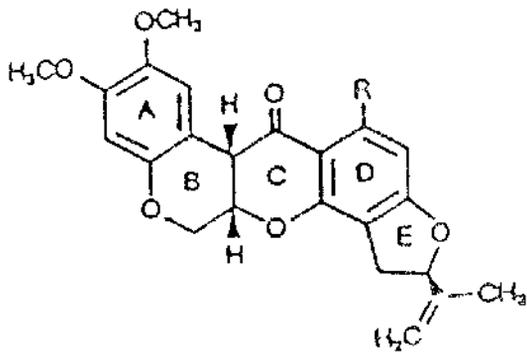
Fig 1.1 The structures of some of the earlier insecticides sometimes called the "first generation" insecticides.



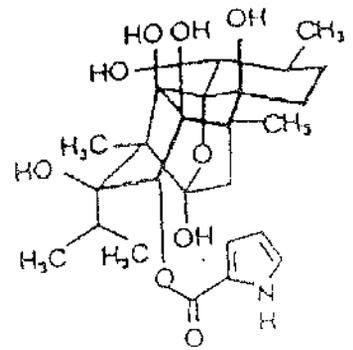
Pyrethrin I



Nicotine



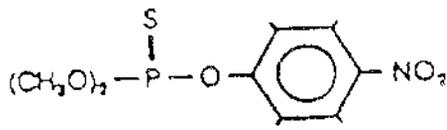
Rotenone



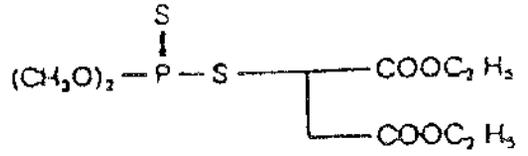
Ryanodine

Fig 1.2 The structure of some of the so-called "second generation" insecticides.

- a) Organophosphorus esters**
- b) Carbamates**
- c) Chlorinated hydrocarbons**

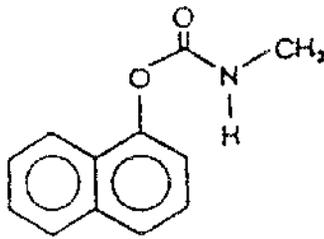


Methylparathion

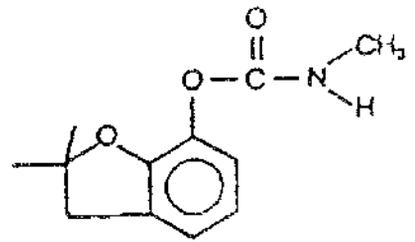


Malathion

a

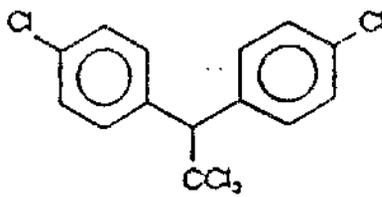


Carbaryl

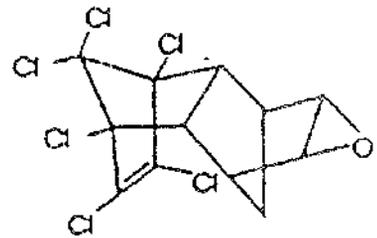


Carbofuran

b



DDT

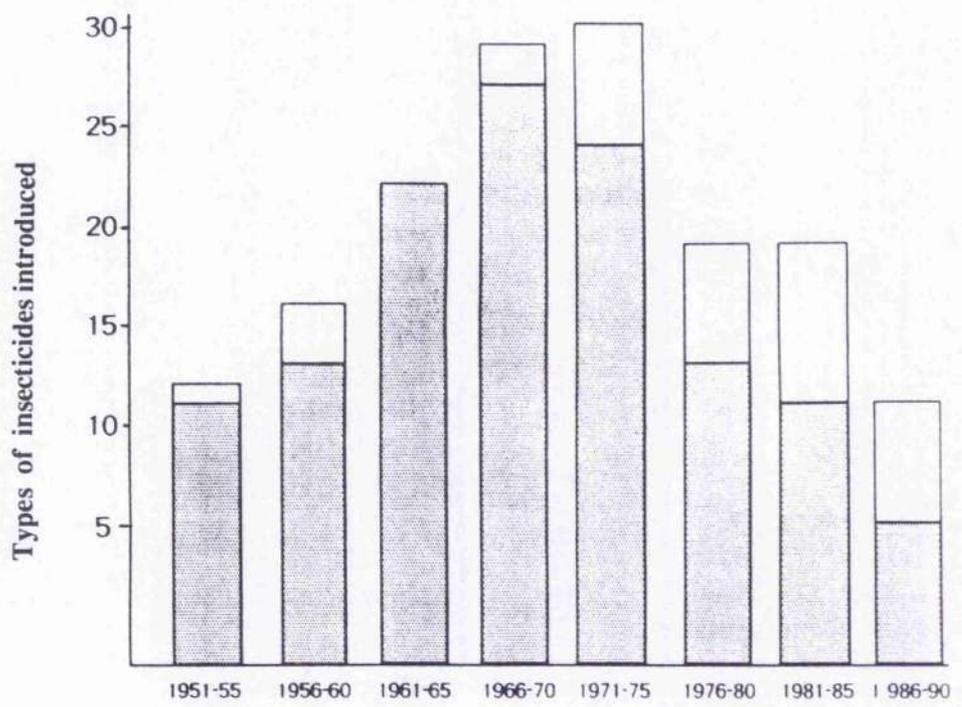


Dieldrin

c

Fig. 1.3 Numbers and types of insecticides/acaricides introduced between 1950 and 1990. Data from Voss and Neumann, (1992).

-  Neurotoxicants
-  Others



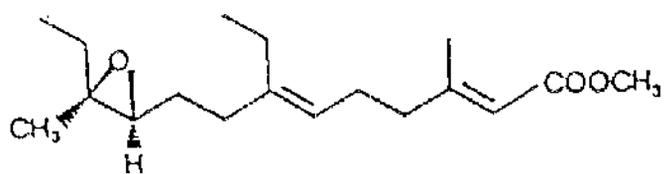
Organophosphate insecticides have been used in crop production and live-stock husbandry worldwide for over 45 years. Initially, the broad spectrum activity and high potency was important, but later other aspects such as reduced toxicity against vertebrates and environmental stability became desirable. After the achievement of potency and efficacy, research began to focus more on selectivity and environmental safety and the challenge then became health and the environment.

Then the "third generation" of insect control agents, such as growth regulators like juvenile hormone (JH I) and β -ecdysone, and dimilin and precocene II was the outcome of research, (Fig 1.4). These compounds, based on the unique biology of insects, are generally selective and environmentally safe, but act slowly, allowing the insects to consume considerable amount of their host plant before they die. They have so far made a limited commercial impact.

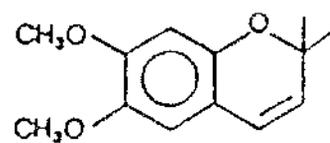
1.2.1.2. Synthetic pesticides and human health

For a number of reasons including risk to human health, the past practice of neurotoxic pesticide use cannot be sustained. Pesticides are no exception to the experience of mankind that beneficial inventions are never free of risks. Overuse or misuse of pesticides compromises human health, contaminates water and soils, and damages the ecosystems (Islam, 1996). It has been reported that exposure to pesticides and agrochemicals constitutes one of the major risks faced by farm workers. Developing countries consume more than 20% of the world's production of agrochemicals, which are responsible for approximately 70% of the total cases of acute poisoning in the working population, i.e. more than 1.1 million cases (Taqi, 1996). The World Health Organisation, a UN agency, estimates the total cases of pesticide poisoning worldwide at between 2 and 5 million workers each year, of which 40,000 are fatal (press release, 1997). The use of organophosphates can cause accidental intoxication in unprotected and uneducated users especially in developing countries.

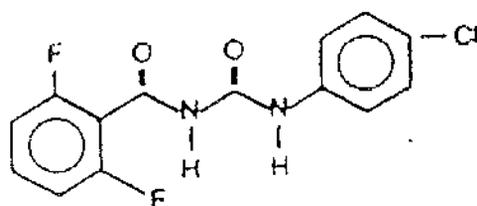
Fig 1.4 The structures of some insect hormones or compounds that interfere with the normal development of insects, sometimes named as "third generation" insecticides.



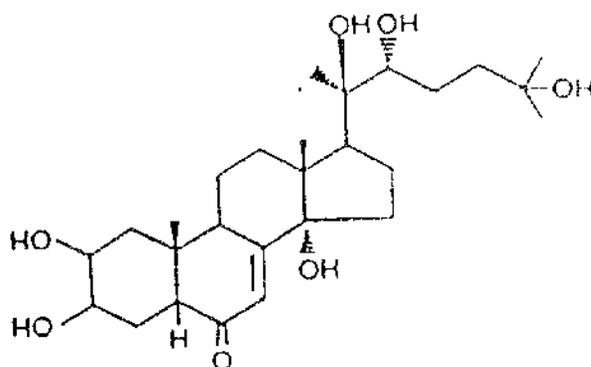
JH I



Precocene II



Dimilin



β -Fedysone

Not only in developing countries but in developed countries as well, the use of pesticides is a cause of threat to human health. In Britain for example, sheep dips are classed as veterinary medicines in which organophosphates are a major component. There are 95,000 sheep farms in the U.K. Despite the advice on protective clothing by the manufacturers of sheep dips, a study produced by the Institute of Occupational Medicine of sheep-dipping practices in the UK found that, very few operators wore the protective clothing and many workers appeared to get soaked in sheep dips (Richards *et al.*, 1996). According to a report, organophosphates are capable of producing several subacute, delayed and chronic neurological neurobehavioural and psychiatric syndromes in Britain (Jamal, 1997). Similarly in USA, according to a study by an Environmental Working Group, the drinking water of 4.3 million Americans had concentrations of pesticides exceeding the standards of acceptable risk, and the worst example was in Ohio, where one glass of water contained estimatable amount of 10 toxic chemicals (Kennedy, 1997).

1.2.1.3. Synthetic pesticides and environmental safety

The long-term use of these neuro-active insecticides can also affect aquatic and terrestrial wildlife, and particularly kill beneficial insects such as bees, pest-parasites and pest-predators. For example, in USA, honey bee pollination is 60-100 times more valued than the honey they produce, and the bees are responsible for pollination of one third of food crops of American diet. In California, the pesticides are reducing the bee population by 2%/year. More than half of the bee population has been lost in the last 50 years and 25% in the last 5 years, due to pesticide exposure (Abramovitz, 1997).

1.2.1.4. Synthetic pesticides and insect resistance

Resistance against synthetic insecticides, is a worldwide phenomenon and one of the consequences of excessive use of pesticides. In 1990s many synthetic pesticides are being withdrawn because of an increase in the number of insects developing resistance

against them (Ruskin, 1992), or due to environmental health hazards (Jonsson, 1996). Ahn, Y.J. et al., (1986) reported an extremely high level of DDT and pyrethroid resistance in the Japanese housefly, *Musca domestica*. In Indonesia, 56 insecticides are banned from being used on rice by the Government because of resistance development in the leaf-hopper, *Nilaparvata lugens* infesting rice by overuse of pesticides (Dinham, 1996). DDT was banned in USA in 1972 due to insect resistance, and concern about health and environmental effects (Sierse, 1997). In Sweden the use of herbicides, pesticides and fungicides has been cut by 70% since 1985 for the same reasons (Jonsson, 1996).

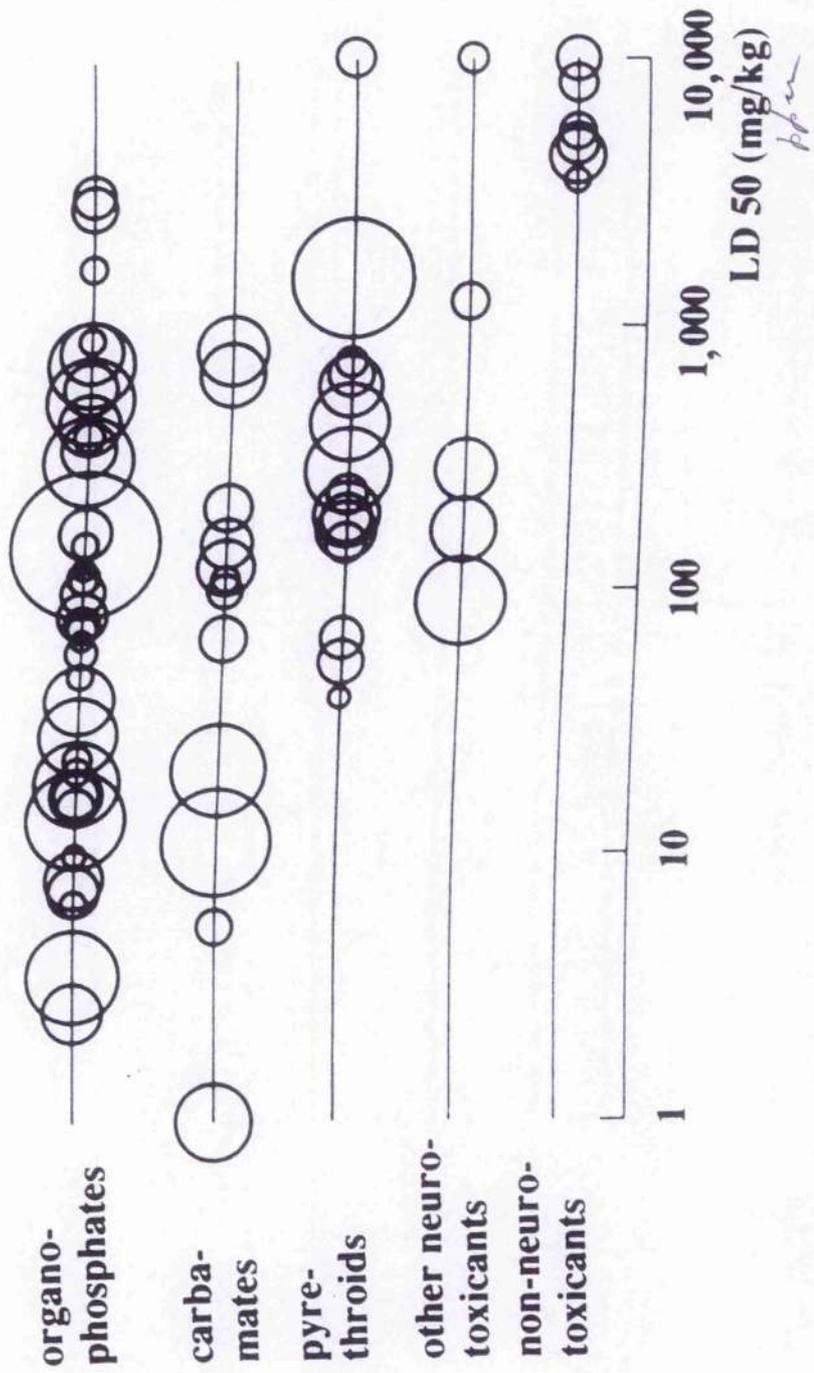
1.2.2. Alternative measures

The genetic and biochemical adaptability of insects threatens to undermine the effectiveness of existing pesticides, so there is a need to discover safe, biodegradable substitutes for the synthetic pesticides. The media have driven public belief that most crop protection chemicals can be replaced by less toxic technologies, including biological products (Jacobson, 1988), and the crop protection industry has shifted its efforts to these new methods.

The ongoing shift to new insecticides, however, contrasts with the present situation in the pesticide market as reported in Pesticide Manual 1990 by CIBA-GIEGY marketing, (Fig 1.5). It was estimated that the non-neurotoxic insecticides have not gained even a 10% share of the worldwide insect control market. The large number of neuro-active insecticides continued to dominate the market until 1990 (Neurotox, 1991), but real safety problems limit the efficacy of neuro-active insecticides.

Research on insecticides having passed through several "ages", is now in a renaissance of integrating chemical and biological methods for sustainable pest control, coupled with human safety. For example, to lessen dependence on synthetic chemicals, an Integrated Pest Management Management (IPM) approach for grasshopper control has

Fig 1.5 Insecticides and their acute mammalian toxicities, (circles: proportional to sale). (Voss and Neumann, 1992).



been encouraged in the United States with emphasis on biological control as an important component (Prior and Streett, 1997).

1.2.3. Natural pesticides

1.2.3.1. History of natural pesticides

Since the neurotoxic synthetic pesticides are proving to be such a mixed blessing, there has in the last few years been a revived interest in more traditional plant metabolites. Plants have an excellent track record in providing novel leads for crop protection, particularly in the field of insecticides (Rice *et al.*, 1998). It is well known that some insecticides of plant origin have been in use for a long time, such as pyrethrum obtained from the flowers of *Chrysanthemum cinerariifolium*, whose useful property was known during the time of the Persian king, Darius the Great (521-486 B.C.), (Schmutterer, 1995). The plant kingdom is a vast store house of chemical substances manufactured and used by plants for defence from attacks by insects, bacteria and birds (Jacobson, 1988). Many plant-derived compounds from several chemical classes have been found to possess antifeedant and/or repellent activity against a number of insects. The Chinese are credited for discovering the insecticidal properties of Derris species (Feinstein, 1952). In 970 A.D. The Arab scholar Abu Mansur listed 584 natural materials possessing pharmacological and poisonous properties, many with insecticidal qualities (Schmutterer, 1995). Similarly, tobacco plant preparations, containing nicotine, have been used for nearly two centuries (Frear, 1943). Approximately 700 plant species have reportedly been used in different parts of the world for pest control, with uncertain efficacy (Secoy and Smith, 1983). A list of approximately 2400 plant species, having pest control properties has been compiled (Grainge and Ahmed, 1988).

1.2.3.2. Recent research and natural pesticides

Research on the biological activity and chemistry of plant origin antifeedants has been emphasized because of their biodegradable nature and relative safety towards beneficial organisms in the environment, (including, of course, Man), (Norris, 1986). During past few years some of plants that have shown potential in pest control are, for instance: Chinaberry, (*Melia toosenden* L. and *M.azedarach* L.); Neem, (*Azadirachta indica*); West Indian mahogany, (*Swietenia mahogani* Jacq); French Marigold, (*Tagetes patula* L); African Marigold, (*Tagetes erecta* L); Custard Apple, (*Annona squamosa* L) and Thunder Gold-vine, (*Tripterygium wilfordii* Hook). Few of these are of commercial interest, although some have their place in the rather short list of commercially significant pesticides of natural origin (Table 1.1).

Among these plant products, azadirachtin is most effective of many terpenoid compounds from the seed extract of a tree called "Neem", *Azadirachta indica*, or the Indian lilac. The tree itself displays a wide, and still growing, variety of favourable properties, which are suitable for agriculture, industrial and commercial exploitation. The major part of research activities concern its use in agriculture, especially its effects on insects, (Schmutterer, 1995). At present, the use of such extracts from different parts of the tree is largely restricted to local farmers in semiarid, tropical areas around the globe where the tree grows, although there is an increase in commercial use of neem products in Sweden, Switzerland, Italy and USA.

1.3. Traditional applications

Neem's traditional use for pest control has been continued in south Asia over many generations. Now there is a need to make use of centuries of knowledge learned from practices of our forefathers. It is observed that in last thirty five years there is a worldwide awakening, especially among researchers, to try to understand traditional customs, values and practices.

**Table 1.1 Natural compounds as commercial pesticides in world markets
(Naumann, 1994)**

Natural product	Origin	Example of application
Pyrethrum	plant	garden pests
Rotenone	plant	garden pests
Nicotine	plant	green house
Avermectin	bacterial	spider mites
B.T. toxins	bacterial	rice fungal pests
Azadirachtin	plant	fungal pests garden pests & agricultural pests etc.

This is a wealth of knowledge, western scientists want to tap. Researchers learned neem's pest-control properties by observing such practices.

1.3.1. Neem in Pakistan

Coming as I do from Pakistan, I am aware since childhood, of the many uses to which neem extracts have been traditionally put. In the two warmer provinces of Pakistan, (Punjab and Sindh), the neem is traditionally used to protect stocks of wheat, rice or other stored grains from insects at farm levels, and as a household remedy for treatment of common ailments since generations untold. Among my personal observations of neem's traditional uses are examples both as an insect repellent and as a folk-medicine.

Traditionally, the following methods are in use to protect food from insects:

- The dry neem leaves are used for pest control in simple grain stores at domestic levels.
- The fresh neem leaves are finely ground with water to make a paste which is spread inside the earthen container used to store the grain. This earthen container is called a "Padolla" in the Punjab and a "Palli" in Sindh. The stored grain remains untouched by insects for a period of six months.
- In extremely hot weather a variety of fresh fruit is packed in baskets lined by neem fresh leaves for display to keep the fruit fresher for a long time in fruit market, giving an indication of antifungal properties.
- The dry neem leaves are burnt by poor people in front of their huts so that fumes can drive away the mosquitoes.
- The leaves are dried under the shade and then used to protect the woollen garments from damage by moth larvae.

The neem tree also serves as the source of several many home remedies, which are thus readily available to the poor villagers who cannot afford to buy expensive medicines. Its medicinal value can be seen in the following home remedies:

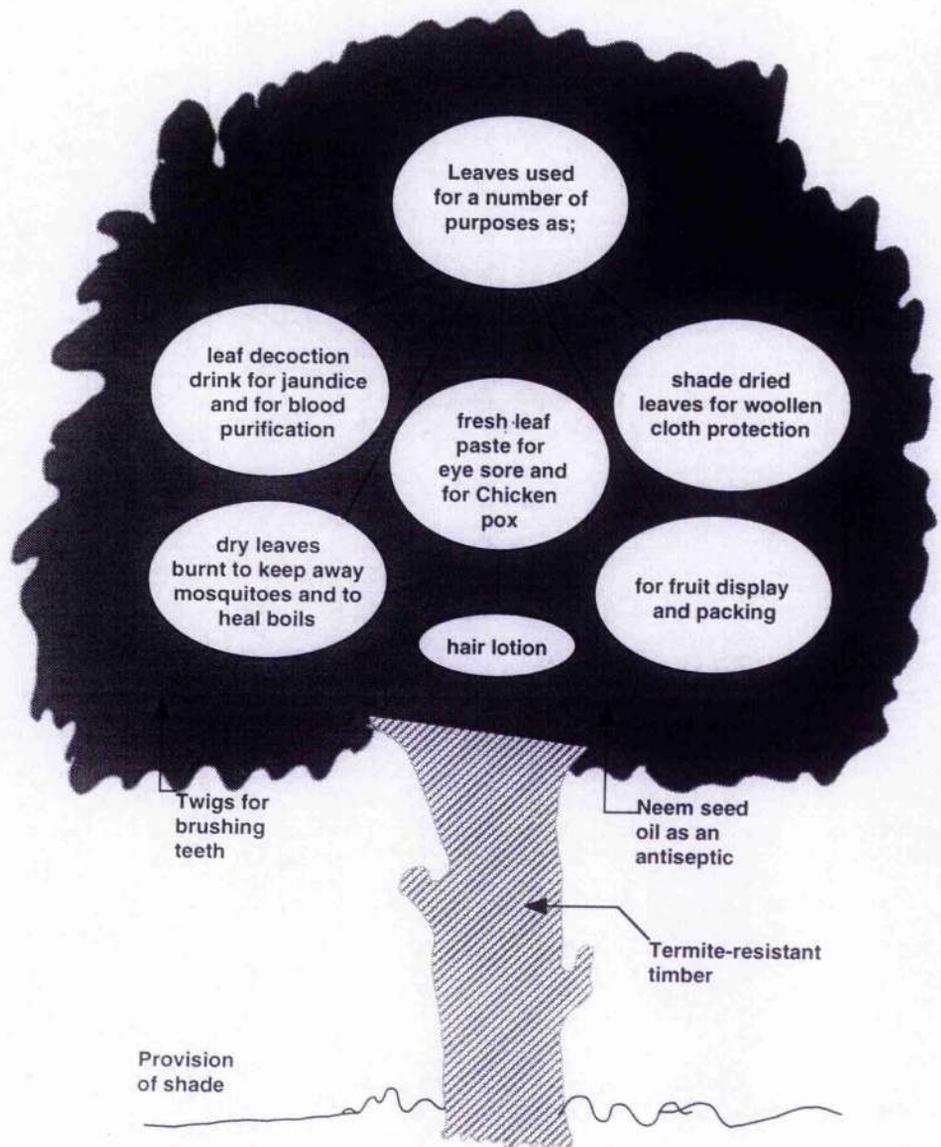
- To drink neem leaf decoction first thing in the morning is thought to be a good remedy to cure jaundice, suggesting some anti-viral properties.
- The fresh neem leaves are boiled in water and the water is then used as shampoo for hair-washing and protection against ectoparasites like head lice, *Pediculus capitis*.
- Green leaves are used as a fodder for cattle, and appear to combat worm infection in livestock serving as an antihelminthic for cattle.
- Neem oil is considered as an antiseptic and used directly against various skin infections, like ringworm and scabies. There is of course a large manufacture of soap from the oil, the use of which safeguards the skin from microbial infection.

The medicinal uses of different parts of the tree are based on, "Tibb-i-Unani" a type of treatment by herbs. The person practicing the herbal and mineral origin medicines, is called a "Hakim", and is an important personality of the small village (Sharma, 1991). Some of these activities are being substantiated by current research. Due to the medicinal significance of neem tree it is considered as "A Village Dispensary" (Fig 1.6). In the following section, the biology and wider uses of the tree will be considered.

1.4. Neem tree (*Azadirachta indica*)

Azadirachta indica A.Juss. (Family Meliaceae), also commonly known as, "Neem". "Neem" (which means "blessing" in Urdu and Hindi), is a subtropical tree native to arid areas of Pakistan and India. The tree provides shade to give solace from the severe summer heat. It may truly be called a tree of south Asia's masses, as every family likes to have at least one tree near the house. It grows well on marginal soils with low precipitation and in intense heat. This might be the reason that the Muslim travellers and settlers who came to India from Western Asia more than a thousand years ago named it as, "*Azad dirakht-i-Hind*" (Free or Noble Tree of India). Neem's botanical name, *Azadirachta indica*, is derived from this title (Ahmed *et al.*, 1989).

Fig 1.6 Traditional uses of Neem tree "A Village Dispensary".



1.4.1. Taxonomic history

Azadirachta indica was first described as *Melia azadirachta* by Linnaeus in his list of species, *Plantarum* (1753). *Azadirachta* was first distinguished from *Melia* by de Jussieu (1830), but both are closely related with only small differences, (Vander *et al.*, 1991). Nowadays, *Azadirachta indica* is considered to belong to the Melieae, one of five tribes of the subfamily Melioidea, within the family Meliaceae.

1.4.2. Neem: origin & distribution

Neem is thought to have originated in Assam and Burma, but now it is well established in at least thirty tropical and semi-tropical countries of the world (Ruskin, 1992). Wherever people from the Indian sub-continent have settled, they have tried to take the tree with them if the climate was suitable.

During this century, it has been introduced into many parts of Africa, especially in East Africa. Now it is widely cultivated in the African continent as in Ethiopia, Somalia, Kenya, Tanzania, Mozambique, Mauritania, Togo, Ivory Coast, Cameroon, Nigeria, Guinea, Ghana, Gambia, Sudan, Benin, Mali, Niger, Burkine, Fasa, Chad, and Senegal particularly in low rainfall regions (Jacobson, 1988). There is even a small neem industry centered in Nairobi, which has just received registration for its first neem-based products (Strang, personal communication). It is currently under cultivation in plantations of West Africa and the Caribbean Islands, as well as parts of central and southern America, Australia and India (Jacobson, 1986).

In the Pacific, it is known that in 19th century immigrants took the tree from the Indo-Pak region to Fiji and now it has spread to other islands in the south Pacific (Jacobson, 1988). The Philippines has also begun wide scale plantings of neem for fuelwood and pesticide production

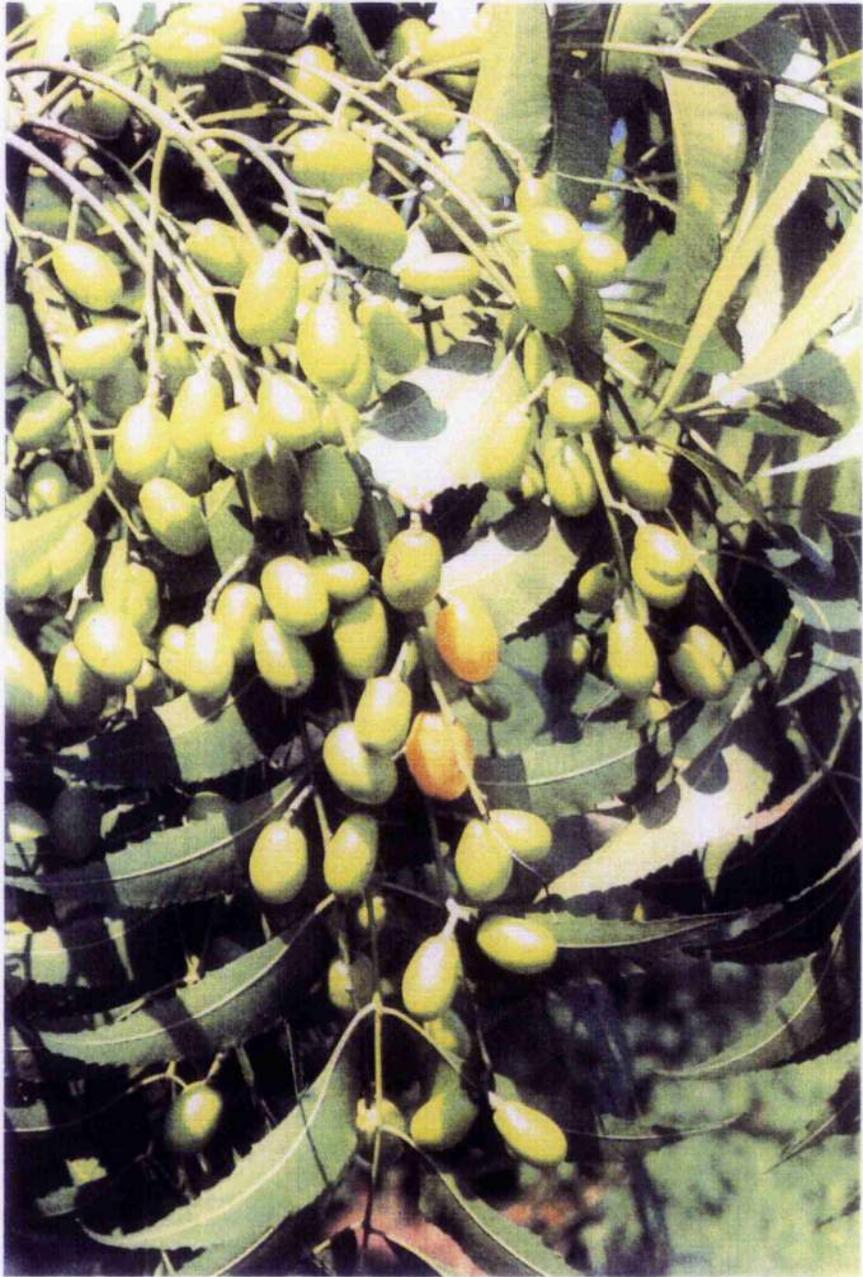
The neem tree was chosen for large scale planting on the Plains of Arafat near Mecca in Saudi Arabia where 2-3 million muslim pilgrims congregate annually during the Hajj, for pilgrimage to Mecca. It is probably the world's largest neem plantation

Fig 1.7 Neem tree in the plains of Arafat, Saudi Arabia.

This is the place where the prophet Mohammed (pbuh) delivered his farewell sermon some 1,400 years ago. A city of thousands of tents spring up each year to accommodate 2-3 million muslim pilgrims who camp there annually for the Hajj. The tree provides shade and relief from the intense heat.



Fig 1.8 Ripe (yellow) and immature (green) fruits of *Azadirachta indica* (Schmutterer, 1995).



Neem grows well from sea-level to over 670m (2000 ft) and can be established in hot and dry regions without irrigation. It grows where maximum shade temperature may be as high as 49°C (120°F) but it does not stand excessive cold (Ruskin, 1992).

1.4.4. Commercial formulations of neem

The most important neem products used in numerous trials are as follows: neem seed oil and neem seed cake; aqueous neem seed kernel and leaf extracts; alcoholic seed kernels and leaf extracts; enriched, formulated seed kernel extracts (Fig 1.9). Almost all of these have been used from the most ancient times. The use of large amounts of neem oil for soap-making dates from after the Second World War. As the science of neem has progressed, there is increasing use of pure and semi-pure extracts. Neem seed oil is pressed from neem seeds in the cold by using an oil presser or prepared by extraction with alcohols or less polar solvents. Small amounts of oil can be obtained by kneading neem seed powder by hand after adding some water (Dreyer, 1987). The very large-scale oil extraction in South India depends on high temperatures (>100°C) to ensure good recovery of the oil. As azadirachtin has been shown to be heat-sensitive (Strang, unpublished results), oil produced by such large-scale methods has little or no azadirachtin. This may account for the low and variable concentrations found in many of the poorer commercial products from India.

Neem seed cake is the residue of seed kernels after the oil has been removed. This cake is a useful organic fertilizer, containing some nitrogen (2-4%) as well as some azadirachtin and other active compounds. It has been reported to reduce nitrogen loss from the soil and to encourage nitrogen fixation when applied to rice fields in India, and in Pakistan. It has also been used to maintain the soil fertility when added to the water in rice fields in Pakistan (Akhter *et al.*, 1998). The first commercial product from neem was "Margosan-O" (W.R. Grace & Co., Cambridge, MA, USA), based on the oil, and registered in USA in 1985. Several commercial formulations and semi-commercial preparations are now available Table 1.2.

Fig 1.9 Some of the commercial products of neem. The figure indicates different applications of neem-based products.

Commercial neem products

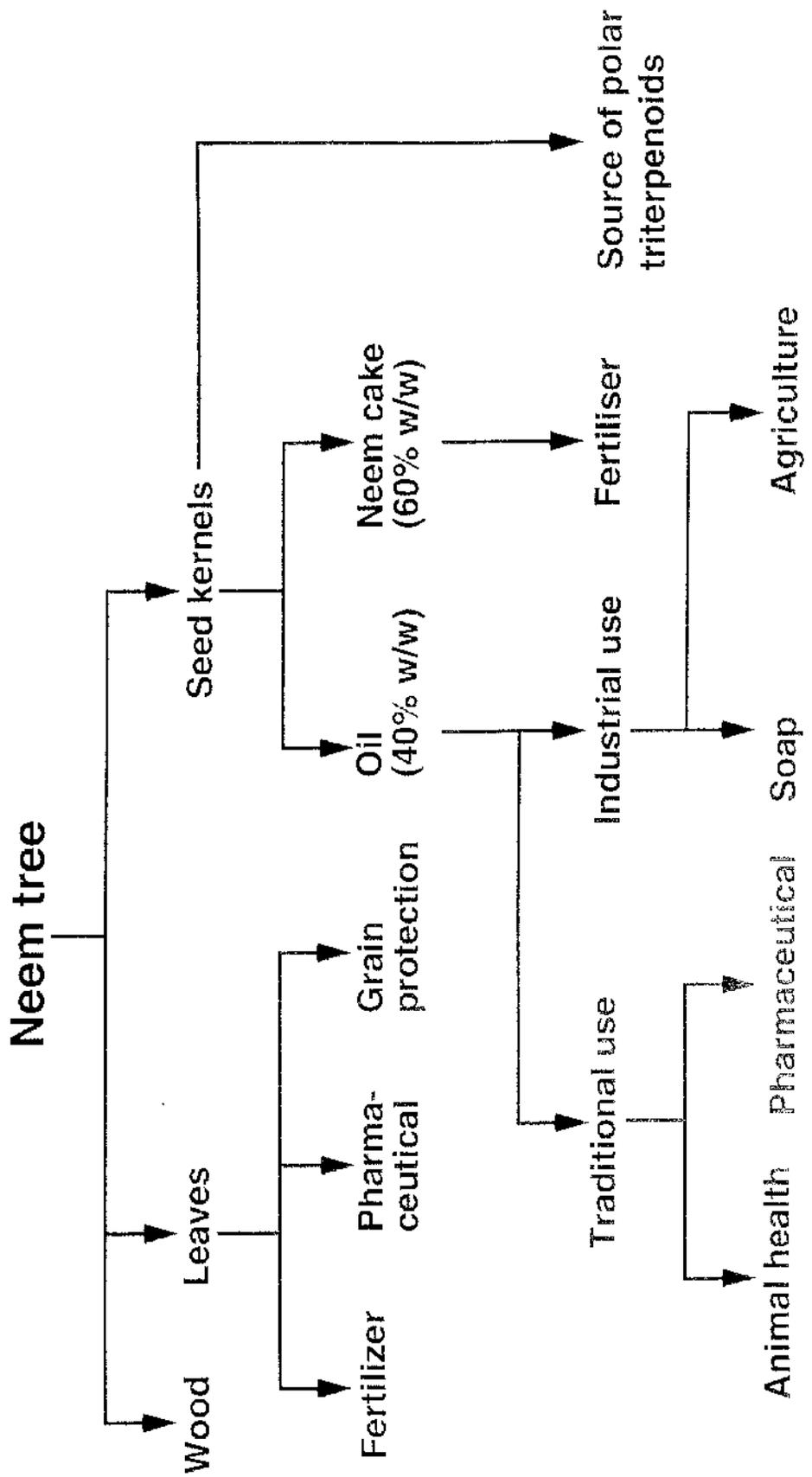


Table 1.2 Commercially produced neem-based pesticide products (Parmar *et al.*, 1993; Schmutterer, 1995).

Trade Name	Active ingredient or source	Application
Morgosan-O	Azadirachtin	Insecticide
NeemAzal	Azadirachtin	Insecticide
Azatin-EC	Azadirachtin	Insecticide
Mecn	Azadirachtin	Antifeedant & IGR*
Field Marshal	Azadirachtin	Antifeedant
Moskit	Oil	Mosquito repellent
Neemgold	Azadirachtin	Antifeedant
Nimba	Kernel based	Pesticide
Wellgro	Kernel powder	Antifungal and antiviral
Align TM	Azadirachtin	Insecticide
Azatin [®]	Azadirachtin	Insecticide
Turplex TM	Azadirachtin	Pesticide

* IGR: insect growth regulator.

Neem seed oil is often a starting material for such insecticides and its biological activity is closely related to its azadirachtin content (Isman *et al.*, 1990). As indicated above, this is one of the drawbacks of the oil, as the azadirachtin content of oil is often low, and it certainly varies from batch to batch. Several companies in Germany, Canada, India, Thailand, Australia and the United States have, however, developed neem oil products for feed crops (Schmutterer, 1995). Margosan-O has been registered by EPA in 1993 for food crops.

In the last few years an enriched neem product has been developed by Trifolio-M GmbH (Lahnau, Germany), using a simple water-extraction method to enrich the extract for the most potent compound azadirachtin, and this will be further discussed below. A crude concentrate called NeemAzal, containing 30% azadirachtin, is formulated into a variety of products with defined contents of azadirachtin. Formulations are currently in use in Switzerland, where it is registered, and it will soon be also registered in Germany (Schulz *et al.*, 1997). Examples of some commercial products are shown in Table 1.2.

1.4.5. Neem terpenoid chemistry and bioactivity

What is the nature of the active compounds in the neem extracts? Chemical study of the metabolites of the neem tree, especially the complex terpenoids, dates back to 1880-1890, when Indian chemists took up the isolation of active principles from its seed and other parts (Jacobson, 1988). The tree elaborates a vast array of biologically active compounds, which are chemically diverse and structurally complex. All of the well-characterised compounds identified in neem belong to the class of "triterpenoids" or more specifically, "limonoids". The bitterness of neem is due to the occurrence of these limonoids (Siddiqui *et al.*, 1986). The term, "Limonoid", is derived from Limonin, the first tetranortriterpenoid obtained from citrus bitter principles in 1841. Out of over 300 limonoids known today about one third are accounted for by neem and china-berry alone (Jones *et al.*, 1989).

Neem attracted the attention of a German scientist, Heinz Schmutterer in 1959, during a locust swarm in Sudan, when all the vegetation was destroyed except neem trees (Fig 1.10), (Uvarov, B, 1977; Schmutterer, personal communication). This incident dramatically demonstrated the presence of an antifeedant quality in the tree.

An antifeedant compound prevents feeding on the leaves in which it is present and acts to deter insects (Munakata, 1975). The recent interest in neem extracts as crop protectants dates back to the work of Pradhan *et al* (1962), who reported that diluted aqueous seed extracts completely prevented feeding by the desert locust *Schistocerca gregaria* (Nisbet, 1992). Later in 1968 the most active antifeedant principle from neem seeds was isolated and named "Azadirachtin", (Butterworth and Morgan, 1968), and it was a highly polar compound.

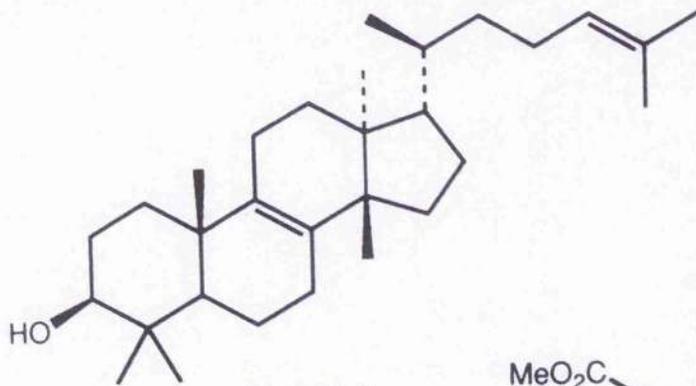
It has long been known that the maximal insecticidal activity of the tree is in the seed kernels (Siddiqui *et al.*, 1942). In the seeds it would appear that oxidised products are formed as ripening of seed proceeds. The most highly oxygenated group of limonoids yet identified, and represented by the azadirachtins, is present in fully ripened seeds (Parmer *et al.*, 1993). So far, at least nine neem limonoids, of very closely related structures, have been given the name of azadirachtins (azadirachtin A-I Table 1.3). These azadirachtins have demonstrated the ability to block insect growth affecting a range of species, including some of the most voracious pests of plants and human health. This group of compounds are said to be "congeners" meaning that they have many structural features in common, and are likely to be derived from a single precursor. This precursor is usually considered to be the simple, terpenoid tirucalol, (Fig 1.11), which has only one oxygen function, although little work has been done to explore the biosynthetic pathway of the azadirachtins. By far the most abundant of the azadirachtins is azadirachtin A, which makes up 80-90% of the group of congeners (Table 1.3). New limonoids are still being discovered in neem, but azadirachtin A, which generally makes up about 10-15% by weight of the total terpenoids of the kernel,

Fig 1.10 In Sudan in 1959, during a locust swarm all the vegetation destroyed except Neem tree (Uvarov, 1977).

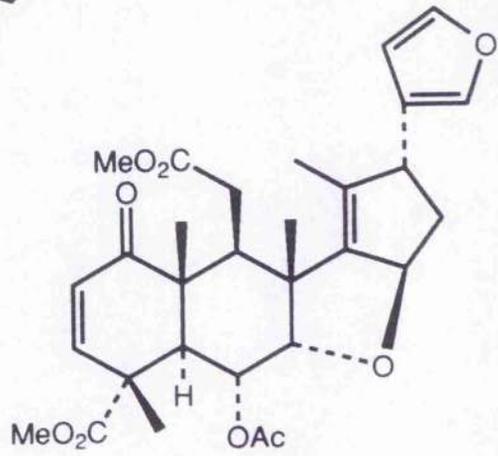
It was an observation of the sort by Dr. Schmutterer in 1959 which started off the interest in neem research in Europe and USA.



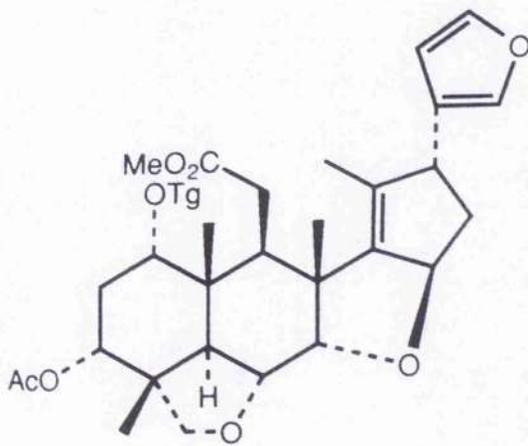
Fig 1.11 Limonoids from neem seeds and their putative biogenetic precursor, tirucallol.



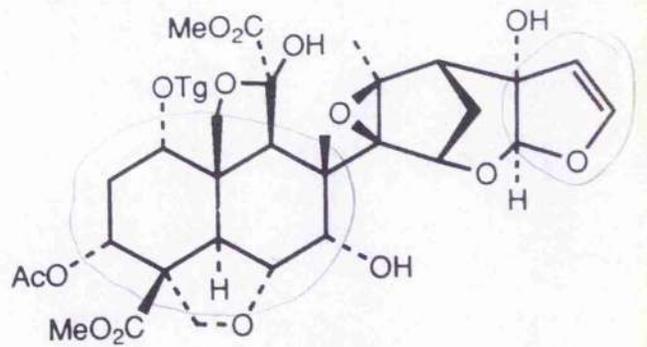
Tirucallol



Nimbin



Salannin



Azadirachtin A

Table 1.3 Congeners of azadirachtin. Pure azadirachtins as isolated from 27 kg neem seeds (Uebel *et al.*, 1979).

Name	Amount (mg)	Fraction (% by weight)
Azadirachtin A	3500	83
Azadirachtin B	700	16
Azadirachtin C	4.7	0.11
Azadirachtin D	3.8	0.08
Azadirachtin E	9.4	0.22
Azadirachtin F	4.5	0.10
Azadirachtin G	3.1	0.07
Azadirachtin H	<1.0	-
Azadirachtin I	<1.0	-

has been shown to be the most potent both as antifeedant and growth regulator in insects. More abundant terpenoids such as salannin and nimbin, may have some biological activity, but are less active (Schmutterer, 1985). In commercial applications such as those listed in Table 1.2, azadirachtin A is generally considered to be the "active principle" for registration purposes etc. The work in this thesis was based solely on the use of pure azadirachtin A whose characteristics will be further considered below.

Of the nine congeners, azadirachtin A comprises the major percentage, azadirachtin B is present at concentrations up to 16% and each of the remaining isomers (C-I) comprise less than 0.2% of the mixture (Ruskin, 1992). In this thesis, wherever the word azadirachtin is used, it is azadirachtin A.

1.5. Azadirachtin A.

Azadirachtin A was one of the first active ingredients to be isolated from neem and has proved to be the tree's main agent for deterring and affecting insects. It appears to cause some 90% of the observed effects on most pests (Parmar *et al.*, 1993). It does not kill insects immediately, but instead it disrupts their growth and reproduction (Mordue *et al.*, 1993). The main source of azadirachtin is the neem seed. Examples of concentrations of azadirachtin in different seed origins are as follows:

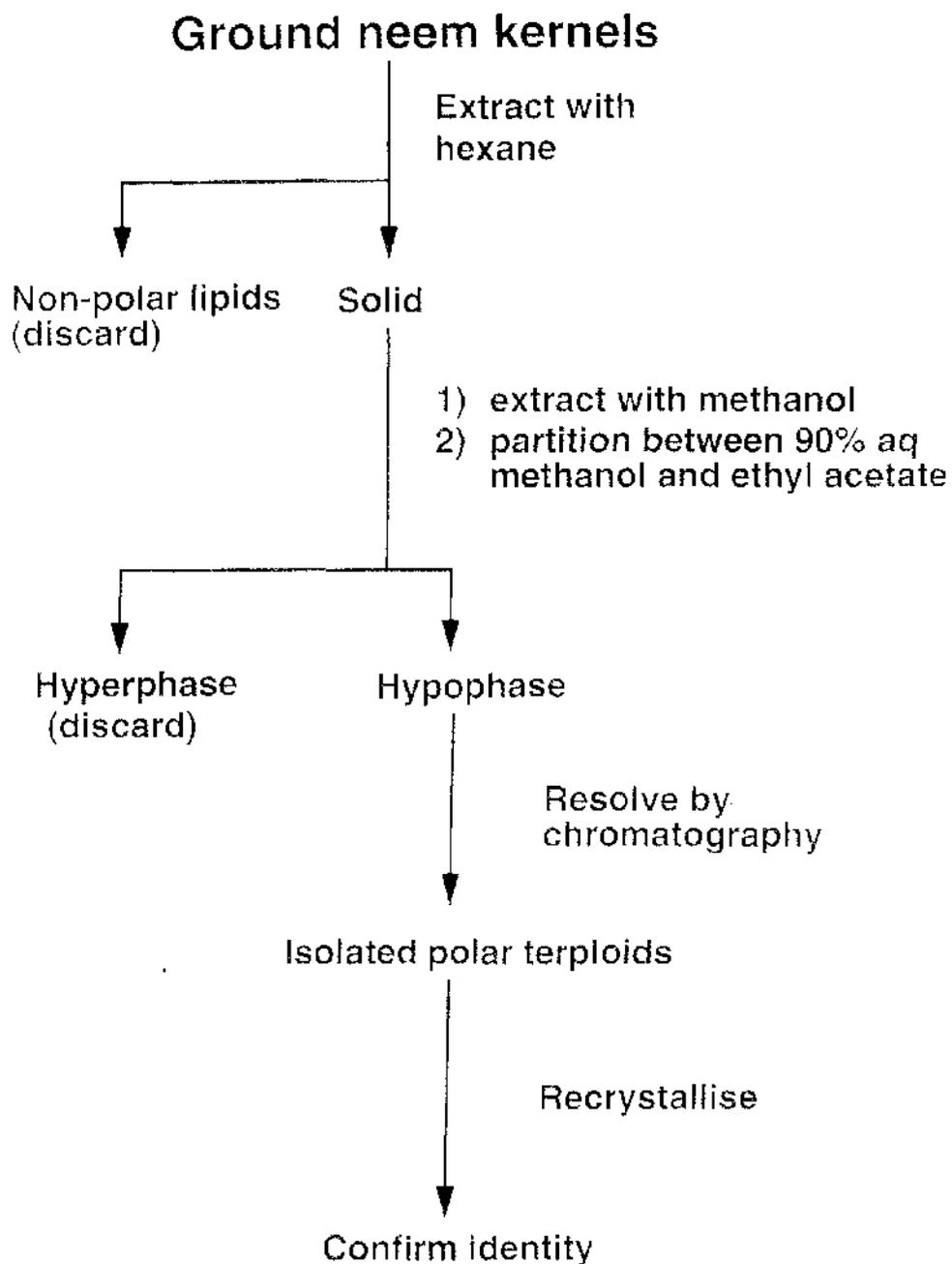
- a) 0.065 % w/w in Srilankan seeds (Paranagama, 1994),
- b) 0.04% w/w in Ghanaian seeds (Nisbet, 1992), and
- c) 0.0015% w/w in Pakistani seeds (Nisbet, 1992).

1.5.1. Isolation of azadirachtin

Several procedures have been published for the isolation of azadirachtin. Most are based on modifications of the original methods of Butterworth, *et al.*, (1968); Nakanishi, (1975); and Yamasaki, *et al.*, (1986). The general principles of the isolation process of the compound are shown in Fig 1.12. Purification has been accomplished by successive partitioning between solvents, and by various methods of column

Fig 1.12 An outline of different procedures involved in isolation of azadirachtin from ground neem seed kernels.

Isolation of pure azadirachtin



chromatography, as well as preparative thin layer chromatography (TLC). The methods of column chromatography include: preparative high performance liquid chromatography (HPLC), (mainly reverse phase); absorption chromatography on silica; and most recently, Supercritical Fluid Chromatography (SFC) (Jarvis *et al.*, 1998).

The isolation of azadirachtin by chromatographic methods is aided by its high polarity in comparison with most of the other terpenoids in the kernels, although azadirachtin A and B are very similar in polarity (Fig 1.13).

1.5.2. Chemical properties of azadirachtin

Azadirachtin A, is a highly oxidized triterpenoid with Relative Molecular Mass (Mr) of 720, (molecular formula $C_{35}H_{44}O_{16}$), and a melting point is $165^{\circ}C$. It is freely soluble in polar organic solvents and sparingly soluble in water (2g/l), (Butterworth *et al.*, 1971). This solubility in water has been made its use in traditional agriculture, in which a plant spray can easily be produced by steeping the ground kernels overnight in water. More recently, water extraction became the basis of a patent by the Trifolio GmbH.

The compounds identified and characterized from neem are triterpenoids and all are derived from the putative parent tetracyclic triterpenoid tirucallool, Fig 1.11 (Jones *et al.*, 1988). From a commercial and biological point of view azadirachtin is currently the most interesting terpenoid from neem. The compound is stable in neutral solution but decomposes rapidly in alkaline medium (Morgan, 1998). It is also susceptible to ultraviolet degradation and decomposes exponentially over time with a half life of 25 hours, when exposed to constant U.V irradiation. However, after decomposition the resulting complex mixture of compounds retained the original growth regulatory properties of azadirachtin (Barnby *et al.*, 1989).

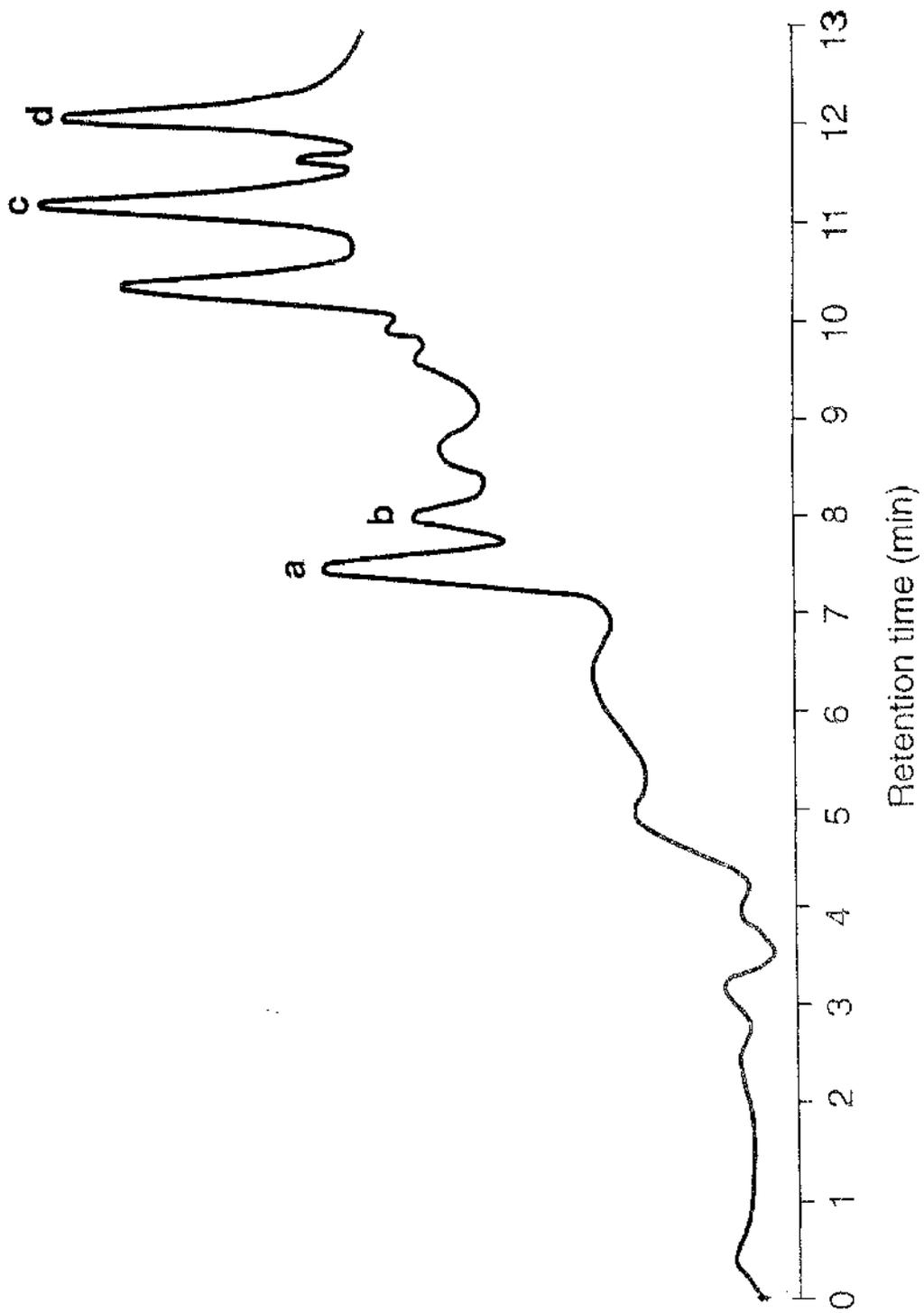
A recent study on the stability of azadirachtin in aqueous and organic solvents suggests that azadirachtin in buffered aqueous solution is most stable between pH 4 and 5, at room temperature, but when compared to other natural insecticides like pyrethrin and rotenone, azadirachtin is a "rather stable substance" (Jarvis *et al.*, 1998).

Fig. 1.13. Resolution by high performance liquid chromatography (HPLC) of the major terpenoids in the neem seed kernels.

The column used was a C6 silica-based reverse phase analytical column. The terpenoids were eluted by means of a linear gradient of methanol in water rising from a starting mixture of 50/50 methanol/water to 100% methanol, over a period of 10 minutes. The rate of pumping was 1ml/min. Detection was by UV light absorbance at 220nm. Some of the major compounds are:

- a: azadirachtin A,
- b: azadirachtin B,
- c: nimbin and
- d: salanin.

In this particular extract, azadirachtin A represents 15% of the terpenoids by weight.



1.6. Effects of azadirachtin on insects

The neem tree has emerged as an important source of insect control agents. The number of insects sensitive to neem products has now increased to more than 400 species (Table 1.4), (Schmutterer, 1995). The important insect orders, Homoptera, Diptera, Heteroptera, Lepidoptera, Coleoptera and Hymenoptera have proved to be neem-sensitive, at least in laboratory experiments. However, the effects varied with tested pest species and the progeny of the material used. For example, lepidopterous species are the most important group of harmful insects in warm climates, so the larvae have been the main targets of neem applications, (Rossner *et al.*, 1987). Reported variations in sensitivities are typical in orthopteroidae, depending on order of insect, species of insect and the manner of application of neem product. Mordue and Blackwell (1993) cite effective concentrations (EC₅₀) applied as an artificial diet between 0.001p.p.m. (*Schistocerca gregaria*) and more than 1000p.p.m. (*Melanoplus sanguinipes*).

The growing accumulation of experience demonstrates that neem products work either as antifeedants or by intervening at several stages of an insect's life instead of killing them instantaneously. In this decade, when many synthetic pesticides are being withdrawn, this kind of pest control approach is seen as more desirable. Pests do not have to be killed instantly if their populations can be incapacitated in ways that are harmless to people and the planet as a whole.

As previously stated, the effectiveness of the many products depends upon azadirachtin. This is the terpenoid, isolated from the neem seed kernels, and is the basis of detailed scientific research work. The effects of azadirachtin on various insects are grouped into two categories, (Fig 1.14):

a) External effects such as :

- deterring feeding,
- repelling larvae and adults,
- deterring females from laying eggs.

Table 1.4 Number of neem-sensitive insect pest species, arranged by order (Schmutterer, 1995).

Order	Number of species tested
Blattodea	6
Dermaptera	1
Caelifera	21
Ensifera	3
Phasmida	1
Isoptera	6
Thysanoptera	13
Phthiraptera	4
Heteroptera	32
Homoptera	50
Hymenoptera	8
Coleoptera	79
Lepidoptera	136
Diptera	49
Siphonaptera	4

Fig 1.14 The effects of azadirachtin on insects are classified into two, external and internal, on the basis of its response in insects.

Effects of azadirachtin on insects



External effects

Antifeedant

Deterrent

Antiovipositional

Internal effects

Secondary antifeedant
(e.g. loss of digestive enzymes in gut?)

Developmental failure
(e.g. loss of developmental peptide hormones)

Loss of "fitness"
e.g. failure to fly and poor resistance to infection
(Loss of lysozyme and other antibacterial proteins?)

Loss of fertility in gonads
(e.g. loss of vitellogenin?)

b) Internal effects such as :

- secondary anti-feedant effects,
- disrupting the development of eggs, larvae, or pupae,
- blocking the molting of larvae or nymphs,
- disrupting mating and sexual communication,
- sterilizing adults, and
- disrupting normal cuticle formation.

Research into the insecticidal effects of azadirachtin has been ongoing for some 35 years. The most striking effects include antifeedant, growth disruption and effects on reproduction.

1.6.1. External effects

1.6.1.1. Antifeedant effects

The antifeedant effect of azadirachtin towards the desert locust was the primary field observation by Schmutterer (1959) and then the laboratory findings of Butterworth and Morgan (1968), which first drew attention to this terpenoid. Its effect is due to the presence of external receptors. Those receptors, mediating the primary antifeedancy must be on the sensory parts of the antennae or the mouthparts.

Published data on the antifeedant effects of azadirachtin and Margosan-O on insect species is enlisted in Table 1.5 (Mordue and Blackwell, 1993). Treatment of insects by azadirachtin does not immediately kill them but often have an antifeedant effect, reducing the amount of food intake, i.e. primary antifeedant effects leading to secondary effects which lower the fitness of the insects, (Blaney and Simmonds, 1995). Concentrations ranging from 0.001% to 4% of various neem seed kernel extracts have generally been found to deter the feeding of most of the insects evaluated so far (Singh, 1993). These figures confirm that different insects possess a variable ability to sense azadirachtin.

Table 1.5 The antifeedant effects of azadirachtin (AZAD) and "Margosan-O" (M-O) (0.3% AZAD) on a selected range of insect pest species in laboratory and greenhouse trials (Mordue and Blackwell, 1993).

Species	Treatment	AZAD Antifeedancy (p.p.m)	
Lepidoptera (larvae)			
<i>Spodoptera frugiperda</i>	AZAD(fd)	1.0	92
<i>Heliothis virescens</i>	AZAD(fd)	1.0	94
<i>Spodoptera littoralis</i>	AZAD(fd)	1.0	95
<i>Choristoneura fumiferana</i>	M-O(ad)	0.3	95
<i>Heliothis virescens</i>	AZAD(ad)	0.07	50
<i>Peridroma plorans</i>	AZAD(ad)	0.4	50
<i>Spodoptera littoralis</i>	AZAD(ad)	10.0	100
<i>Earias insulana</i>	AZAD(ad)	50.0	100
<i>Spodoptera litura</i>	AZAD(t)	50.0	37
<i>Spodoptera frugiperda</i>	AZAD(t)	50.0	43
<i>Peridroma saucia</i>	AZAD(t)	2.4	50
<i>Ostrinia nubilalis</i>	AZAD(t)	24.0	50
<i>Achoea janata</i>	AZAD(t)	1.0	54
<i>Achoea janata</i>	AZAD(t)	10.0	75
<i>Spodoptera littoralis</i>	AZAD(sp)	0.06	50
<i>Macalla thyrsisalis</i>	M-O(sp)	20.0	41
<i>Spodoptera frugiperda</i>	AZAD(sp)	600.0	100

<i>Pieris brassicae</i>	AZAD(syst)	30.0	56
Coleoptera			
<i>Epilachna varivestis</i>	AZAD(t)	500.0	100
<i>Diabrotica</i>			
<i>undecimpunctata howardi</i>	AZAD(sp)	100.0	98
<i>Acalymma vittatum</i>	AZAD(sp)	100.0	98
<i>Lepitinotarsa</i>	AZAD(sp)	600.0	0
<i>decemlineata</i>			
Hemiptera			
<i>Myzus persicae</i>	AZAD(ad)	100.0	80
<i>Rhopalosiphum padi and</i>			
<i>Sitobion avenae</i>	AZAD(t)	250.0	50
<i>Asmetrasca decedens</i>	M-O(sp)	60.0	50
<i>Myzus persicae</i>	AZAD(syst)	300.0	30
<i>Rhopalosiphum padi and</i>			
<i>Sitobion avenae</i>	AZAD(syst)	500.0	18
<i>Asmetrasca decedens</i>	M-O(syst)	60.0	100
<i>Rhodnius prolixus</i>	AZAD	25.0	50
Isoptera			
<i>Coptotermes formosans</i>	M-O(fd)	100.0	49
Diptera			
<i>Liriomyza trifolii</i>	M-O(syst)	10.0	31

Orthoptera			
<i>Schistocerca gregaria</i>	AZAD(fd)	0.07	100
<i>Locusta migratoria</i>	AZAD(fd)	50.0	100
<i>Dissosteira carolina</i>	M-O(fd)	150.0	82
<i>Schistocerca gregaria</i>	AZAD(ad)	0.001	100
<i>Milanoplus sanguinipes</i>	AZAD(ad)	1000.0	0
<i>Eyperpocnemis plorans</i>	AZAD(t)	0.01	44
	AZAD(t)	0.1	85
<i>Diapheromera femorata</i>	M-O(t)	150.0	75
	M-O(t)	600.0	91
<i>Gryllus pennsylvanicus</i>	M-O(t)	150.0	100
<i>Schistocerca gregaria</i>	AZAD(sp)	1.0	50

Treatment applied:

fd-filter paper or glass fibre discs;

ad-artificial diet;

t-topical treatment to leaves or leaf discs;

sp-spray application to crops; syst-systemic application.

* Antifeedancy given in relation to control feeding. 100% represents the total abolition of feeding at the stated concentration.

The antifeedant activity has been assessed by applying a variety of formulations in many different ways against more than 200 species, e.g. to crops; to the insects as sprays; topical applications; by cannulation; by injection; on simple feeding discs or within artificial diets. It is clear that lepidoptera are extremely sensitive to azadirachtin and show an effective feeding inhibition for 1-50p.p.m. of azadirachtin, depending upon species. Coleoptera, Hemiptera and Homoptera are less sensitive to azadirachtin, whereas Orthoptera show an enormous range in sensitivity from the most sensitive species tested, *Schistocerca gregaria* ($EC_{50}=0.05$ p.p.m.), the moderate sensitivity of *Locusta migratoria* ($EC_{50}=100$ p.p.m.) and the extreme insensitivity of *Melanoplus sanguinipes* ($EC_{50} > 1000$ p.p.m.) (Table 1.5).

Powdered seed kernels of *Azadirachta indica* A. JUSS, (5% by weight of the grain) were found to be effective repellents to protect stored wheat and lentil seeds from pests, like grain weevil, *Sitophilus granarius* L., rice weevil, *S. oryzael* L., and cowpea weevil, *Callosobruchus chinensis* L. using an olfactometric method (Ignatowicz and Wesolowska, 1996).

A spray of water and acetone extracts of the leaves of *Azadirachta indica* against the okra shoot and fruit borer, *Earias vittella*, reduced the fruit damage by 20%, indicating feeding inhibition (Shukla *et al.*, 1997).

Neem seed kernel, seed coat and fallen leaves were extracted with water and ethanol (Singh, 1987). The ethanolic extract was re-extracted successively with hexane, chloroform and methanol. These extracts were tested for their antifeedant efficacy against the desert locust, *S. gregaria*. The neem seed kernel extract was most active, followed by seed coat and leaves. The water extract deterred feeding nearly as effectively as the methanol extract indicating thereby that water is used as an effective solvent for extracting the seed kernel. The seed coat which constitutes about 50% of the seed weight exhibited high antifeedant activity. Water and ethanolic extracts of the seed kernel were evaluated against chafer beetle, *Apogonia blanchardi* (Doharey *et al.*, 1989) and found to inhibit the feeding of the beetle at 2.5 and 1% concentrations respectively.

Although only crude extracts were used in the above work, it is clear from the effectiveness of the polar extracts that azadirachtin was the main agent.

The more recent commercial developments have tried to maximise the azadirachtin content. NeemAzal (Trifolio GmbH) normally has 20% azadirachtin content. According to azadirachtin contents, three concentrations of 0.2%, 2% and 10% of NeemAzal were tested in three series with different periods of exposure during the life cycle of cockroaches *Periplaneta americana* L. The reduction in food consumption in larvae was 15%, 35% and 60% respectively against the controls for the above said treatments (Bohm *et al.*, 1997). The antifeedant effect was directly related to the azadirachtin content, and was the cause of retardation of larval growth. The females that developed from these larvae showed a decreased ability to reproduce. In a recent study, fifth-instar of *S. gregaria*, fed on barley seedlings sprayed with different concentrations of azadirachtin and analogues, showed potent antifeedant activity (Mordue *et al.*, 1998).

1.6.2. Internal effects

There is no doubt that azadirachtin is an extremely effective antifeedant for phytophagous insects. On the other hand, haematophagous insects are not responsive for antifeedant effects. The differences in responsiveness relate to differences in the presence of receptors on neurons in the taste sensilla. In some cases, the external effects of azadirachtin could induce starvation in insects, and thus indirectly cause developmental failure (Slama, 1978). For example, *S. gregaria* will die from starvation rather than feed on azadirachtin-treated crops, whereas *L. migratoria* will ingest enough for toxic physiological effects to manifest themselves (Mordue *et al.*, 1996). Similarly aphids ingest sufficient amount which not only inhibits their reproduction but the production of viable nymphs as well (Nisbet *et al.*, 1994; Lowery and Isman, 1994).

Reports of the physiological disorders in insects caused with treatment of azadirachtin are widespread in literature. The reported deleterious effects of azadirachtin include: growth inhibition (insect growth regulatory effect (IGR), prevention of proper function of the gut (which may result in the so-called secondary antifeedant effects), interruption of reproduction, disturbances in larval-adult development, reduction of fecundity, and general, ill-defined, loss of "fitness", usually culminating after some time in actual death. In the following sections, some of the above mentioned effects are described in greater depth.

1.6.2.1. Insect Growth Regulatory (IGR) effect

This biological effect of azadirachtin on insects is often the most obvious one. Azadirachtin, (or unresolved polar extract of seed kernels), when applied or fed to juvenile insects, arrests their growth. The insects are either killed before reaching adult stage or produce malformed and miniature adults, depending on the given dose.

The IGR effects of azadirachtin on different insect species are listed in Table 1.6. McMillan *et al.*, (1969) were the first to report the growth-disrupting effect of a chloroform extract of the leaves of *Melia azedarach*, a close relative of neem, against *Spodoptera frugiperda* and *Heliothis zea*. Later, Gill *et al.* (1971) reported that *Pieris brassicae* larvae fed on foliage treated with neem kernel extract failed to develop to maturity and most of them died during moulting.

The growth-disrupting effects of azadirachtin in *P. brassicae* larvae feeding on cabbage leaves treated with the compound at concentrations which were not antifeedant were reported by Ruscoe (1972). Final instar larvae which had fed on leaves treated with 50p.p.m. azadirachtin were unable to complete their pupal moult.

In Homoptera the growth-inhibitory and disrupting effects of neem derivatives are much more profound than either the repellent or antifeedant effects. Regardless of the biotype, young nymphs of the rice hopper, *Nilaparvata lugens*, were highly sensitive to

Table 1.6 The insect growth regulatory (IGR) effects of azadirachtin on insects after injection, oral cannulation or topical application (Mordue and Blackwell, 1993).

Species	Treatment at the start of instar	Azadirachtin concentration ug/g(body weight)	Moult inhibition mortality (%)
Lepidoptera			
<i>Heliothis virescens</i>	injection	1.7	15
<i>Spodoptera frugiperda</i>	injection	1.5	45
<i>Spodoptera littoralis</i>	injection	1.9	35
<i>Spodoptera litura</i>	injection	1.1	50
<i>Achoea janata</i>	injection	4.1	50
<i>Peridroma saucia</i>	injection	1.2	50
<i>Bombyx mori</i>	injection	1.2	50
<i>Spodoptera frugiperda</i>	oral cann	1.5	50
<i>Spodoptera littoralis</i>	oral cann	1.9	50
<i>Heliothis virescens</i>	oral cann	5.0	100
<i>Spodoptera littoralis</i>	topical appli	1.5	15
<i>Spodoptera frugiperda</i>	topical appli	1.9	20
<i>Heliothis virescens</i>	topical appli	1.7	5
Hemiptera			
<i>Rhodnius prolixus</i>	injection	0.2	50
<i>Oncopeltus fasciatus</i>	injection	0.6	50

<i>Oncopeltus fasciatus</i>	topical appli	0.14	50
Orthoptera			
<i>Schistocerca gregaria</i>	injection	1.7	50
<i>Locusta migratoria</i>	injection	1.3	50
<i>Melanoplus sanguinipes</i>	injection	3.2	50
<i>Melanoplus sanguinipes</i>	oral cann	11.3	50
<i>Melanoplus sanguinipes</i>	topical appli	4.5	50

Treatment applied:

oral cann: oral cannulation

topical appli: topical application

neem and fewer than 10% reached adulthood on rice plants sprayed with 3% neem oil. None became adults at higher concentrations of neem oil (Saxena *et al.*, 1981).

Moulting failures were observed in young pea aphids, *Aphis pisum*, exposed to bean plants sprayed with neem seed kernel extracts (Schauer, 1984). Third instar *Myzus persicae* and *Nasonovia ribisnigri* exposed to 0.5% neem oil moulted successfully to adults but they were generally much smaller in size than controls (Lowery, 1992). Koul *et al.*, (1987) found that final instar *Bombyx mori* larvae injected with azadirachtin experienced a dose-dependent reduction in weight gain resulting in a failure to pupate in those larvae which did not reach a threshold weight of 2.5g. A severe reduction in the development of ovaries of females of the earwig *Labidura riparia* was observed after azadirachtin treatment (Sayah *et al.*, 1996). The effect was dose-dependent and not linked to an absence of feeding.

The growth-disrupting effects of neem seed extracts have been reported for forty-one species of insects covering almost every economically important order (Jacobson, 1986; Warthen, 1989). Mexican bean beetle, *Epilachna varivestis* is a phytophagous coccinellid with considerable economic importance on legumes in America. It has a short life cycle, and consumes considerable amounts of food in the larval and adult stages. The growth-disrupting effects of azadirachtin on larvae of *E. varivestis* are typical of many other species (Rembold, 1988). When larvae feed on treated substrates, they may remain in the larval state without progressing to the pupal moult. This species has been often used as a bioassay of azadirachtin and neem in general.

In Lepidopterans, disruption of the larval-pupal moult is frequently reported (Schmutterer *et al.*, 1983; Koul and Isman, 1991). Depending on the time of treatment and dose, larvae treated with azadirachtin or neem failed to initiate the larval-pupal moult or moult into larval-pupal moult intermediates (Jagannadh and Nair, 1992). In noctuids e.g *Perdroma saucia*, *Heliothis virescens*, *Spodoptera exempta*, pupation is disrupted by azadirachtin at doses as low as 0.5-1.0µg per larva. Qadri and Narsaiah (1978) injected

azadirachtin in acetone into the haemolymph of last instar male and female nymphs of *Periplaneta americana*. Moulting was delayed by 2 weeks, and accompanied with disturbances during moult and subsequent mortality. Laboratory bioassays proved that topical neem application on hoppers of *L. migratoria* and *S. gregaria* result in prolonged nymphal development (Schmutterer, 1995). A long delay was caused when 3rd instar nymphs of *S. gregaria* were kept in neem-oil-sprayed cages. A prolonged development period of *S. gregaria* was recorded in semi-field trials, similar to that in the laboratory (Langewald and Schmutterer, 1992).

One striking fact from the data in Table 1.6, is that the insects tested have a high sensitivity to this effect of azadirachtin, and the effective concentrations fall within rather narrower limits compared to the antifeedant effects.

1.6.2.2. Effect on reproduction

The effect of neem products on the reproduction of insects have been known since 1975, when it was reported for the first time that the number of eggs produced by *Epilachna varivestis* decreased after the intake of active principles from neem seed kernels and of azadirachtin (Steets and Schmutterer, 1975). After feeding for 5 days on residues of azadirachtin at 50p.p.m. on bean leaves in the laboratory, treated females laid less than 10% eggs of the untreated controls. Not only were the number of eggs reduced, the fertility of eggs was also affected. The reproduction-reduction effects of neem products have been reported in insects of other orders, such as Caelifera, Heteroptera, Homoptera, Hymenoptera, Lepidoptera and Diptera (Schmutterer and Rembold, 1995).

In the African migratory locust, *Locusta migratoria*, injection of 10mg azadirachtin/female, 2-10 days after the last moult, prevented egg-production (Rembold and Sieber, 1981). Singh *et al* (1987) found that females of *S. gregaria* laid significantly fewer eggs in sand, treated with 0.1, 0.5 and 1% concentration of neem kernel suspension than the untreated controls.

In Heteroptera, in laboratory bioassays the females of the stainer bug, *Dysdercus fasciatus*, derived from 5th instar nymphs treated topically with methanolic neem seed kernel extracts, laid on the average only 59% of the number of eggs of untreated controls (Ochse, 1981). Females of the large milkweed bug, *Oncopeltus fasciatus*, were completely sterilized by topical application of 8 and 16mg of azadirachtin /female (Dorn *et al.*, 1987). Females of the melon fly *Bactrocera cucurbitae* laid significantly fewer eggs than controls when treated as larvae and pupae with 1.85p.p.m. azadirachtin in the diet (Stark *et al.*, 1990).

A remarked reduction in the number of deposited first instar nymphs of green peach aphid, *Myzus persicae*, was recorded, when 250p.p.m. of Margosan-O was applied to *Vicia faba* plants in laboratory trials (Grothe, 1992).

Treatment of 3rd instar larvae of *Adoxophyes orana* with NeemAzal-F (apple leaves dipped in solutions containing 3p.p.m.) resulted in 50% sterile egg-masses after treatment of male larvae and 37% after treatment of female larvae (Jakob, and Dickler, 1994). In *Periplaneta americana*, NeemAzal does not lead to increased mortality but may result in a substantial reduction in the fecundity, so that the following generation may be reduced below an economic threshold level (Schmutterer, 1990 & 1992).

In a recent study, the ovicidal effects of various formulations of azadirachtin against the mosquitoes, *Culex tarsalis* and *Culex quinquefasciatus* were investigated by Su and Mulla (1998). The ovicidal activity was influenced by concentrations of azadirachtin and the age of the egg rafts.

Not only azadirachtin A and its congeners reduce the fecundity of Lepidopterous insects, but so do the volatiles of seed kernels. The exposure of adults of the moth *Earias vitella* to volatile substances of neem oil and neem leaves resulted in a reduction of the fecundity of females and an increase in egg-sterility, compared with controls (Pathak and Krishna, 1986). A pronounced reduction of egg output and egg viability occurred in rice moth, *Corcyra cephalonica*, following exposure to neem oil vapour emanating from paper discs containing 160µl of oil. The total number of eggs laid per

treated female was 245.4 as against 401.2 in the control. The mean number of viable eggs laid per treated and untreated females was 231.6 and 360.6 respectively (Pathak *et al.*, 1985).

1.6.2.3. Effect on mortality

There are several reports on the mortality of adult or developmental stages of insects after azadirachtin treatment. The reported effects are not only for pure azadirachtin but for different commercial formulations used in both, field and laboratory trials. For example, Margosan-O (1% aqueous suspension) tested in the laboratory, against European leafroller, *Archips rosanus* showed 100% larval mortality 48hours after treatment. In a field trial, the same concentration was effective after 3 weeks of application (Ali *et al.*, 1997).

The *Spodoptera mauritia* eggs when treated topically with 5, 10 and 20µg of azadirachtin, there was no effect on egg hatchability, but the hatched larvae showed high rate of mortality during development (Jagannadh and Nair, 1996).

In a pilot study in the Seychelles, by Schroder and Kahnert (1997), short-term tests on mosquito larvae with NeemAzal formulations containing two different concentrations of azadirachtin, caused significant deaths of the treated larvae. At 15 p.p.m. of azadirachtin, 50% of the larvae died within 48hours and the mortality rate was 100% at 30p.p.m., or after three days at 15p.p.m. respectively. On the basis of these results in protected natural conservancy of Seychelles, neem is recommended for biological control of mosquitoes and other blood-sucking insects.

Spider mites (Tetranychidae) have a broad host range, causing damage to many plants in Russia, such as: cotton, fruit, ornamental and vegetables crops, and of greenhouse flowers. Many populations of spider mites have become resistant to synthetic insecticides. *Tetranychus urticae* Koch. is one of the most destructive species of spider mites, 0.1, 0.3 and 0.5% of NeemAzal-T/S was evaluated for eggs, larvae and adults of *T. urticae*, sprayed on fig leaf disks. In adults, there was 53.3, 72.8 and 98%

mortality, in larvae it was 38, 98 and 100% respectively, and only at 0.5% of NeemAzal, the hatching from eggs was 27% less in comparison to the controls (Mironova and Khorkhordin, 1997).

A high demand exists for biological control of the Colorado potato beetle, *Leptonotarsa decemlineata* Say, because it has developed resistance against synthetic pyrethroids during recent years (Schmutterer, 1995). It has again become a major pest of potatoes in parts of America and in some countries in Eastern Europe. Both the larval instars and adults are destructive consuming considerable amounts of the foliage of potato plants. Biological control by azadirachtin seems worthwhile, looking for eventually long term effects on development to control the beetle (Reibold, 1995). A field spray test of 0.1% NeemAzal near Frankfurt showed 71% mortality in larvae (Basedow and Peters, 1997). In another study, in a field trial of Align™ a single spray in organic gardening systems of Pine Ridge Indian reservation, South Dakota U.S.A, showed 94% of mortality for *L. decemlineata* (Baumgart *et al.*, 1997).

Injection of 1µg azadirachtin into red cotton bug, *Dysdercus koenigii* caused 50% mortality by the seventh day of treatment. The surviving insects showed a significant effect on reproduction behaviour, and generally no eggs were laid due to failure of vitellogenesis. Topical application of azadirachtin severely impaired embryogenesis (Koul, 1984).

Neem oil, when tested against larvae of *Aedes aegypti* and *Culex quinquefasciatus* in the lab showed 100% mortality at a concentration of 0.02% and mortality was 63% at a concentration of 0.01% after an exposure for 96 hours (Sinniah *et al.*, 1994).

1.6.2.4. Effect on 'fitness'

The effects of azadirachtin within the insects, described above are quite well-defined, if not understood. There are a number of less obvious effects, for example changes in biological fitness such as loss of flying ability, immunodepression, enzyme synthesis inhibition and splitting of biological rhythms caused due to azadirachtin treatment.

These changes lower the biological activity of the insect, and perhaps hasten its death. In this respect the orders studied are; Caelifera, Heteroptera, Homoptera, Coleoptera, Lepidoptera and Diptera. In many cases, the loss of activity due to neem products may have lethal consequences in the long run as affected insects cannot survive against adverse environmental conditions as do normal, healthy individuals. The pest insects with reduced activity may be caught much easier by natural enemies compared with unaffected insects (Schmutterer, 1995). For example in *S. gregaria*, by application of neem oil, the eyes of the locust have reduced sense of sight and so, orientation may also be disturbed (Nicol and Schmutterer, 1991). Similarly in *Phormia terraenovae*, an injection of 0.5µg of azadirachtin into the head capsule caused irreversible visual impairment (Wilps and Gade, 1996).

After topical application with neem oil (0.25-1ml), gregarious nymphs of the locusts *S. gregaria* and *L. migratoria* show a dose-dependent decrease of their crawling, jumping and climbing ability, and a general trend towards solitarization (Schmutterer and Freres, 1990; Freisewinkel and Schmutterer 1991; Nicol and Schmutterer, 1991). The adult locusts topically treated with neem oil containing 0.2% azadirachtin while resting on ground, had 50% reduced flight performance, and this reduction was maintained throughout the life span of *S. gregaria* (Wilps *et al.*, 1992). In consequence of this effect, the treated locusts should not be able to form swarms or, in case of treated swarms, the locusts should not be able to fly to long distances (Wilps *et al.*, 1991a, b).

Recently, Kollien *et al* (1998) has reported a decrease in the number of *Trypanosoma cruzi* in fifth instars of *Triatoma sordida* bugs, fed with azadirachtin-supplemented blood in comparison to the bugs fed with non supplemented blood. These findings indicate the decreased ability of the vector to transmit the parasite by simply reducing the number of parasites and the number of vectors developed into adults. Similarly the ability of *Myzus persicae* to transmit potato leaf roll luteovirus (PLRV) was studied and it was found that at a dose range of 320-2560 p.p.m. of

azadirachtin, 55-90% of transmission was inhibited. Larval growth and mortality were also affected in a dose-dependent manner (VanhenHeuvel *et al.*, 1998).

Another effect, which was the subject of some of the experimental work in this thesis, is to lower the immune response in insects after azadirachtin treatment. Insects are subject to attack by microorganisms and parasites, and have immune reactions employing a variety of mechanisms, including haemocytes for phagocytosis or encapsulation, a prophenoloxidas, and a range of antibacterial proteins. In *Rhodnius prolixus*, apart from the prophenoloxidase systems where melanin production was not reduced, it was found that azadirachtin at a concentration of 1.0mg/ml in the blood meal caused a reduction in immune response within 6 days in fifth instars challenged with *Enterobacter cloaca* B12 strain, thus reducing the fitness of the insect, (Azambuja *et al.*, 1991).

1.6.2.5. Endocrine effects

The hormonal regulation of insect growth, development and reproduction works in a heirarchic manner. This means that neuropeptides from the central nervous system control the synthesis of steroid (moulting) and sesquiterpenoid (juvenile) hormones in peripheral glands. The most important hormones are ecdysone and 20-hydroxyecdysone as moulting hormones, and the juvenile hormones 0-III involved in developmental biochemistry and physiology of insects (Schmutterer, 1995).

The haemolymph hormone concentrations, as an endocrine signal, has to be carefully controlled. The juvenile hormone balance is achieved by two types of neuropeptides, allatotropin and allatoinhibin, which are synthesised in the brain and then released into the insect haemolymph. The brain and its neurohemal organs play a dominant role in controlling the hormone titres and metabolism.

A major action of azadirachtin is to modify haemolymph ecdysteroid titres, and to cause major disorders in some phytophagous insects. Similar morphogenetic defects could also be induced by synthetic hormone mimics and it was concluded that

azadirachtin might function like the ecdysteroids (Rembold, 1988).

There is a pronounced effect of azadirachtin on control of ecdysteroid titre and it was first observed in fifth instar of *L. migratoria* (Sieber *et al.*, 1987). This effect was an interference of the compound with the neuroendocrine system of the larvae and this argument is supported by histological studies (Rembold *et al.*, 1981 and Sieber *et al.*, 1987). Inhibition of ecdysis in azadirachtin-treated larvae is due to interference with the hormonal control of moulting. The ecdysteroid titre in the treated larvae reveals a close relationship between endocrine conditions and morphogenetic processes. A shift, or even complete disappearance of the moulting-hormone titre is the result of azadirachtin application (Sieber *et al.*, 1987). Sieber *et al* also demonstrated that, although the overall ecdysteroid titre in azadirachtin treated larvae was not reduced, the contribution of 20-hydroxyecdysone to the peak was diminished. The peak ecdysteroid level in *Tenebrio molitor* pupae injected with azadirachtin was reduced by more than 50% (Macro *et al.*, 1990). In adult females of the carwig *Labidura riparia*, Sayah *et al* (1998) reported the effects of endocrine and neuroendocrine system after azadirachtin treatment. There was a drastic reduction in the ovarian ecdysteroid levels in azadirachtin treated insects. The reduction in ecdysteroid was dose-dependent and responsible for the inhibition of vitellogenesis.

1.7. Targets of azadirachtin

The azadirachtins are the biologically most active neem seed components against insects. On the organ level they may be directed primarily against the endocrine system, while on the cellular level they may interfere with the expression of polypeptide patterns in a distinct way. On the molecular level no direct interference with protein synthetic machinery has been found in the range of its physiological mode of action (Schmutterer, 1995). Our present knowledge of the mode of action of azadirachtin speaks in favour of the presence of highly specialized receptor molecules for the drug. As a consequence of

its effects at the cellular level, the effect on activity and behaviour makes it possible that neuro-transmitter pools are affected by azadirachtin treatment (Banerjee and Rembold, 1992).

The antifeedant activity has been demonstrated for several insects, and it was found by the quantitative *Epilachna* bio-assay that the feeding was inhibited in a dose-dependent manner, and diminished with reduced concentrations, and was even absent at the concentrations which still can induce malformations and growth inhibition (Rembold, 1988). Azadirachtin inhibits feeding at much lower concentrations in hemimetabolous than in holometabolous insects. On the other hand, even in the heteropteran *Rhodnius prolixus*, only high doses of azadirachtin caused an antifeedant effect when given through a blood meal, whereas a single dose of a minute amount of the drug when fed through a blood meal, completely inhibited growth of *Trypanosoma cruzi*, the casual agent of Chaga's disease in its host, *R. prolixus* (Garcia *et al.*, 1989). Very little is known about the molecular target of azadirachtin. The present view is that in azadirachtin-sensitive insects, a highly specific receptor protein is present, which is irreversibly inactivated by minute amounts of this compound (Schmutterer and Rembold, 1995). Application of high resolution two-dimensional gel electrophoresis demonstrated for a tissue-specific modulation of the polypeptide patterns in *S. gregaria* (Annadurai and Rembold, 1993). All the studied tissues, i.e., brain, haemolymph, suboesophageal ganglion and *corpus cardiacum* show a reduced number of polypeptides in the treated individuals, which was most distinct in the brain. An analysis even 45 days after treatment showed the persistent effect of azadirachtin most dramatically in the haemolymph pattern. This effect of azadirachtin on the polypeptide pattern suggests the blockade of secretion of neuropeptides by low doses of azadirachtin, achieved on the regulatory level (Rembold, 1995). This view is supported by the results of studies with *L. migratoria* in which 2p.p.m. concentration is a physiological dose for an ED_{50} *in vivo*, but for an *in vitro* study with the isolated fat body, the protein synthesis was inhibited at a concentration of 200p.p.m. (Sieber and Rembold, 1983).

An other promising result was obtained when insect cell line Sf9 showed its high sensitivity for azadirachtin, compared with other limonoids and with a mammalian cell line (Rembold and Annadurai, 1993). This means that azadirachtin interacts in a high structural specificity with an insect-specific recognition system, the molecular basis of which is unknown.

1.8. Aims of the project

From the above sections, it is clear that azadirachtin and other neem derived terpenoids act against many systems and tissues of insects, but there is still a great deal of confusion about the details of effects on tissue level. There could be many reasons for this and the obvious one is the multiplicity of effects they produce even in one species. In this connection, they contrast with pyrethrin and synthetic pesticides whose main and often only immediate targets are nerve cells.

Azadirachtin has shown diverse biological effects against locusts including antifeedant, oviposition deterrent, growth regulatory (IGR) and sterilitant effects (Mordue, 1993). The three major types of locusts have significant effect on the world's agriculture. These are: the desert locust, *Schistocerca gregaria*, the red locust, *Nomadocris septemfasciata*, and the migratory locust, *Locusta migratoria* (Chapman, 1976).

S. gregaria has been used previously for studies on neem and azadirachtin in particular. There are several reasons for this, for example, by application of neem oil, the nymphs of harmful gregarious phase of the desert locust *Schistocerca gregaria* can be transformed into the harmless solitary phase (Schmutterer, 1995). This may gain considerable importance for locust control in future. An *in vitro* study of the effects of azadirachtin on sperm motility of the adult male desert locust suggests the preferential binding of azadirachtin to sites on the organelles associated with maturing sperm tails,

causing a reduction in the motility of sperm-bundles (Nisbet *et al.*, 1997). A recent report on cytological study of testes of fifth instar of *S. gregaria*, removed after 20 days of treatment showed arrested spermatogenic meiosis at Metaphase I (Linton *et al.*, 1998). A simple preganatical reason for the selection of *Schistocerca gregaria* is its large size which allows aliquot tissues for *in vitro* studies on the effects of azadirachtin on the synthesis of identified proteins in isolated tissues.

This project was aimed to examine the action of azadirachtin more clearly in specific tissues of *Schistocerca gregaria* in connection with protein biosynthesis. This is a continuation of work of Paranagama (1994), who showed effects of azadirachtin on unspecified proteins in various tissues. The present work aims to make this more exact by examining specific identified proteins, which can be induced by defined stimuli both *in vivo* and *in vitro*.

The identified/selected target proteins were lysozyme and vitellogenin. As indicated in Fig 1.14, the loss of these proteins may result in some of the important internal effects of azadirachtin. The effect of azadirachtin was studied on their synthesis (or release of already synthesised ones), in the specific tissues of adult female locust *Schistocerca gregaria*. The general strategy was to choose proteins, whose synthesis could be induced by an identified single compound. Experiments done *in vivo* were to be confirmed *in vitro* to obviate as much as possible unknown hormonal and other physiological effects.

Chapter 2

Effect of azadirachtin on the rate of incorporation of [³H] leucine into the proteins of specific tissues of the female adult locust, *Schistocerca gregaria*.

2.1. Introduction

As discussed in the Introduction the broad underlying aim of the work reported in this thesis was to explore the idea that the action of azadirachtin in insects is to interfere with some aspects of protein biosynthesis, in either a specific or general manner. The toxicology of azadirachtin is not well understood, for example the ecdysis inhibitory effects occur both in insect species which do not show a strong feeding inhibition as well as in those insects which are strongly deterred by the compound. Rembold (1988) suggested that the primary action of azadirachtin was on neurosecretory systems, involving the production or release of neuropeptides, and the effects observed in the rest of the body were all derived from this. Sieber and Rembold (1981) reported an inhibition of egg development in adult females of *Locusta migratoria* after azadirachtin treatment. The work of Paranagama (1994) suggested that the results on protein biosynthesis are likely to be much more general, consistent with the uptake of the azadirachtin into most of the body tissues of *Schistocerca gregaria*. The major tissue of protein biosynthesis in locust is the fat body. Many of the proteins formed by this tissue are secreted into the haemolymph including the two most closely examined here: vitellogenin and lysozyme. Before proceeding to more exact studies of the individual proteins, the effect of azadirachtin was evaluated for the rate of incorporation of a radio-labelled amino acid into acid-precipitable protein in the fat body and haemolymph of adult female *S. gregaria*

2.1.1. Protein metabolism of fat body

The fat body is often the most conspicuous object in the body cavity of the insect. It consists of a loose meshwork of lobes, invested in delicate connective-tissue membranes, so as to expose the maximum of surface to the blood. It performs many of the functions of the vertebrate liver, including amino acid metabolism and making amino acids available for protein synthesis by fat body itself and other tissues such as

ovary and haemolymph. It is important in the storage of reserves of fat, glycogen and proteins and plays an important role in intermediary metabolism (Clemants, 1959).

The pattern of incorporation obtained for the various precursors show that the fat body is able to use a wide range of precursors for the formation of proteins. The work presented in this chapter is to study the effects of azadirachtin in connection with protein biosynthesis in fat body of the desert locust. As the haemolymph proteins are synthesised by fat body, so any change in protein synthesis in fat body would in turn affect the haemolymph protein concentration.

2.1.2. Proteins of the haemolymph

In the body of insects, there is only one tissue fluid, the haemolymph, occupying a single cavity the haemocoel, and it implies to a combined function of the blood and lymph. The haemolymph of an insect accounts for between 16-20% of its total weight (Patton, 1963). The haemolymph is rich in proteins (Wigglesworth, 1984). Insect haemolymph proteins attract a great deal of attention as biochemical model systems (Law and Wells, 1989). The main haemolymph proteins, lipophorin (LP), storage proteins (SP) and vitellogenins (Vgs) are common to most insects. They have special functions for development, metamorphosis and reproduction. The change in concentration of the haemolymph proteins depends on the developmental stages, physiological conditions and reproductive state of insects (Chinzei *et al.*, 1994). For example, during moulting, haemolymph appears to act as a reservoir of proteins with an increase of over 100% in its volume, decrease or increase of 50% in volume with dietary levels of water or there could be a decrease of 25% in haemolymph protein concentration with one day of food deprivation (Chapman, 1990). In insects, fluctuation in haemolymph proteins during the developmental stages provide an excellent material to study the regulation mechanisms related to not only to reproduction physiology but other cellular events of protein biosynthesis as well.

2.1.3. Azadirachtin and haemolymph proteins

Many of the reported manifestations of azadirachtin treatment of insects may be explained in terms of the effects of the terpenoid on proteins of the specific tissues of insects (Mordue *et al.*, 1986). A large amount of data is available detailing effects of azadirachtin on insects but reports on azadirachtin induced changes in the haemolymph proteins of the desert locust *S. gregaria* are scarce. This insect has been used for a long time as an experimental model to study the physiological and biochemical effects of azadirachtin. However, the effects of azadirachtin are often apparent over periods of days rather than minutes, so the rate and extent of metabolism is obviously important to study the effect of azadirachtin on general protein biosynthesis in fat body of *S. gregaria*.

2.1.4. Cycloheximide and protein metabolism

Cycloheximide has been shown to inhibit the protein synthesis in the systems that utilise ribosomes of the 80S type (Sisler *et al.*, 1967; Abu-Hakima and Davey, 1977). It acts mainly through blocking translation (Sass and Kovacs, 1980). Cycloheximide also inhibits both breakdown and reassembly of polyribosomes in rat liver systems (Trakatellis *et al.*, 1965), and in hamster cells (Stanners, 1966). The effect of cycloheximide was evaluated on the *in vivo* incorporation rate of [³H] leucine into haemolymph and fat body proteins of the adult female desert locust *S. gregaria* for comparison to the effect of azadirachtin.

2.1.5. Aims

The aim of this preliminary study was to determine if in fact azadirachtin did reduce the incorporation of a radio-labelled amino acid into the proteins of fat body and haemolymph of *S. gregaria*. The tracer chosen was [³H] leucine, which has been previously used, and found to be a suitable amino acid for this purpose to study the effects of cycloheximide in mealworm, *Tenebrio molitor* (Soltani and Soltani, 1995).

2. 2. Materials and methods

2. 2.1. Insects

Adult *Schistocera gregaria*, were purchased from Blades Biological Ltd., Edenbridge, Kent, and were maintained under laboratory conditions in metal cages at a temperature of 26°C, and a light/dark cycle of 16/8 hours. The insects were fed on wheat grains and fresh washed cabbage leaves. Fresh tap water was also supplied. Cages were regularly cleaned. Female locusts were used for all experiments.

2.2.2 Insect saline

Saline containing 10mM KCl; 140mM NaCl; 2mM CaCl₂; 10mM glucose; and 100mM sucrose was used as a simple locust physiological medium (Paranagama, 1994).

2.2.3. Treatment of insects

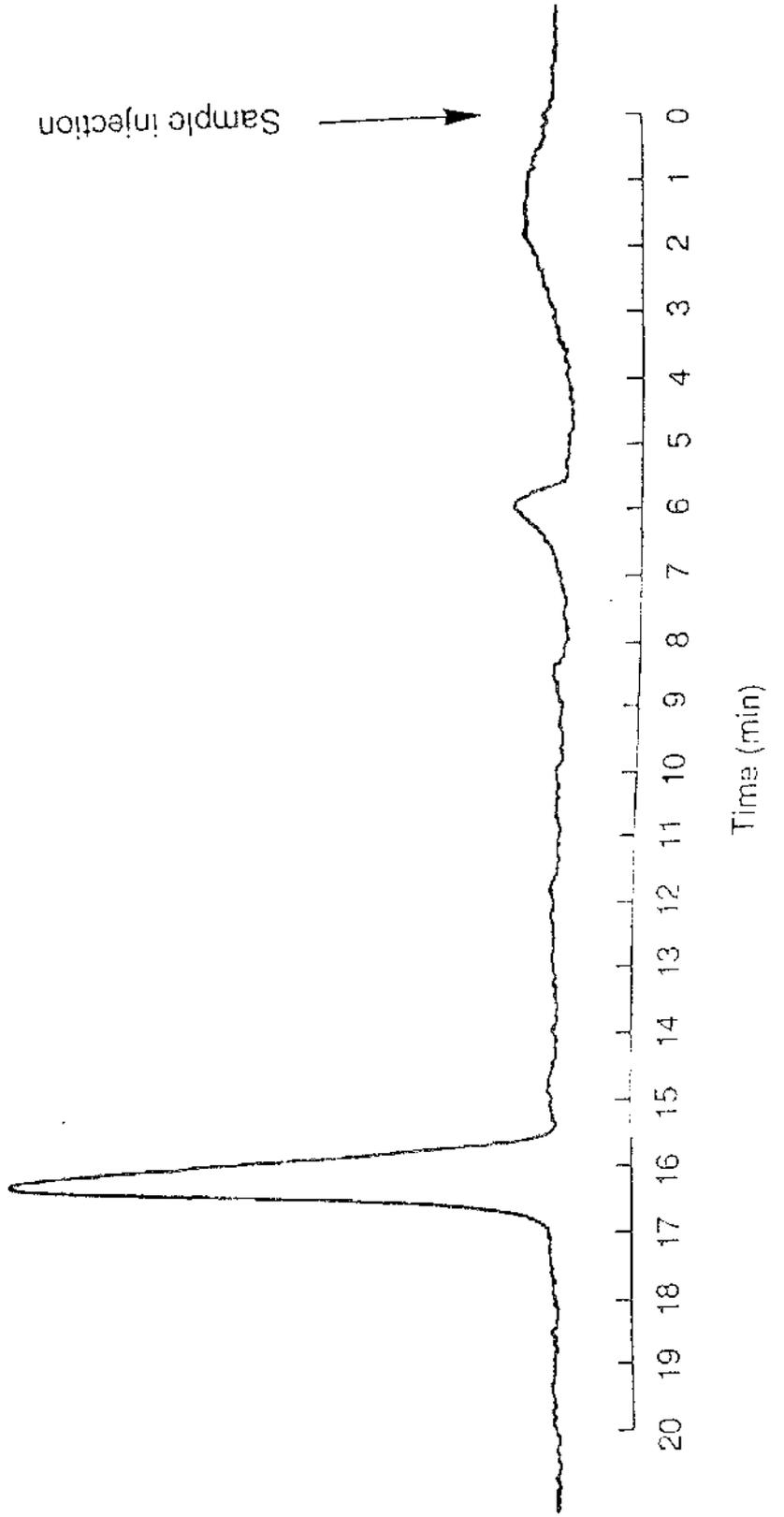
2.2.3.1. Azadirachtin treatment

Azadirachtin, >95% pure, was obtained from the Vittal Mallya Science Research Institute, KR Road Bangalore, India. When this was run on reverse phase High Performance Liquid Chromatography, (HPLC) only one peak was present, as shown in Fig 2.1. Azadirachtin was stored dry at -20°C, during the experimental period, under which conditions no deterioration was apparent.

Azadirachtin was usually dissolved in ethanol, and then diluted to 10% and 5%(v/v) with water and injected in 10µl volumes to give a dose of 5µg/locust into the body of adult insects (Annadurai and Rembold, 1993), into a group of four locusts. This dose was physiologically enough to significantly affect protein synthesis, as shown previously (Paranagama, 1994). Injections were made through the abdominal intersegmental membrane using a Hamilton syringe (20µl) (Hamilton Company Nevada.), 16hrs before the administration of tritiated leucine. Control insects received a similar dose with 10% ethanol-water (v/v) alone.

Fig. 2.1. Analytical high performance liquid chromatogram (HPLC) of azadirachtin.

The figure represents the chromatography of azadirachtin by reverse phase HPLC, the solvent was 50:50 methanol water (v/v). The arrow indicates the point of sample injection.



2.2.3.2. Incorporation of tritiated leucine

Tritiated leucine was obtained from Amersham International Aylesbury, Bucks. The specific activity was 58TBq/mmol , in 2% (v/v) ethanol aqueous solution. Adult females of *S. gregaria*, from controls, azadirachtin and cycloheximide treated, were injected with $2\mu\text{Ci}$ of $[^3\text{H}]$ -leucine, in a volume of $20\mu\text{l}$ diluted by insect saline, after 16hrs of azadirachtin treatment and after 2hrs of cycloheximide treatment. The radiolabelled precursor was injected into the body of the locust with a Hamilton syringe ($25\mu\text{l}$). Injections were made through the abdominal intersegmental membrane.

During the whole period of each treatment the locusts were fed on fresh cabbage and kept under standard rearing conditions.

2.2.3.3. Cycloheximide treatment

Cycloheximide (Sigma Chem Co.), was dissolved in sterilised insect saline (2.5mg/ml) and kept frozen at -20°C until further use. An amount of $25\mu\text{g/insect}$ of cycloheximide, in a volume of $10\mu\text{l}$ of insect saline was injected 2hrs before the injection of tritiated leucine, with Hamilton syringe, into the body of the female locusts.

2.2.4. Uptake of radiolabelled leucine by haemolymph and fat body

In a preliminary experiment, the rate of incorporation of radioactivity was measured over a period of 24 hours at intervals of 2 hrs. The aim was to find out a time course for the maximum incorporation rate of radiolabelled amino acid into the proteins of haemolymph and fat body of untreated female locusts. The amount of incorporated leucine into the proteins of both haemolymph and fat body, reached at its maximum value by 6 hrs, as shown in the result section. Following the initial experiment, the rate of incorporation of $[^3\text{H}]$ -leucine into the haemolymph and fat body was measured over 6 hours to investigate precisely the effect of azadirachtin on individual tissues. As mentioned earlier cycloheximide, a protein synthesis inhibitor, was also evaluated at the

same time to compare the effects of terpenoid. The tissue collection in each case was done using a method described under the following section.

2.2.5. Tissue sampling

2.2.5.1. Simultaneous collection of haemolymph and fat body

The insects were anaesthetised by 10 minutes cooling at -20°C , and then kept in ice until used. The insects were carefully decapitated, without severing the oesophagus, and the posterior tips of the abdomen then cut off, which allowed the entire gut to be removed from body cavity. The body of the insect was then placed in the filtration device indicated in Fig 2.2. Low speed centrifugation at 400-800 rpm (Beckman Model TJ-6 Centrifuge) for 2 minutes allowed the separation of the fat body. The fat body was retained on the filter, and the whole haemolymph passed into a preweighed centrifuge tube. The total volume of haemolymph was determined by weighing.

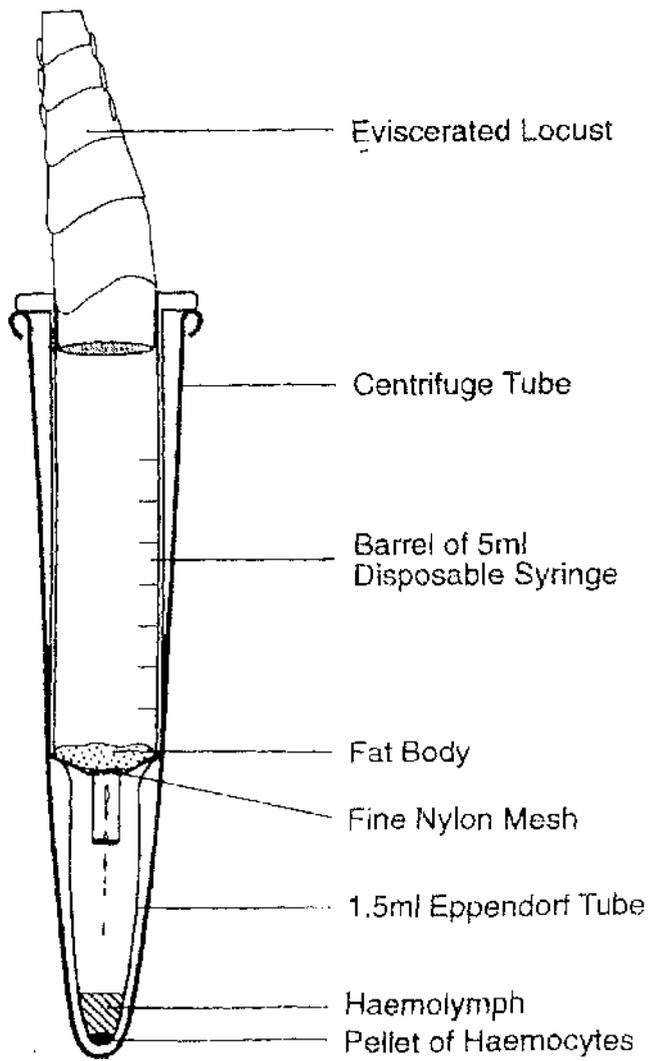
The isolated fat body from each locust was transferred to a pre-weighed plastic centrifuge tube, which was then weighed again to determine the weight of fat body. It was then homogenised in 500 μl of phosphate buffer, pH 6.2, by a few passes of a teflon homogeniser, surrounded by ice slurry and then sonicated with a 3mm Dawe Soniprobe (Type 1130 A; Lucas Dawe Ultrasonics Ltd., London.) for 30 seconds. Finally it was centrifuged for 2-3 minutes at 13,000g. Centrifugation yielded 3 layers, a fatty plug on the above surface, a clear middle supernatant layer and a pellet at the bottom. Samples were carefully removed from the clear middle layer and processed further for extraction of proteins.

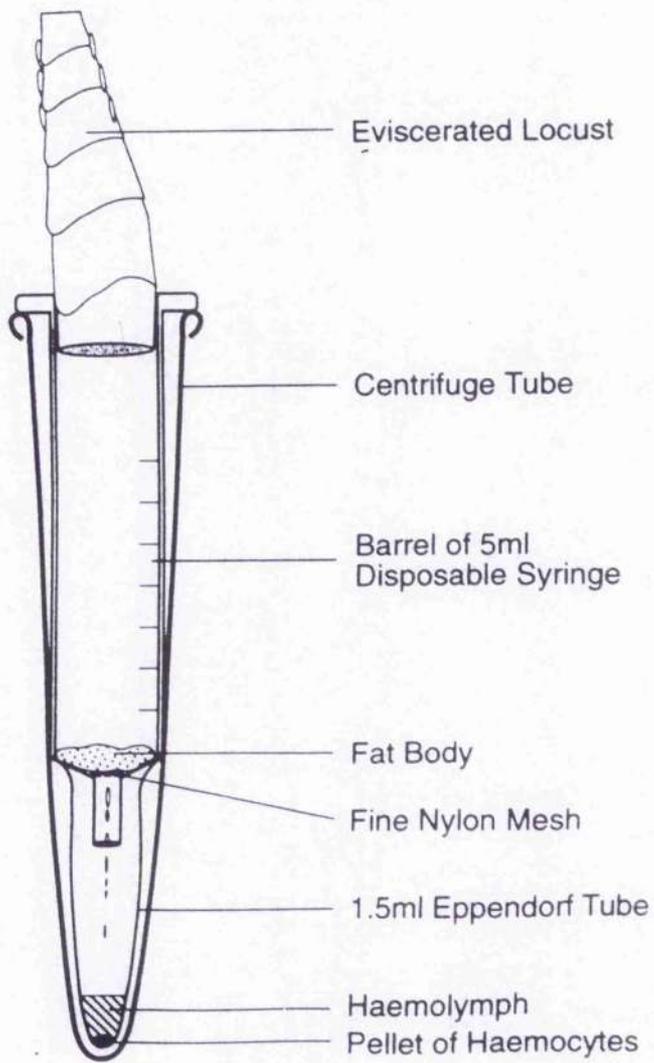
2.2.6. Determination of protein amounts and radioactivity

2.2.6.1. Extraction of proteins from haemolymph

The individual samples of haemolymph were measured and mixed with equal volumes of 10 % (w/v) trichloroacetic acid (TCA). Samples were kept deep frozen in 5 % TCA until further processing as required.

Fig. 2.2. A device for simultaneous collection of fat body and haemolymph from the body of the locust.





After thawing, the homogenate was centrifuged for 5 minutes at 13,000 g, then supernatant was removed, and the pellet washed 3 times in 5 % TCA by resuspension. This process removed all the radioactivity not incorporated into the haemolymph proteins. The protein pellet was redissolved in 0.2ml of 0.1M NaOH containing 0.1 w/v SDS. The samples were incubated overnight at 30°C before the estimation of specific activity.

2.2.6.2. Extraction of proteins from fat body

The isolated fat body samples were processed exactly in the same way as described for haemolymph except,

- i) initially it was washed 3 times by 5 % TCA
- ii) homogenised for 30 sec in 0.5 ml of TCA (5 % w/v) using an ultrasonic microprobe, and
- iii) centrifuged for 5 min at 13,000g.

2.2.6.3. Estimation of protein

When required, protein estimation of the tissue extracts were made from duplicate or triplicate samples by the Coomassie Blue using bovine serum albumin (BSA) as a standard (Bradford, 1976). The concentration of BSA was 1mg/ml (w/v) in a solution of 0.15M NaCl.

The reagent was prepared by the following procedure:

Coomassie Brilliant Blue G-250 (Sigma Chem Co.) (100mg) was dissolved in 50 ml of 95% ethanol. To this solution 100 ml of 85% (v/v), phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre with water and filtered twice by Whatman No.1 filter paper.

The standard protein solution (BSA 1mg/ml) containing 10, 20, 40, 60, 80, and 100µg of protein was pipetted into 12x100mm test tubes. The volume in each test tube

was adjusted to 0.1ml with 0.15M NaCl. Then five millilitres (ml) of protein reagent was added into each tube and contents were mixed by inversion of tubes.

The absorbance at 595nm (Ultrospec II Spectrophotometer LKB, Biocheome), was measured after 2 minutes and before 1 hr, in 3ml plastic cuvettes against a reagent blank prepared from 0.1ml of 0.15M NaCl and 5ml of protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve. This curve was used to determine the amount of protein present in the haemolymph or fat body samples of individual locusts of controls, azadirachtin and cycloheximide treated groups.

2.2.7. Determination of radioactivity into haemolymph and fat body proteins

The haemolymph and fat body samples were prepared as described in sections 2.2.6.1 and 2.2.6.2, and incubated for 24 hrs at 30^oC. After incubation, the final residue of each sample was divided into three parts. One part was used for determination of protein levels by the Bradford method (1976), (2.2.6.3). The remaining two parts were used to measure the rate of incorporation of tritiated leucine into the proteins of each sample.

The incorporation of labelled leucine into the proteins of haemolymph and fat body was measured in the remaining two parts as duplicate samples from each locust for each tissue. The radioactivity was counted after addition of 4ml of scintillation liquid/cocktail (Ultima-Flo.AF LSC-Cocktail, Packard.). The samples were counted at 65% efficiency using a WALLAC 1209 Rackbeta Liquid Scintillation Counter.

The specific activity of the acid-precipitable proteins in each tissue was estimated as dpm.mg⁻¹ of protein (with reference to the protein content).

2.3. Results

2.3.1. Time course of incorporation of [³H]-leucine into the haemolymph and fat body proteins of locust *S. gregaria*

The time course for the maximum incorporation of [³H]-leucine was determined for periods of 2, 4, 6, 8, 10 and 12hrs in the haemolymph and fat body of untreated female adult locusts. The incorporation of [³H]-leucine into both tissues was almost linear for at least 6hrs and then there was a decrease after this time period (Fig 2.3).

It was obvious that the incorporation of labeled amino acid into the TCA precipitable proteins followed the same time scale with a maximum at 6hrs. This maximum time of incorporation of tritiated leucine was used to assess the effects of azadirachtin and cycloheximide on haemolymph protein concentration and fat body protein biosynthesis, in later experiments.

2.3.2. Effect of azadirachtin and cycloheximide on incorporation of the [³H]-leucine on the haemolymph proteins

The course of the *in vivo* incorporation (dpm.mg⁻¹ of proteins) of tritiated leucine into haemolymph proteins after 6hrs was determined respectively in controls, azadirachtin and cycloheximide treated locusts (Fig 2.4). In controls the rate of incorporation increased during the examined period.

In the azadirachtin injected locusts the incorporation of radiolabelled amino acid into the haemolymph was reduced as compared to the controls. The incorporation level of azadirachtin treated locust haemolymph was reduced 25% only to that of controls and these values were not significantly different from each other. On the other hand, the treatment of cycloheximide caused a statistically significant ($p < 0.05$) decline (i.e 37% that of controls), in the rate of incorporation of tritiated leucine into the haemolymph proteins of locusts.

Fig. 2.3. The rate of incorporation of [³H]-leucine into the haemolymph and fat body proteins of the untreated locust, *S. gregaria*.

The time course of incorporation of [³H]-leucine into the haemolymph and fat body proteins of the locust after injection of 2μCi/insect into the body of the adult locust was measured for 2-12hours. The values represent the mean (±SD) of four locusts.

The specific activity is at its maximum after 6 hours of injection.

-----○ Haemolymph
——● Fat body

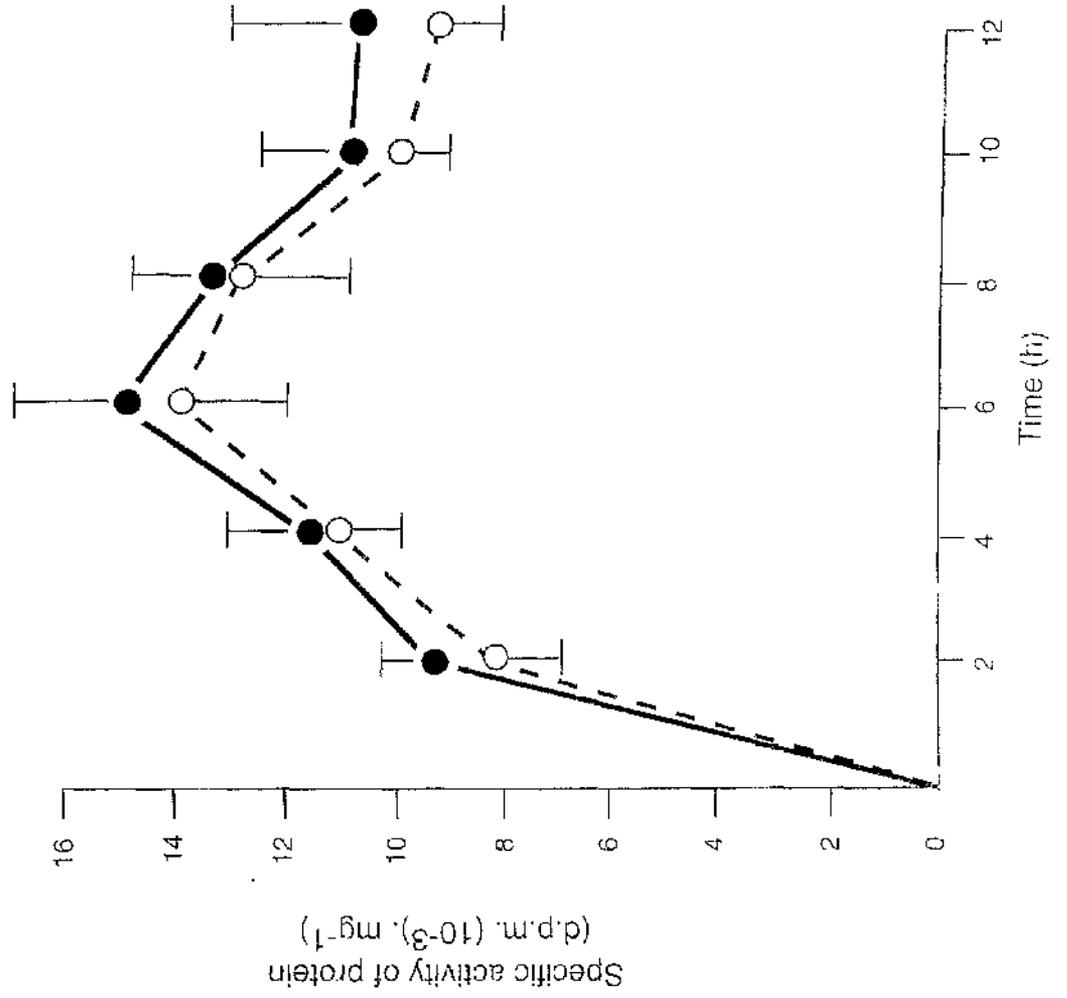


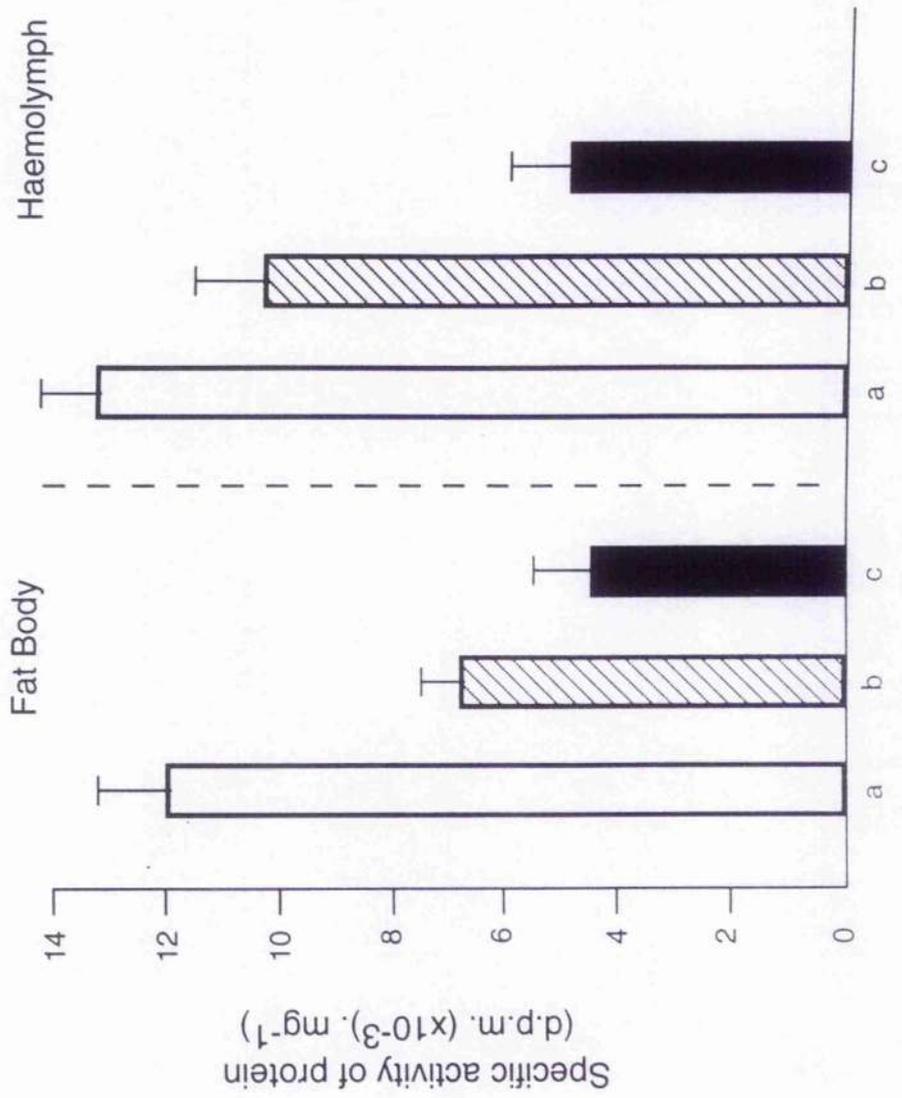
Fig. 2.4. The effect of azadirachtin and cycloheximide on the rate of incorporation of [³H]-leucine into the proteins of haemolymph and fat body of the female adult locust, *S. gregaria*.

The experimental locusts were injected 5µg of azadirachtin/locust, (10µl volume), 16 hours before the injection of radiolabelled amino acid. The controls received the same volume of 5% (v/v) ethanol-water. Another group of locusts was injected with 25µg of cycloheximide/insect 2 hours before the injection of [³H]-leucine. After a period of 6 hours of the injection of 2µCi/locust into the body of each locust from each group, the rate of incorporation into the haemolymph and fat body proteins was measured.

The values represent the mean (\pm SD) of four locusts.

For both haemolymph and fat body,

- a) controls
- b) azadirachtin treated and
- c) cycloheximide treated.



2.3.3. Effect of azadirachtin and cycloheximide on incorporation of [³H]-leucine on the fat body proteins

The incorporation of [³H]-leucine into the fat body proteins was measured over 5 hours, and results are shown in Fig 2.4. Generally the level of incorporation into fat body proteins was slightly lower compared to the haemolymph proteins. In azadirachtin-treated locusts, the incorporation of radiolabelled amino acid was not as efficient as in control locusts. There was a reduction of 42% in the rate of incorporation of [³H]-leucine into fat body proteins after azadirachtin treatment which is statistically significant ($p < 0.05$). The treatment of cycloheximide also caused a significant decrease, 64% ($p < 0.05$) in the rate of incorporation of tritiated leucine into the fat body proteins of female adult locusts as compared to the control ones.

2.4. Discussion

Studies of the incorporation of radiolabelled amino acids into proteins can provide very useful data on the pathways of biosynthesis and metabolism. Mordue and Highnam, (1973) used the uptake of ³⁵S-cysteine into the neurosecretory proteins of adult female locust *Schistocerca gregaria* as a mean to study the different physiological conditions of locusts. Radiolabelled incorporation of cysteine demonstrates that the azadirachtin treated locusts have a very low turnover of neurosecretory proteins in corpus cardiacum, while in controls the transport of labelled protein was at a higher level (Subrahmanyam *et al.*, 1989b). Radiolabelled leucine and glycine were more readily incorporated into fat body proteins than the other precursors in the locusts (Clements, 1959). A considerable rate of incorporation of tritiated leucine into the proteins of the fat body of the *Rhodnius prolixus* was observed (Vanderberg, 1963). Tritiated leucine also has been used as a precursor for fat body proteins in adult migratory locust, *Locusta migratoria*, (Gellissen and Wyatt, 1980). It was also used to study the storage proteins by the fat body of the larvae of greater wax moth, *Galleria mellonella* (Miller and Silhacek, 1982).

When a physiologically effective dose of azadirachtin was injected into the body of the female adult locust, it induced an inhibitory effect on the rate of incorporation of radiolabelled leucine into the proteins of the identified tissues like haemolymph and fat body, suggesting a general inhibitory effect of azadirachtin on protein metabolism. The method of protein precipitation employed here was intended to contain most labelled amino acid which has been incorporated into the proteins of haemolymph and fat body, and previously has been used successfully in our laboratory (Paranagama, 1994).

In untreated locusts, the sequence of incorporation of tritiated leucine into the proteins of haemolymph and fat body was different from the one previously reported by Hill, (1965) for glycine in the locust *S. gregaria*. The previous author found a clear progression of label first into fat body then haemolymph, but our results suggest nearly an equal level and time labelling for both haemolymph and fat body. The time course for the maximum incorporation of tritiated leucine was nearly same as that one observed for a maximum label for the same amino acid in the larval fat body of *Galleria mellonella*, (Miller and Silhacek, 1982).

The profound effect of azadirachtin on reducing the incorporation of label into the fat body is likely to be a direct effect on that tissue. This would help to explain the fact that the observed clearcut reduction of protein synthesis by azadirachtin in fat body is not apparent in haemolymph. This observation is in agreement to the result of incorporation of ^3H -glycine into the proteins of haemolymph and fat body of locusts (Paranagama, 1994). This could be due to the fact that fat body is the site of protein synthesis while in haemolymph the proteins are sequestered, already synthesised by fat body. These findings made a basis to proceed further with the effects of azadirachtin and to work with some identified or purified proteins, like lysozyme and vitellogenin synthesised by fat body.

Chapter 3
Suppression of lysozyme production by azadirachtin
in specific tissues of
the desert locust, *Schistocerca gregaria*

3.1. Introduction

Although the primary mode of action of azadirachtin in insect tissues is not known, the work in Chapter 2 and other sources show an effect on general protein biosynthesis in the fat body of *S. gregaria* (Paranagama, 1994; Annadurai and Rembold, 1993). It is still not clear if the effect is general or specific to only a few proteins. However, it is quite clear that azadirachtin has a general inhibitory effect on protein biosynthesis in differentiated tissues of the locust, including fat body.

One of the reported effects of azadirachtin in insects is on the biological "fitness" of insects, or more specifically the suppression of their immunity (Su and Mulla, 1998). The term, "immunity" in Webster's Dictionary is defined as, "resistance to or protection against a specified disease; or power to resist infection and ability to resist bacterial infection is related to the biological fitness". The work reported in this chapter is about the effect of azadirachtin on the suppression of antibacterial protein synthesis in the desert locust *S. gregaria* using lysozyme as an identified antibacterial marker protein.

3.1.1. Insect immunity

The primary defences of insects against bacterial infections are the passive physical barriers to the penetration, like exoskeleton, tracheal system, foregut and hindgut, and the peritrophic membrane surrounding the food bolus within the midgut lumen (Gupta, 1989). However, if these barriers are ruptured, opportunistic bacteria may enter the body cavity, multiply and produce infection (Russel and Dunn, 1990). There is evidence that insects defend themselves effectively from infection by combination of two types of responses, cellular and humoral (Boman and Hultmark, 1987). Insects synthesise a battery of potent antibacterial proteins when invaded by bacteria (Hultmark *et al.*, 1981; Steiner *et al.*, 1981). The antibacterial proteins synthesised in a response to bacterial infection, constitute an important part of the insect humoral immune system. It is generally accepted that in insects, these proteins are synthesized in the fat body and

certain hemocytes, then secreted into the haemolymph, where they eliminate the invading microorganisms. For bacteria, which have gained access to the body cavity of insects, to multiply and colonize the hemocoel, they must survive the action of a set of active antibacterial defences produced by hemocytes and/or fat body. An outline of these defences is given in Fig 3.1, which is based on the main sources of synthesis of these proteins, produced in the result of response. Normally all these proteins are not present in haemolymph but are promptly synthesized by hemocytes and/or fat body and then secreted into the haemolymph when insects are in acute phase. Most of these proteins are small molecules of high pI values, exhibiting a broad spectrum of activity against Gram-positive and/or Gram-negative bacteria (Natori, 1994).

3.1.1.1. Sources/Types of immune responses

3.1.1.1.1. Humoral response

There are a number of factors contributing to the evolutionary successes of insects, and the ability to remove microorganisms and parasites from their circulation is one of them. The humoral and cellular defence systems cooperate in a more or less integrated way to decrease the chance of the microorganisms becoming pathogenic. The humoral factors are a battery of antibacterial peptides which are induced by bacterial infections, and include cecropins, attacins, lysozyme, dipterocins, defensins and other factors (Feder *et al.*, 1997). In practice the term "peptide" is rarely used for molecules larger than 10kDa, and the word "protein" is rarely used for molecules smaller than insulin.

Induction and biosynthesis of the major humoral components involves the recognition of elicitors of the insect immune system. The recognized elicitors of the insect immune response are bacterial cell wall components, e.g. peptidoglycan and lipopolysaccharide (LPS), (Boucias *et al.*, 1994). It is suggested that in the *Manduca sexta*, after injection of soluble peptidoglycan from a Gram-positive bacterium, plasma lysozyme hydrolyses bacterial peptidoglycan and produces a signal for the synthesis of antibacterial proteins in fat body (Kanost *et al.*, 1988).

Fig 3.1. An outline of the different types of immune responses in insects.

Immune response in insects



Humoral response

Cecropin synthesis

Attacin formation

Lysozyme production

Cellular response

Phagocytosis

Encapsulation

Nodule formation

Prophenoloxidase activation

Lysozyme production

3.1.1.1.2. Cellular response

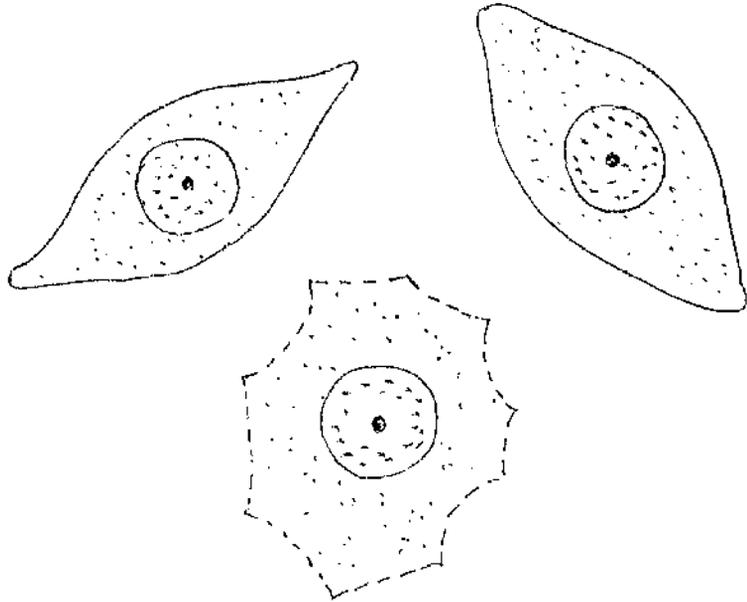
Insects respond to a bacterial infection with the stimulation of a cellular defence system in addition to the humoral one. Cellular reactions include phagocytosis, nodule formation, in some cases encapsulation, production of lysozyme and activation of the prophenoloxidase cascade. Prophenoloxidase is present as an inactive precursor in haemolymph under normal physiological conditions and is activated in response to bacterial peptidoglycan (Morishima *et al.*, 1992). According to Price and Ratcliffe (1976), who have designed a classification scheme for blood cells in insects and recognized six cell types on the basis of an investigation of haemocytes of 15 orders of insects. These are such as:

- a) prohemocytes
- b) plasmatocytes
- c) granulocytes
- d) coagulocytes
- e) spherulocytes and
- f) oenocytoids.

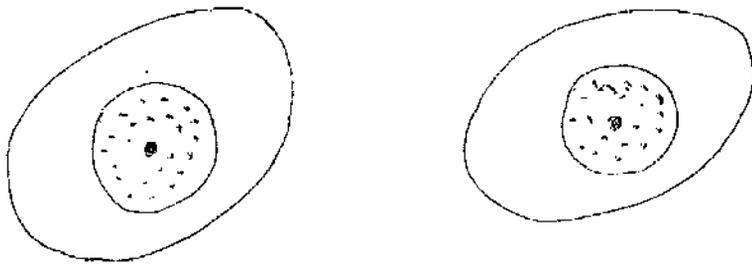
There is a diversity of appearance in these cells (Fig 3.2). Different types of blood cells or haemocytes have important roles in the protection of insects against invading microorganisms. Small agents such as bacteria or unicellular fungi are phagocytised, while larger aggregates of foreign cells are encapsulated by the cooperation of many haemocytes. Finally, nodule formation occurs as a response to a large number of foreign cells (Boman and Hultmark, 1987). The main types of cells involved in phagocytosis are plasmatocytes and granular cells. Plasmatocytes also participate in capsule and nodule formation. However, the core of a nodule is formed from the material released by granular cells.

Both defence systems of insects combine to fight the infections. In some investigated cases, the phagocytized bacteria were not killed, but continued to live in a

Fig 3.2. Variation in the shape of the different types of haemocytes in *Rhodnius prolixus* (Wigglesworth, 1984).



Plasmatocytes



Oenocytoids

steady state for up to 1 week (Abu-Hakima and Faye, 1981). The next step is the synthesis of RNA and specific proteins, which gives rise to increasing antibacterial activity in the haemolymph. This activity continues to increase for a certain time period which varies according to the type of infection, the age/stage and species of insect, and then it gradually declines. For example, in the immunized giant silkworm moth *Hyalophora cecropia*, the overall protein pattern and profile of newly synthesized antibacterial haemolymph proteins was analysed by gel electrophoresis and grouped into three: lysozyme, cecropins and attacins. Similar immune response has been found in the tobacco hornworm *Manduca sexta*, in a beetle and several flies (Boman and Hultmark, 1987).

3.1.2. Variations in immune response

Bacterial diseases of insects have profound effects on the growth, development, productivity and survival of insect populations. Therefore much research has been focused on the active defenses whereby insects detect the presence of bacteria within the body cavity. Generally, the primary active haemolymph-based antibacterial defences of naive insects are a series of sequestration processes mediated by the circulating haemocytes. In addition to mobilizing haemocytic defenses, the presence of bacteria in the body cavity also induces the synthesis of a suite of antibacterial proteins which accumulate in the plasma (Dunn *et al.*, 1987).

Previously infected insects with elevated levels of haemolymph bactericidal peptides were able to survive bacterial infections of a magnitude that would kill the naive insect (Dunn and Drake, 1983). Feder *et al* (1997) reported the effect of 15-45 days starvation, in a significant reduction of cecropin-like and lysozyme activities in the haemolymph of *Rhodnius prolixus* larvae when challenged with *Enterobacter cloacae* B12.

3.1.2.1. Haemolymph immune response in insects after infection

The changes in haemolymph proteins could be a direct result of an infection or a physiological response, secondary to infection. The cellular and humoral immune system of the insects and their response to infection is not limited to bacteria only. The immune responses to fungal, viral or parasitic infections have also been studied extensively. Morphological changes in haemocytes were reported in response to these infections. In the migratory grasshopper, *Melanoplus sanguipes*, morphological changes were observed in haemocytes, as cells had extensive pseudopodia after infection with an *Entomopxvirus* (Miranpuri *et al.*, 1992). Similarly in *M. sexta* larvae, certain abnormalities in haemocytes were reported when parasitized by *Cotia congregata*. The plasmatocytes failed to assume their normal spindle-like shape and the granulocytes failed to attain their normal "fried egg" like appearance (Lavine and Beckage 1996). Andersons *et al* (1990), examined the immune response of *Trichoplusia ni* to injection with bacteria and/or a nuclear polydnavirus (NPD). There have been observed alterations in haemolymph protein patterns and some breakdown of haemolymph proteins including induced bactericidal proteins.

3.1.2.2. Induction of haemolymph antibacterial proteins

Studies of the regulations of the haemolymph antibacterial protein synthesis in *M. sexta* larvae have suggested that bacteria which enter the haemolymph, are attacked by haemocytes and haemolymph lysozyme, which is present at low constitutive levels in naive larvae. This process leads to the release of fragments from the bacterial outer envelope. These fragments in turn induce the synthesis and secretion of the suite of proteins like lysozyme, families of cecropin like bactericidal peptides, bacteriostatic attacin-like proteins and the hemolin (Russel and Dunn, 1996). It is thought that these proteins are synthesized by haemocytes and/or fat body and then released into haemolymph (Kanost *et al.*, 1988). The injection of bacterial lipopolysaccharid caused the production of a high titre of lysozyme activity in the haemolymph of beet

armyworm, *Spodoptera exigua* (Boucias *et al.*, 1994). According to Kubo *et al* (1984), in the haemolymph of larvae and adult of flesh-fly *Sarcophaga peregrina*, there was an induction of lectin and three bactericidal proteins when their body wall was injured with a hypodermic needle, and fat body was the likely site of synthesis of these proteins. In *Hyalophora cecropia* and *Manduca sexta*, lysozyme was one of the major proteins induced after bacterial infection (Faye and Hultmark, 1993).

The inoculation of bacteria in *Rhodnius prolixus* induced an increase in haemocyte numbers and nodule formation and the appearance of antibacterial peptides (Azambuja *et al.*, 1991). There has been reported an increase in the lysozyme activity in the haemolymph of *Bombyx mori* after injection of not only bacteria but also some other foreign substances (Powning and Davidson, 1973). Lysozyme, cecropins and attacins constitute the main antibacterial components of the humoral immune system in *Cecropia*, induced by bacterial infection or injury (Sun *et al.*, 1991). Faye and Wyatt (1980) showed that cecropins in *Cecropia* silkworm pupae were synthesized in the fat body.

3.1.3. Azadirachtin and antibacterial proteins in insects

Limited information is available regarding the effect of azadirachtin on haemolymph immune responses in insects, such as total or differential haemocyte numbers, production of specific humoral substances, or changes in identified haemolymph proteins. It has been recorded that azadirachtin influenced immune reactivity of the haemolymph protein in larvae of *Rhodnius prolixus* when *Enterobacter cloacae* B12 strain were inoculated into the insects. Azadirachtin affects the immune reactivity as shown by the following:

- a) significant reduction in numbers of haemocytes and nodule formation after challenge with *E. cloacae*,
- b) decreased ability of insect to produce antibacterial and lysozyme activity in the haemolymph when inoculated with bacteria,

c) a reduction in ability of azadirachtin-treated insects to destroy the primary infection caused by inoculation with *E. cloacae*.

An explanation of the above effects of azadirachtin could be that *R. prolixus* may have a diminished ability to undertake haemolymph protein synthesis (Azambuja *et al.*, 1991). In our study the lysozyme was chosen as an easily-assayed antibacterial protein. The effect of azadirachtin on the induction of lysozyme synthesis was studied in the identified tissues of the female adult locust *S. gregaria*, when challenged with bacterial lipopolysaccharide.

3.1.4. Lysozyme

Lysozyme (E. C. No: 3.2.1.17), is the general name given to a group of low Mr enzymes, showing a range of Mr from different organisms of approx 3-15 (Boman and Hultmark, 1987; and Boucias *et al.*, 1994). The enzyme was first discovered by Alexander Fleming in 1922. It is a mucolytic enzyme with antibiotic properties. The name lysozyme was derived from its capacity to lyse bacteria. It is a basic protein, consisting of a single polypeptide chain of 129 amino acids, crosslinked by disulfide bridges (Stryer, 1988).

Lysozymes have been found in bacteriophages, bacteria, lower fungi, higher plants and both invertebrates and vertebrates. In the human body, lysozyme is present in the secretions of eye, nasal mucus, saliva and blood serum. It is most abundant in the white of chicken eggs and this is the major source of lysozyme used commercially. A number of the lysozymes from different sources have been isolated and characterized. These enzymes are one of the most investigated areas of comparative biochemistry. Since its discovery, lysozyme has been extensively studied and used as a model system for investigating the structure of proteins, mechanism of enzyme action and molecular evolution.

3.1.4.1. Action of lysozyme

The bacterial cell wall is made up of two kinds of sugars, N-acetylmuramate (NAM), and N-acetylglucosamine (NAG). All glycosidic bonds of the cell wall polysaccharide have a β -configuration. So the cell wall polysaccharide is an alternating polymer of NAM and NAG residues joined by β 1—4glycosidic linkages (Fig 3.3). Lysozyme catalyses the hydrolysis of the linkage between NAM and NAG in the bacterial cell wall peptidoglycan of Gram-positive bacteria, as indicated in Fig 3.3 (Stryer, 1988).

The susceptibility of *Micrococcus lysodeikticus* makes it particularly suitable for its detection/assay.

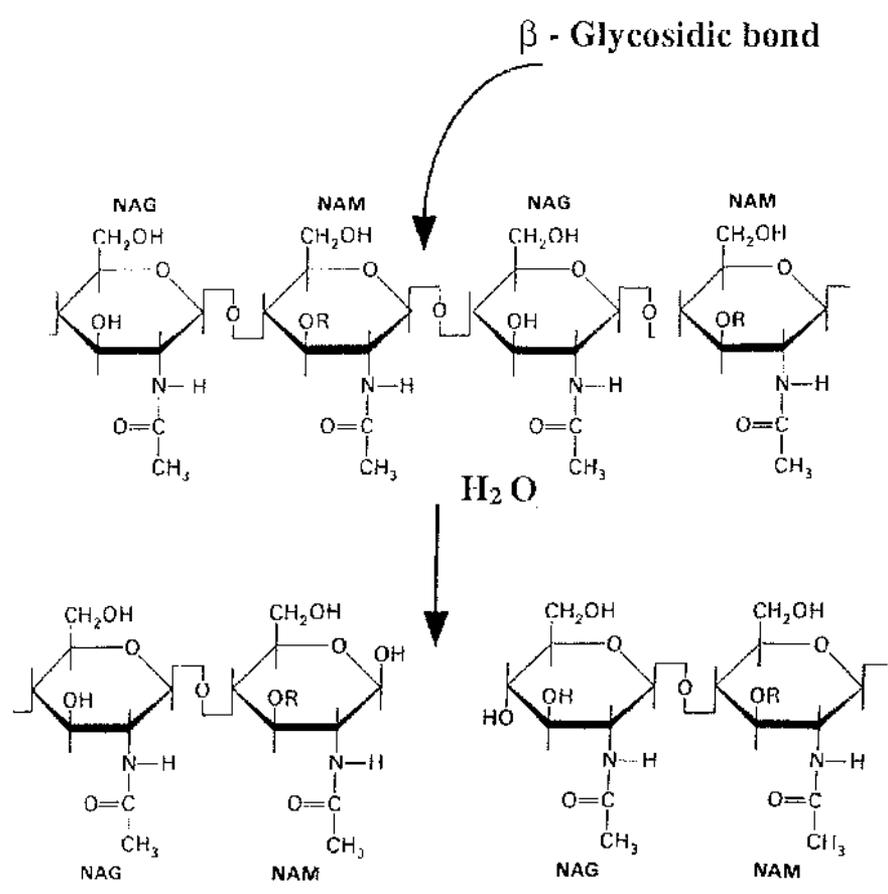
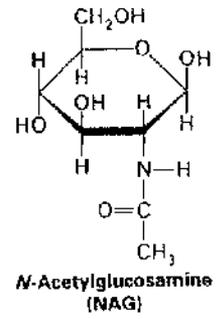
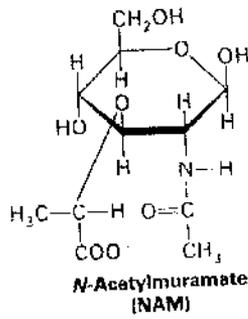
3.1.4.2. Function of lysozyme

The function of lysozyme is to protect the organisms against bacterial invasion by catalysing the hydrolysis of glycosidic bond of the peptidoglycan present in bacterial cell wall causing cell lysis (Lemos *et al.*, 1992).

3.1.4.3. Occurrence & distribution of lysozyme in insect tissues

It has been known for a long time that the production of lysozyme in response to bacterial invasion is a major defence mechanism in most insect species, especially in lepidopterans such as *Galleria mellonella* and *Bombyx mori* (Powning and Davidson, 1973; Dunn, 1986). It has normally been found to be present in low activities in the haemolymph of many insects such as in *Spodoptera eridania*, originating from the haemocytes and/or fat body (Anderson and Cook, 1978; Morishima *et al.*, 1994). The introduction of bacteria or their associated lipopolysaccharides or cell wall fragments into the body cavities of some insects elicit synthesis and release of lysozyme into the haemolymph. Several lysozymes have been identified and isolated from insects, but the one best studied is from giant silkworm, *Hyalophora cecropia* (Sun *et al.*, 1991).

Fig 3.3. Lysozyme hydrolyses the β 1-4 glycosidic bond between c₁ of N-acetylmuramate (NAM) and c₄ of N-acetylglucosamine (NAG), (Stryer, 1988).



In spite of the wide distribution of lysozyme in insects, little is known about the exact origin of this enzyme. The haemolymph, haemocytes and fat body of various insect larvae have been reported to possess lysozyme-like activities (Dunn *et al.*, 1985). In orthopterans, the involvement of lysozyme-like activity is reported in the early response against Gram-positive bacteria (Zachary and Hoffman, 1984). Russel and Dunn (1990), reported fat body as a primary source of haemolymph lysozyme in *Manduca sexta*.

To study the effect of azadirachtin on hemolymph induction of lysozyme in *Sch. gregaria*, the actual site of lysozyme synthesis was investigated. The *in vivo* work was followed by experiments done *in vitro* with the fat body of the female mature locusts in an attempt to confirm the suppression of LPS induced lysozyme-like activity, by azadirachtin.

3.2. Materials and methods

3.2.1. Insects

Female mature adults of the desert locust *S. gregaria*, were used as the experimental insects. They were fed and maintained as described in Chapter 2.

3.2.2. Insect saline

Saline containing: 10mM KCl; 140mM NaCl; 2mM CaCl₂; 10mM glucose; 100mM sucrose; pH6.9, was used as a simple locust physiological medium (Paranagama, 1994).

3.2.3. *In vivo* experiments

3.2.3.1. Antibacterial response of the locust

To explore the nature of the antibacterial response in *S. gregaria*, a series of experiments were planned using elicitors, as detailed in following sections.

3.2.3.1.1. Induction of response

To induce the synthesis of antibacterial proteins, two methods were adopted:

a) Injection of heat-killed culture of *E. coli*

Escherichia coli strain α DH5 was cultured in 10mM glucose salts medium at 37°C for 24 hours in a shaker incubator. From this culture 2ml was centrifuged at 13,000g for 5 minutes to separate the cells. The pellets were resuspended in 1ml of insect saline, and autoclaved. A volume of 10 μ l of this solution was injected into each locust. Injections were made through the abdominal intersegmental membrane with the aid of a Hamilton syringe (Hamilton Company, Nevada).

Control insects received only sterilised insect saline. After injection, the insects were returned to the standard rearing conditions and kept for a period of 24hours before tissue sampling.

b) Injection of Bacterial Lipopolysaccharide (LPS)

Commercially-available lipopolysaccharide from *Escherichia coli* serotype 026: B6 (Sigma Chem Co), was injected into the body cavity of locusts in an attempt to elicit increased activity of lysozyme. The lipopolysaccharide was dissolved in insect saline and subsequently sterilised by filtration (0.2 μ m Sterile Filter Assembly, Gelman Sciences, U.K). This dissolved LPS was stored at -20°C until used.

Volumes of 3-10 μ l of LPS solution (10 μ g/ μ l) were injected into the body of locusts, as described above. Each locust received LPS up to 30-100 μ g LPS /insect, (Anderson and Cook, 1979; Boucias *et al*, 1994). Control insects received only sterilised insect saline. After injections, locusts were returned to the standard maintenance conditions.

3.2.3.2. Tissue sampling

3.2.3.2.1. Haemolymph collection

Initially, whole haemolymph samples (5 μ l), containing both haemocytes and plasma, were taken from the base of thoracic leg of the locust by means of a calibrated 5 μ l micro

pipette (L.I.P Equipment and Services), and the lysozyme activity was estimated immediately. No attempt was made to separate the haemocytes.

3.2.3.2.2. Collection of haemolymph, haemocytes and fat body

The haemolymph and fat body samples were collected in the same way as described in Chapter 2, except that the haemolymph was centrifuged to separate the haemocytes.

The total volume of haemolymph was determined by weighing. Haemolymph was then centrifuged at 13,000g in a microcentrifuge (Micro-Haematocrit Centrifuge, Hawksley England.) for 3-5 minutes to sediment the haemocytes (Zachary and Hoffmann, 1983; Boucias *et al.*,1994). After that the haemolymph was carefully removed from the haemocytes by means of a pasteur pipette (volume of haemolymph/locust was in a range of 50-200 μ l). The pellet of haemocytes was gently resuspended by trituration with a pasteur pipette in isotonic insect saline to wash the cells. The haemocytes were finally sonicated for 30 seconds or homogenised in phosphate buffer, pH 6.2, with a specially designed teflon homogeniser (the volume of the buffer was same as that of the original haemolymph sample).

3.2.3.2.3. Preparation of fat body sample for lysozyme assay

The isolated fat body from each locust was transferred to a pre-weighed plastic centrifuge tube, which was weighed again to determine the wet weight of fat body. It was homogenised in 500 μ l of phosphate buffer (PB), pH 6.2, by a few passes of the teflon homogeniser, surrounded by ice slurry and then sonicated with a 3mm Dawe Soniprobe (Type 1130 A; Lucas Dawe Ultrasonics Ltd., London) for 30 seconds. Finally it was centrifuged for 3-5 minutes at 13,000g before the supernatant was assayed for lysozyme activity. Centrifugation yielded 3 layers, a fatty plug on the surface, a clear middle layer and a pellet. Samples were carefully removed from the clear middle layer for estimation of lysozyme activity.

3.2.3.3. Growth inhibition of *E.coli* (α DH 5)

In order to see if the injection of lipopolysaccharide or bacteria causes the locusts to produce bactericidal compounds, as previously has been shown for lepidoptera in *Spodoptera exigua* (Boucias *et al.*, 1994), the previous method was used with a slight modification. The experiment was conducted for both male and female locusts as to find the differences in the response or the differences in the type of treatment.

E. coli (α DH 5), was cultured for 24 hours in a glucose salts medium, and 100 μ l of this culture was poured onto autoclaved nutrient agar (Oxoid Ltd., Basingstoke, Hampshire England), in petri dishes under sterile conditions. Then, the bacteria were spread evenly over the surface of the agar by means of a sterile glass spreader. Wells were made in the surface of the agar with the sterile tip of a Pasteur pipette. Into these wells were placed aliquots of haemolymph from locusts which had been injected with bacterial lipopolysaccharide or with a suspension of heat-killed *E. coli* 24hrs before. Control wells contained haemolymph samples from locusts which had received insect saline. To ensure a positive control, one well on each plate contained a few crystals of the antibiotic, ampicillin (Sigma Chem Co). The plates were incubated at 37°C for 24 hours. After this time, an even lawn of bacteria had covered the agar, except the area where growth of bacteria was inhibited.

In each experiment a batch of four locusts was used for control and treated ones and the haemolymph samples were collected and pooled before testing for growth inhibition.

3.2.3.4. Assay of lysozyme activity

3.2.3.4.1. Agar gel lysoplate assay

A semi-quantitative and visual way to detect the lysozyme-like activity was to observe the degree of clearing in an agar gel lysoplate containing a suspension of *Micrococcus lysodeikticus*. A 1.5% (w/v) solution of bacteriological agar (Oxoid Ltd., Basingstoke, Hampshire.) was made up in phosphate buffer pH 6.2, containing 2mg/ml dried *M.*

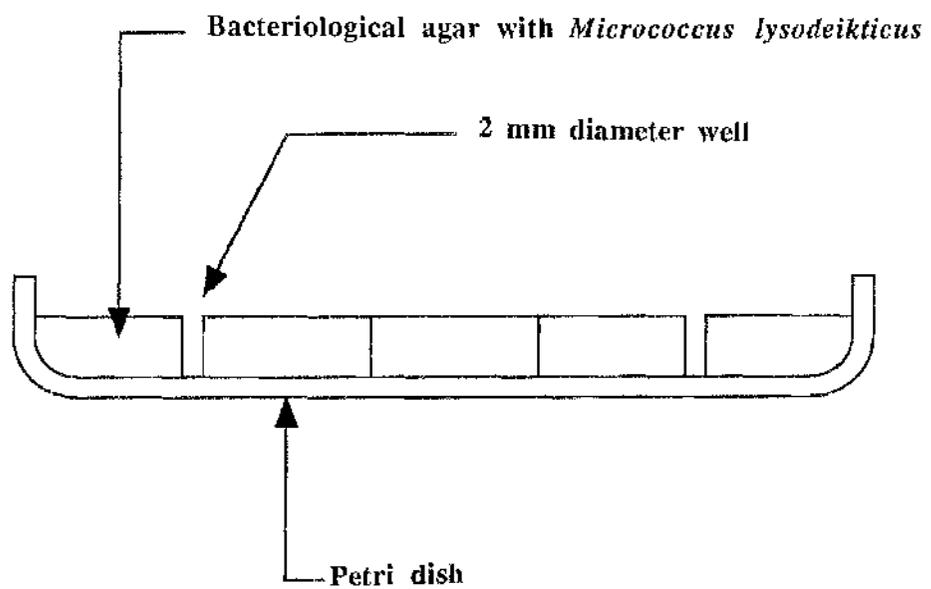
lysodeikticus cells (Zachary and Hoffmann, 1983). This was sterilised by autoclaving at 126°C (Prestige Medical Clinical Autoclave, England.), for 15 minutes, and allowed to cool, before pouring in 30ml aliquots into sterile plastic 8.5cm diameter petri dishes. Wells were made in the agar plate with the sterile tip of a pasteur pipette (Fig 3.4). Into the wells were pipetted the same volumes but different units (0.25, 2.5, 25 and 250U/well) of solutions of commercial lysozyme of known activity. Control wells contained samples of boiled enzyme. Similarly, aliquots (5µl) of extracts from locusts, thought to contain lysozyme, were placed into the wells of other plates. The plates were placed in a 37°C incubator for 24hrs. At the end of incubation period the lysozyme had created a zone of lysis around the well. Plates were recorded by scanning on UMAX Powerlook II after manipulation in Photoshop v.3.05. The recorded lysed zones in agar gels are shown in the results section.

3.2.3.4.2. Spectrophotometric method

After initial confirmation of presence of lysozyme-like activity by agar gel method, a more quantitative method was then used to assay the activity of enzyme. Lysozyme activity was estimated by a standard spectrophotometric assay based on the lysis of a suspension of a Gram-positive bacterium (Azambuja *et al*, 1991). Lyophilised cells of *M. lysodeikticus* (ATCC 4698) (0.2mg/ml) were suspended in 0.1M phosphate buffer pH 6.25. The activity of the enzyme was measured at 30°C by the decline in absorbance, (or strictly speaking the light scattering) at 450 nm through this suspension. Fresh samples (5-10µl) of homogenates derived from haemolymph, haemocytes and fat body were added to 1.0ml of the bacterial suspension in a plastic semi-microcuvette, and the change in absorbance followed over a period of 5-10 minutes was recorded, during which time the rate of change of absorbance was linear. If necessary, tissue samples were diluted with insect saline or phosphate buffer pH 6.25, before adding to the reaction mixture.

Fig 3.4. A sketch of bacteriological agar gel lysoplate setting with 1.5% of *Micrococcus lysodiekcticus*.

Wells were made and used for lysis test to estimate the presence of lysozyme by appearance of a zone of clearing around the wells.



3.2.3.4.3. Unit of lysozyme activity

Enzyme activity is given in International Units of Activity (I.U.). One Unit of lysozyme activity was defined as: that amount of enzyme which decreases the absorbance at a rate of 0.001 absorbance unit /min at 30^oC in 1.0 ml of the described medium (Dunn and Dai, 1990). Rates were derived from the linear part of the recorded curve. Final specific activities of the various tissues were quoted as U/ml of haemolymph or U/mg wet weight of the tissue.

3.2.3.5. Estimation of protein

When required, protein estimation of the tissue extracts were made from duplicate or triplicate samples by the method of Bradford, as described in 2.2.6.3 of Chapter 2, and a standard curve was generated. The assay was carried out in triplicate samples of haemolymph from the locusts used in experiments. Each absorbance obtained was converted to mg.ml⁻¹ using the standard curve. The value reported is the average of these three protein concentrations.

3.2.3.6. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

To identify the induced individual proteins particularly in fat body, and locust lysozyme in particular, SDS-PAGE was used. Electrophoresis was carried out under denaturing conditions using a Bio-Rad Agar Mini Protean II Cell.

Under denaturing conditions, analytical gel electrophoresis of proteins of haemolymph, haemocytes and fat body was carried out in 15%, and 20% gels at pH8.8, using a discontinuous system, with stacking gel of 3%, pH6.8. The acrylamide/bisacrylamide ratio was 29:1. Samples (5-100µg protein) were solubilised in a volume of Laemmli denaturing buffer, containing 0.1 M tris/HCl pH6.8, 20% (v/v) glycerol, 2 % (w/v) SDS, 0.02 % (w/v) Bromophenol blue and 5 % (v/v) β-

mercaptoethanol (which was added just prior to use) and the mixture was immediately boiled for 3-5 min at 100°C. The gels were run at 200 mV until the tracker dye (Bromophenol blue) was leaving the gel (electrophoresis buffer was containing 25mM Tris, 250mM Glycine and 0.1% SDS).

Commercial lysozyme and reference proteins (Bio-Rad) Table 3.1 of known molecular weights (kDa), were also used for comparative detection of induced proteins in the tissues of treated locust against controls.

3.2.3.6.1. Coomassie blue staining of proteins after SDS-PAGE

All gels were stained with 0.25%(w/v) Coomassie Brilliant Blue R250, in 10%(v/v) acetic acid and destained in 10%(v/v) methanol 10%(v/v) aqueous acetic acid. After destaining, the stained gels were scanned electronically on UMAX Powerlock II after manipulation in Photoshop v.3.05 to make a permanent record.

3.2.3.7. Azadirachtin

Azadirachtin >95% pure, was obtained from the research institute as described in Chapter 2, and stored in the same way as mentioned earlier.

3.2.3.7.1. Administration of azadirachtin to locusts

Usually, azadirachtin was dissolved in ethanol, diluted to 10%(v/v) with water and injected in 10µl volumes to give a dose of 5µg/locust into the body of adult locusts (Annadurai and Rembold, 1993). Injections were made through the abdominal intersegmental membrane using a Hamilton (10µl) syringe, 16 hrs before the further treatment of LPS. Control insects received a similar dose with 10%(v/v) ethanol-water alone. It was found that 10%(v/v) ethanol, when used as a solvent to dissolve azadirachtin, itself caused the apparent induction of lysozyme-like activity. So, in another experiment 5%, and 1% of ethanol was used to administer the azadirachtin into the body of locust. Control locusts received only the solvent.

Table 3.1. Reference proteins of known Molecular weight.

Name Of Protein	Mr (kDa)
Phosphorylase b	97
Scrum albumin	66
Ovalbumin	45
Soybean Trypsin	20.1
Lysozyme	14.6

As will be reported in the results section, an alternative method of administering the terpenoid was also tried. Perrow, (personal communication, 1996) had used a solution of cyclodextrin in order to solubilise non-polar terpenoid, azadirachtin, in an aqueous medium. To overcome the apparent induction of lysozyme-like activity by ethanol, β -cyclodextrin (20mg/ml) or methyl- β -cyclodextrin (100mg/ml) was used instead of ethanol. Controls received cyclodextrin solution only.

Finally, it was concluded that 5% ethanol should be used as a solvent in future experiments for azadirachtin administration to locusts. Sixteen hours after the locusts had received the azadirachtin, they received the dose of 100 μ g of LPS as described above. The control insects received insect saline. 24hours after LPS treatment, tissues were collected and then assayed for lysozyme activity using the protocols mentioned in 3.2.3.3. During the whole period of treatment the locusts were fed on fresh cabbage leaves and kept under standard rearing conditions.

3.2.3.8. Cycloheximide treatment

Cycloheximide has been shown to inhibit protein biosynthesis in systems that utilise ribosomes of the 80S type, (Sisler *et al.*, 1967; Abu-Hakima and Davey, 1977) and acts mainly through blocking translation (Sars and Kovacs, 1980). Cycloheximide also inhibits reassembling of polyribosomes in rat liver systems (Trakatellis *et al.*, 1965) and in hamster cells (Stanners, 1966). Cycloheximide was evaluated for the *in vivo* inhibition of protein synthesis and more specifically the suppression of induced lysozyme in specific tissues of the desert locust *S. gregaria* against controls.

3.2.3.8.1. Administration of cycloheximide to locusts

Cycloheximide (Sigma Chem Co.), was dissolved in sterilised insect saline (2.5mg/ml) and kept frozen at -20 $^{\circ}$ C until further use. 25 μ g of cycloheximide/insect, in 10 μ l of insect saline was injected through the abdominal intersegmental membrane by a Hamilton (25 μ l) syringe, 2hours before the injection of LPS into the body of each

locust of the group (four locusts/group). The controls received only insect saline by the same route. After two hours of administration of cycloheximide, each locust of both groups received 100µg of LPS in exactly the same way as mentioned in the section 3.2.3.1.1.(a). 24 hours after LPS treatment, tissues were sampled as described in 3.2.3.2.2 and assayed for lysozyme-like activity according to 3.2.3.4.2.

3.2.4. *In vitro* experiments

In vivo experiments were followed by the *in vitro* plan to verify the hypothesis that the fat body is the likely site of lysozyme synthesis.

3.2.4.1. Incubation medium

The tissue culture medium (GIBCOBRL, Life Technologies) was used as the incubation medium for the *in vitro* experiments.

3.2.4.2. Fat body preparations for *in vitro* incubation

A group of 10 female adult locusts was precooled at -20^o C for 15 minutes, and each surface-sterilized with 70% aqueous (v/v) ethanol before dissection. The fat bodies were dissected out from all locusts, pooled under sterile conditions, rinsed and washed in sterilized insect saline, to remove the presence of any traces of haemolymph. The pooled fat body was divided in equal amounts in 4 wells of a six-welled cell culture cluster dish, (Coaster Corporation Cambridge MA, U.S.A). The remaining two wells were used for a negative control.

3.2.4.3. Chemicals

3.2.4.3.1. Lipopolysaccharide

Lipopolysaccharide, from *E. coli* serotype described in section 3.2.3.1.1 of this chapter was used for the *in vitro* experiments and the amount of LPS used was 400µg/well.

3.2.4.3.2. Cycloheximide

Cycloheximide (Sigma Chem Co.), dissolved in sterilized insect saline (2.5mg/ml) was kept frozen at -20° C until further use.

3.2.4.3.3. Ampicillin

Ampicillin (Sigma chem Co.) (100µg/well), was used *in vitro* experiments to minimise the chances of bacterial infection.

3.2.4.3.4. Effect of azadirachtin on lysozyme production in fat body

To investigate the effect of azadirachtin on the production of lysozyme, isolated fat body samples were incubated with 50µg/incubation of azadirachtin for a period of 24 hours. The lysozyme activity was assayed in the tissue and incubation medium as well.

3.2.4.4. Incubation of fat body

The *in vitro* incubations of fat bodies were done three times in three steps in the tissue culture medium. Each one is described in the following sections separately.

3.2.4.4.1. Lysozyme induction in fat body by LPS

Initially, the effect of LPS on the induction of lysozyme activity in adult female locust fat bodies was investigated and this has been shown in Table 3.2.

3.2.4.4.2. Effect of azadirachtin on the LPS-induced lysozyme in fat body

In second step, the effect of azadirachtin was investigated on the induced lysozyme by LPS. The experiment was designed as shown in Table 3.3.

Table 3.2. Step I: Induction of lysozyme by LPS in fat body

No. of well	Medium (1ml)	Ampicillin (100µg)	Fat body	LPS (400µg)
1	+	+	-	-
2	+	+	-	-
3	+	+	+	-
4	+	+	+	-
5	+	+	+	+
6	+	+	+	+

LPS: Lipopolysaccharide

Table 3.3. Step II: Effect of azadirachtin on the LPS-induced lysozyme in fat body.

No of well	Medium (1ml)	Ampicillin (100 μ g)	Fat body	LPS (400 μ g)	AZA (50 μ g)
1	+	+	+	-	-
2	+	+	+	-	-
3	+	+	+	+	-
4	+	+	+	+	-
5	+	+	+	+	+
6	+	+	+	+	+

LPS: Lipopolysaccharide

AZA: Azadirachtin

3.2.4.4.3. Effect of cycloheximide on the LPS-induced lysozyme in fat body

In third step the effect of cycloheximide was studied on the induction of lysozyme by LPS in the fat body of locust, for comparison, as shown in the result section. Table

3.4 indicates the layout of the experiment. For steps II and III, the number of locusts in each experiment was increased to 12.

In each experiment, the tissue-culture plate was covered with parafilm and incubated at 30°C with vigorous shaking, (Eberbach shaker, 200rpm) for a period of 24 hours (Verno *et al.*, 1984), at which time the samples were transferred to individual sterile 1.5ml polypropylene centrifuge tube to prepare the tissue for lysozyme assay.

3.2.4.5. Assay for lysozyme in fat body

3.2.4.5.1. Spectrophotometric method

The fat body samples from each well and for each treatment was homogenized by a teflon pestil, sonicated for 30sec by an ultrasonic microprobe in ice and then centrifuged for 6min at 13,000g. The lysozyme-like activity was assayed for duplicate samples of 50µl each from the middle layer.

Lysozyme activity was estimated by the assay described in section 3.2.3.4.2 and enzyme activity units were measured according to 3.2.3.4.3. Final specific activities were quoted as U/ml of the homogenised samples.

The lysozyme for step II and III was assayed for both fat body tissue and the incubation medium separately, to confirm the evidence of fat body supposed to be a source of induced synthesis of haemolymph lysozyme-like activity, *in vivo*, which was increased in the presence of LPS.

Table 3.4. Step III: The effect of azadirachtin and cycloheximide on the LPS-induced lysozyme in fat body.

No of well	Medium (1ml)	Ampicillin (100µg)	Fat body	LPS (400µg)	AZA (50µg)	Cyclo. (25µg)
1	+	+	+	+	-	-
2	+	+	+	+	-	-
3	+	+	+	+	+	-
4	+	+	+	+	+	-
5	+	+	+	+	-	+
6	+	+	+	+	-	+

LPS: Lipopolysaccharide

AZA: Azadirachtin

Cyclo: Cycloheximide

3.3. Results

3.3.1 Growth inhibition of a Gram-negative bacterium

Antibacterial activity induced by whole *E. coli* cells or LPS on the growth of Gram-negative bacterium was tested for the haemolymph of female adult locusts. Fig 3.5 shows that there was no inhibition of growth of *E. coli* in nutrient agar, while there was a clear inhibition of growth with the antibiotic ampicillin. The results for both female and male locusts and for both types of treatments were the same, indicating no effect on the growth of a Gram-negative bacterium. The result for the haemolymph of LPS-treated female adult locusts against controls is shown in Fig 3.5. There was no evidence of induction of cecropin-like antibacterial proteins.

3.3.2. *In vivo* experiments

3.3.2.1. Agar gel lysoplate assay for lysozyme activity using *Micrococcus lysodeikticus* as a substrate

The estimation of lysozyme activity is based upon the lytic action of lysozyme, causing a zone of clearing as it diffuses outward from a well in the agar. A range of known activities of the standard commercial egg-white lysozyme were used in the agar gel lysoplate assay. From the results in Fig 3.6, it was obvious that there was a linear relation between the \log_{10} amount of lysozyme and the lysed area around the well. Afterwards this technique was applied to estimate the *E. coli* or LPS induced lysozyme activity in the haemolymph of locusts as described in the following sections.

3.3.2.1.1. Agar gel lysoplate assay of *E.coli*-induced lysozyme activity in locust haemolymph

The agar gel lysoplate assay was used to give a preliminary indication of the distribution of lysozyme in the female locust haemolymph. Fig 3.7 shows the differences in lysed zones between the *E.coli*-treated haemolymph and controls. Both of these observations

Fig 3.5. Growth inhibition test for a Gram-negative bacterium in nutrient agar gel by haemolymph of locust.

A group of four adult female locusts was injected by 100µg of LPS, haemolymph was collected after 24 hours, pooled and used to test for the inhibition of growth of a Gram-negative bacteria. Controls received insect saline.

Into wells 1 and 2 was placed 5µl of haemolymph from controls, wells 3 and 4 have the same volume of treated haemolymph and well 5 received crystals of ampicillin as positive control.

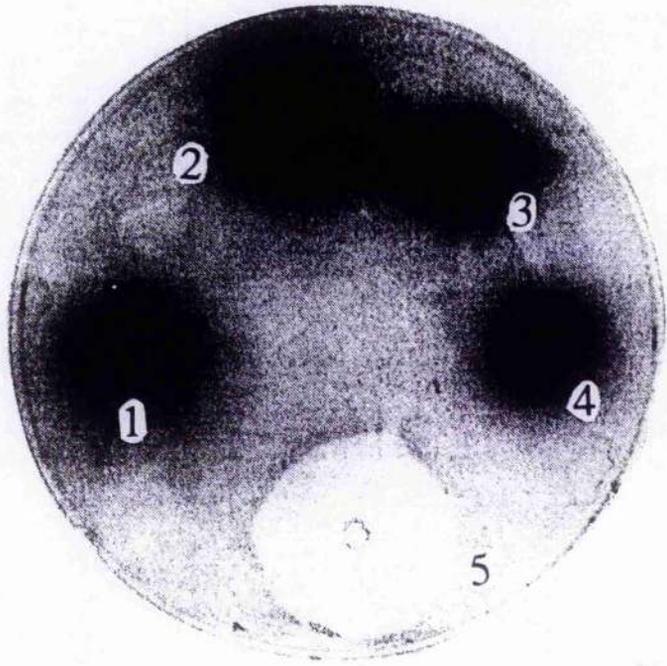


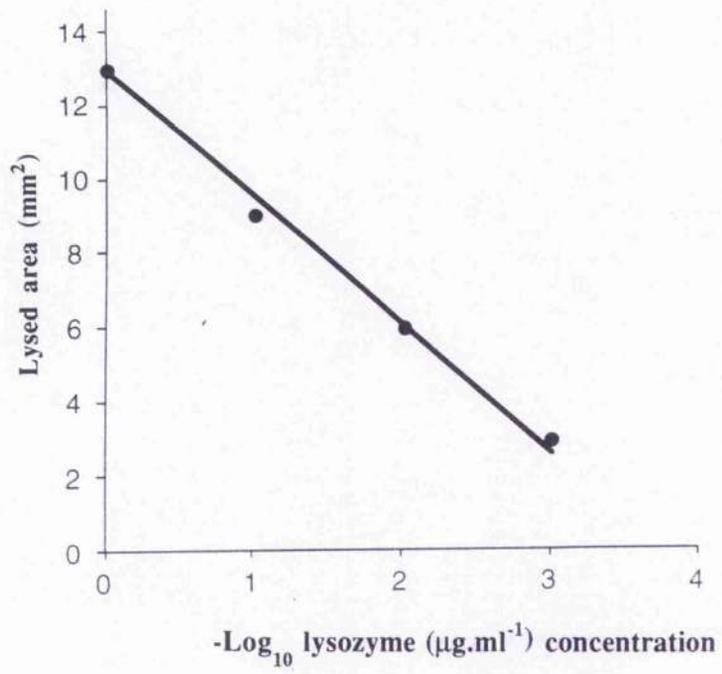
Fig 3.6. Lysozyme activity indication by lysis of *Micrococcus lysodeikticus* in an agar gel lysoplate assay.

(a) The agar contained a concentration of 1.5% *M. lysodeikticus*.

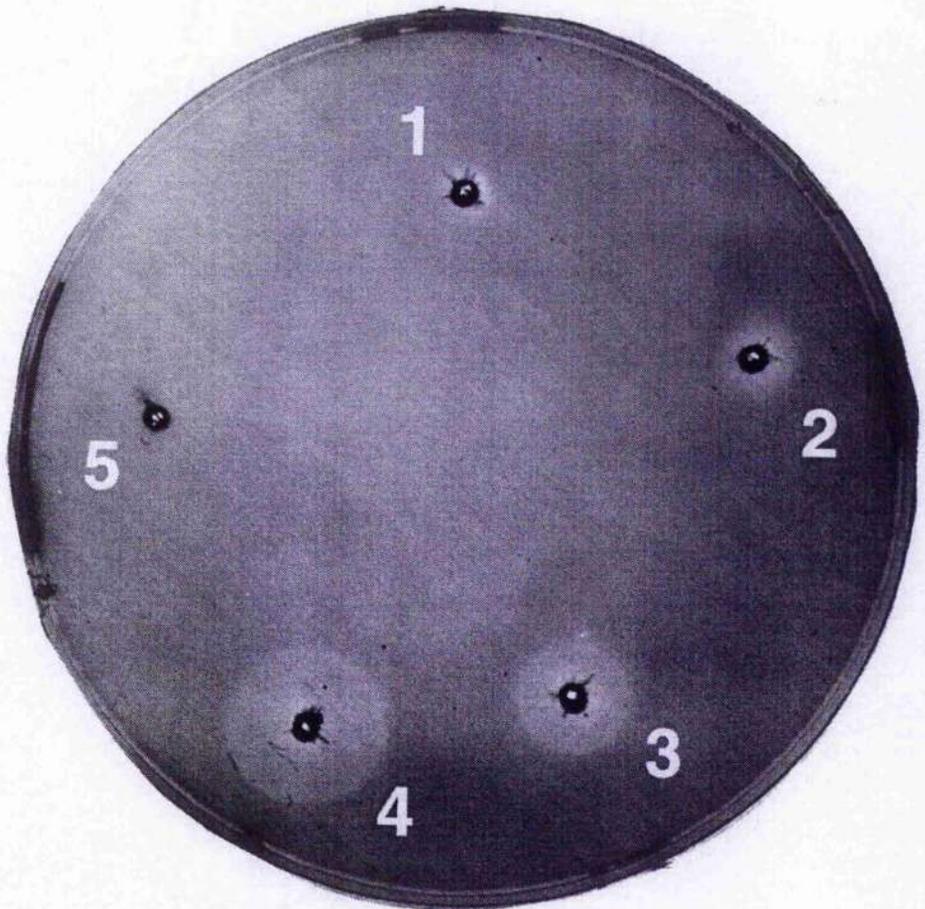
Into each well was placed 5 μ l of a solution of commercial egg-white lysozyme ranging in concentration from 0.25U(1), 2.5U(2), 25U(3) and 250U(4). Well 5 represents a control of boiled enzyme. The light area around the well represents lysis of the suspended bacteria.

(b) The areas of lysis are plotted against the \log_{10} of the amount of lysozyme in (a).

Each point is a mean of four values.



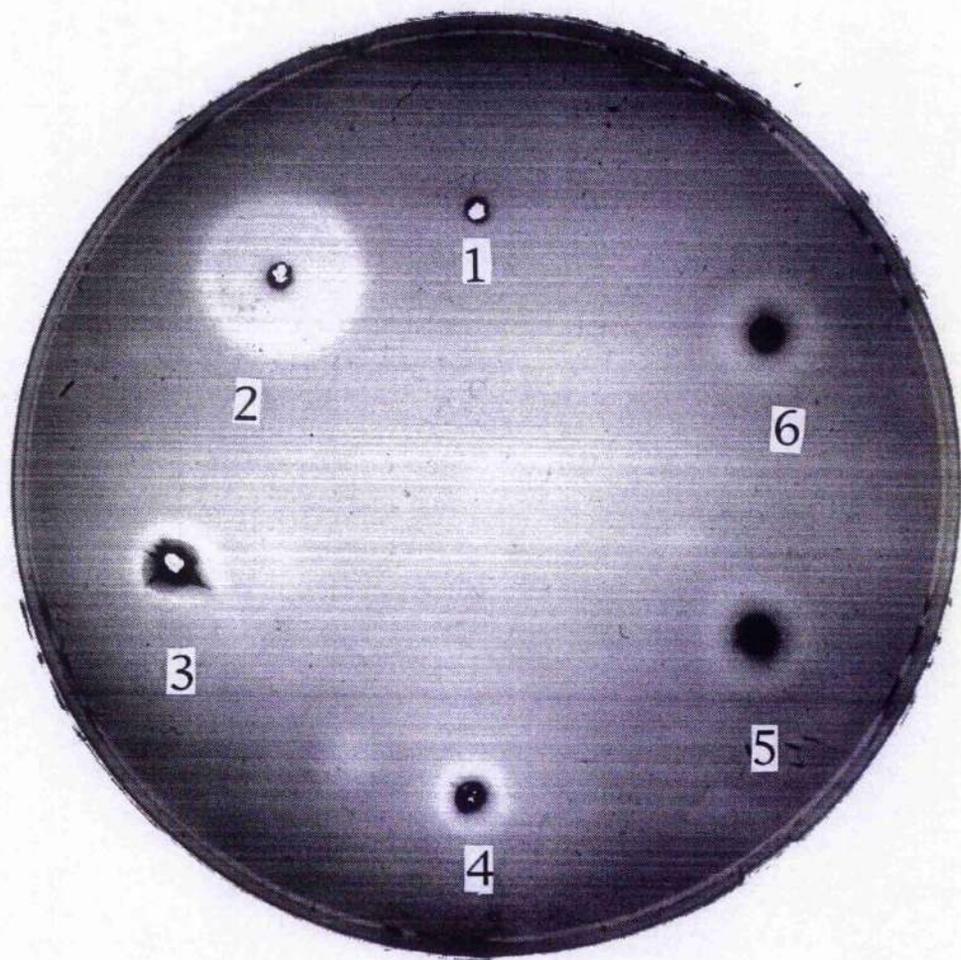
(a)



(b)

Fig 3.7. The lysis of *Micrococcus lysodeikticus* in an agar gel by haemolymph lysozyme of female *S. gregaria*. The locusts were injected by heat-killed *E. coli* 24 hours before.

A preliminary indication of *E. coli* induced lysozyme distribution in the haemolymph of female locust. Controls received insect saline. The agar contained 1.5% of *M. lysodeikticus*. Well 1 received boiled commercial lysozyme and well 2 commercial egg-white lysozyme (1250 units). Into wells 3 and 4 was placed 5 μ l of control haemolymph while in wells 5 and 6 was placed same volume of haemolymph of treated locust. In each experiment, the haemolymph from four locusts was pooled.



were different from the one for commercial egg-white lysozyme and the one with denatured lysozyme. There was no lytic activity at all in the well containing boiled commercial egg-white lysozyme (well 5 Fig 3.7). Although there was some quantitative relation in the lysed zones indicating lysozyme activity differences, the method was considered to be insufficiently precise for quantitative studies.

The experiment was repeated for haemolymph of adult male *E.coli*- treated locusts, and there were not any significant difference in the results of both sexes (results not shown).

3.3.2.1.2. Agar gel lysoplate assay for haemolymph lysozyme of LPS-treated locusts

When the agar gel method was used to investigate the lytic action of haemolymph lysozyme in LPS-treated locusts, it showed clear induction in the lytic activity after LPS treatment (Fig 3.8). Although the method was not wholly quantitative, still it was obvious that there were differences in the lysed areas of wells received samples from controls and LPS-treated haemolymph. The lytic activity was different for the standard and boiled commercial enzyme. There were differences in the lysed areas for haemolymph lysozyme activity and egg-white one. It was difficult to standardise the activity of lysozyme from different sources, and there was a need to adopt a more precise method to estimate the activity of lysozyme.

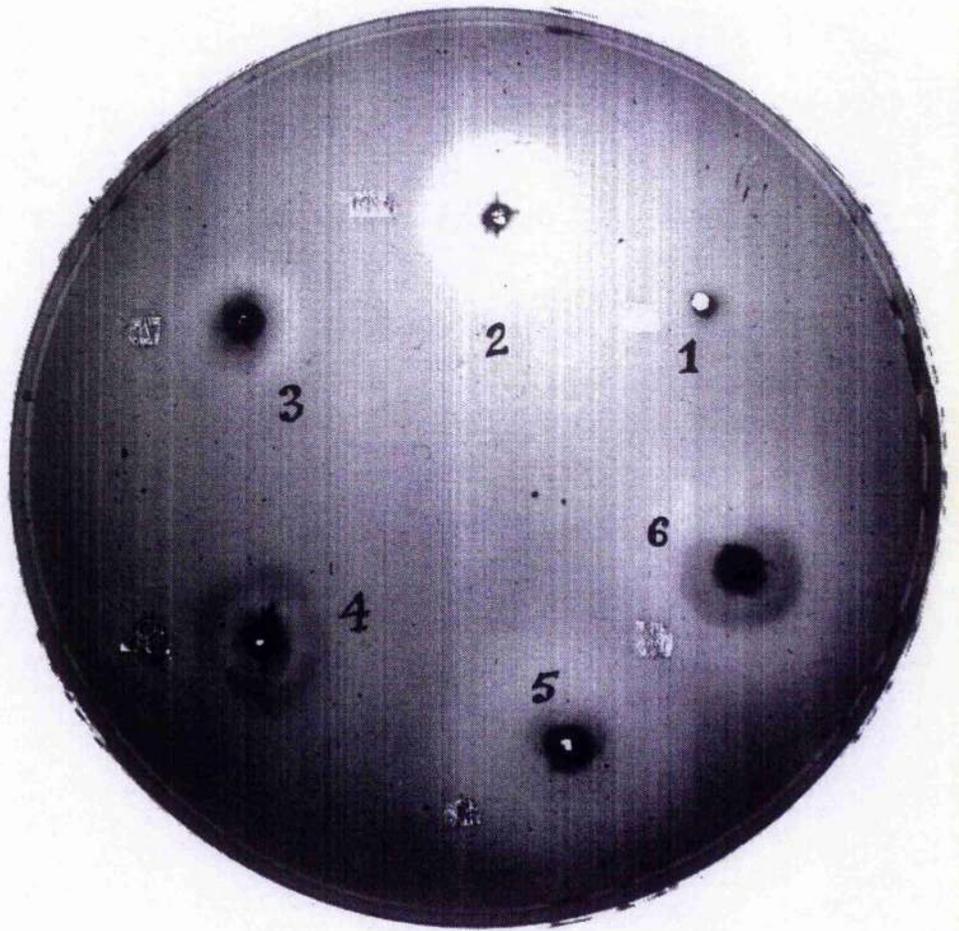
The adult male locusts were also used to study the effect of LPS treatment to induce the lytic activity of haemolymph lysozyme (results not shown).

3.3.2.2. Changes in lysozyme activity in response to bacterial LPS treatment

The above results confirmed the occurrence of changes in humoral lysozyme activity in response to injection of bacterial lipopolysaccharide in mature females of the desert locust *S. gregaria*. In future experiments, LPS was used as an elicitor to study the

Fig. 3.8. The lysis of *Micrococcus lysodeikticus* in an agar gel by haemolymph lysozyme of female *S. gregaria*. The locusts were treated with (100µg/locust) bacterial lipopolysaccharide (LPS) for a period of 24 hours.

A preliminary indication of LPS induced lysozyme distribution in the haemolymph of female locust. Controls received insect saline. The agar contained 1.5% (w/v) of *M. lysodeikticus*. Well 1 received boiled lysozyme and well 2 commercial egg-white lysozyme (1250 units). Into wells 3 and 4 was placed 5µl of LPS-treated haemolymph while in wells 5 and 6 was placed same volume of haemolymph from control insects. In each experiment, the haemolymph from four locusts was pooled.



lysozyme activity changes in specific tissues of *S. gregaria*. The lytic action of *Micrococcus lysodieticus* in agar gels was considered to be only a semi-quantitative method for lysozyme activity estimation, so in subsequent experiments an alternative quantitative method was applied to assay the lysozyme activity using the same substrate i.e spectrophotometry as detailed in materials and methods.

3.3.2.2.1. Humoral lysozyme-like activity estimation by spectrophotometry

Investigations on humoral immunity in *S. gregaria* by agar gel method showed that the inoculation with bacterial LPS induces lysozyme activities in haemolymph.

The effects of injecting LPS into the mature females of locusts are shown in Fig 3.9. No difference was observed between control insects which had received only an injection of insect saline and those receiving 100µg of LPS until more than 20 hours after the injection. At 24 hours however, although both control and experimental insects showed an increase in lysozyme activity, that shown by the LPS-treated insects was 80% above the normal, while the control was only raised by 30%. Both of these increases in activity were statistically significantly different from the normal resting level of $1600 \pm 210 \text{ U.ml}^{-1}$ ($P < 0.01$), and the higher level in the LPS-treated insects was significantly different from the control ($P < 0.01$). Thereafter, by 48 hours, the levels of activity of the enzyme in the haemolymph of both control and experimental insects returned to the normal. The results showed that even the simple process of injecting sterile saline into the locusts produced a small increase in the activity of circulating lysozyme, but this was doubled by bacterial lipopolysaccharide. The maximum response of LPS-treated locusts in lysozyme activity was at 24 hours after treatment. Subsequent measurements of enzyme activity were made at 24 hours after treatment.

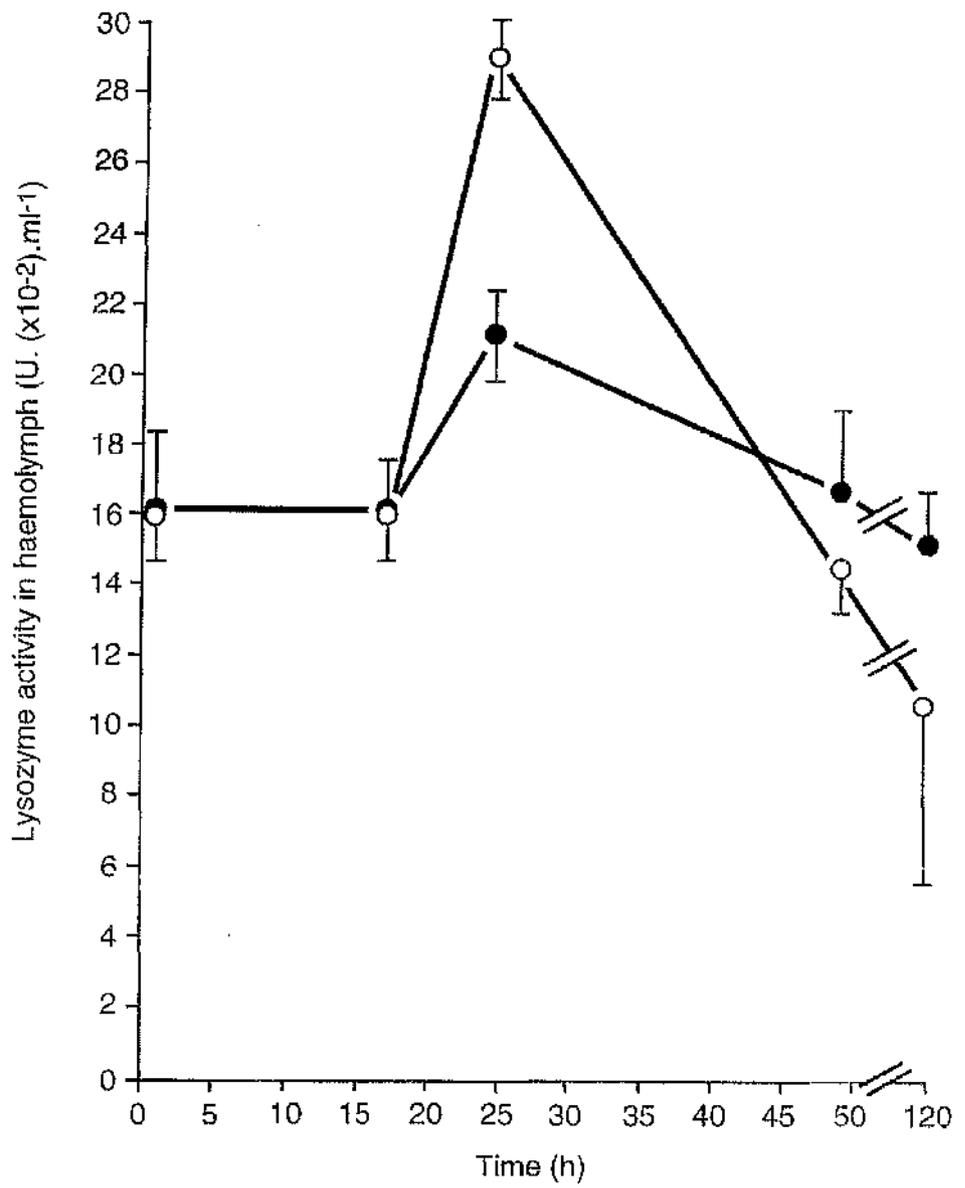
Fig 3.9. The activity of lysozyme in the haemolymph of mature *S. gregaria* after injection with bacterial lipopolysaccharide.

Each insect was injected at 0 time with 100µg of LPS in 10µl of sterile insect saline. Controls received only saline. Samples (5µl) of haemolymph were taken over a period of 5 days, and the activity of lysozyme in the whole haemolymph was assayed immediately.

-----● Control haemolymph

——○ LPS-treated haemolymph

Each point is an average of 4 values and the vertical bars represent the \pm SD.



3.3.2.2. Lysozyme activity changes in fat body after 24 hours of LPS injection

To find out the source of circulating lysozyme in haemolymph, fat body of mature female LPS-treated *S. gregaria* was assayed for the enzyme activity after 24 hours of injection. The activity of lysozyme found in homogenates of fat body (Fig 3.10) indicates that the level of activity found in the tissue is low compared to haemolymph. It seemed that fat body did not respond like haemolymph in the induction of lysozyme-like activity. Although the level of activity rises significantly after LPS treatment, it only increases by 32% above the untreated locusts.

3.3.2.2.3. Lysozyme activity changes in haemocytes and plasma of locusts after 24 hours of LPS injection

It was anticipated that the haemocytes may make up a proportion of the total lytic activity in the haemolymph. Initially no attempt was made to separate the cellular fraction of lysozyme activity from the plasma, but later haemocytes were also assayed for lysozyme activity (Fig 3.11).

The enzyme activity of haemocytes represented between 20-30% of the total lysozyme activity of haemolymph. The cellular response to LPS was almost a doubling of the resting level of enzyme activity, while there was no significant change in activity in the haemocytes of controls receiving insect saline. The LPS response of plasma lysozyme activity was in an increase of 24%. In locust haemolymph as a whole, 70-80% of enzyme activity was that of plasma.

Fig 3.10. The activity of lysozyme in the fat body of mature *S. gregaria* after 24 hours of injection with bacterial lipopolysaccharide.

Each insect was injected with 100µg of LPS in 10µl of sterile insect saline (3). Controls received only saline (2). The results are the means from 4 locusts. Fat body samples were collected, weighed, homogenised and prepared for lysozyme assay as described in materials and methods after 24 hours of injection. The activity of lysozyme in the samples (20µl) was assayed immediately. Another untreated group of locusts was also used to find the basal level of lysozyme activity in fat body (1). Vertical bars indicate the \pm SD.

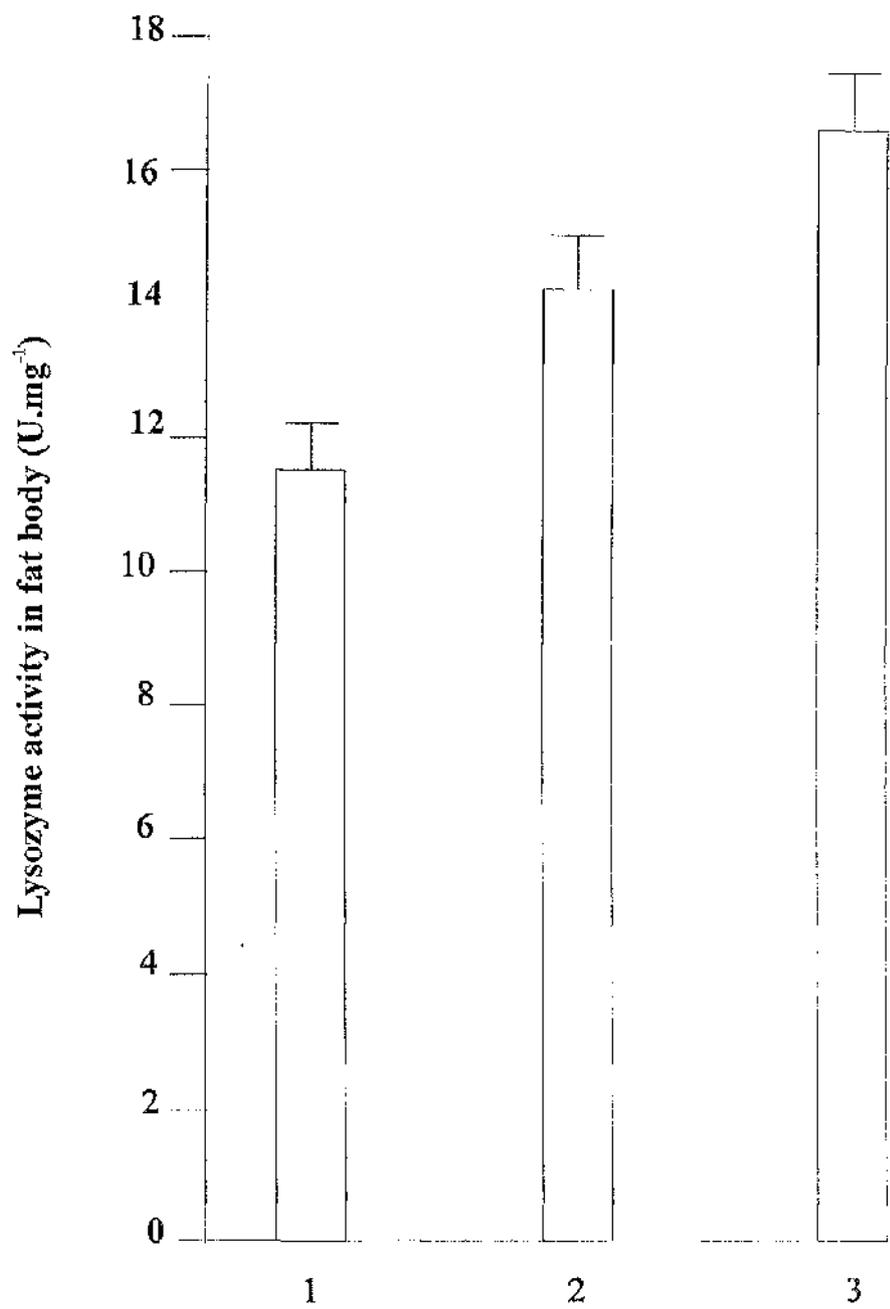
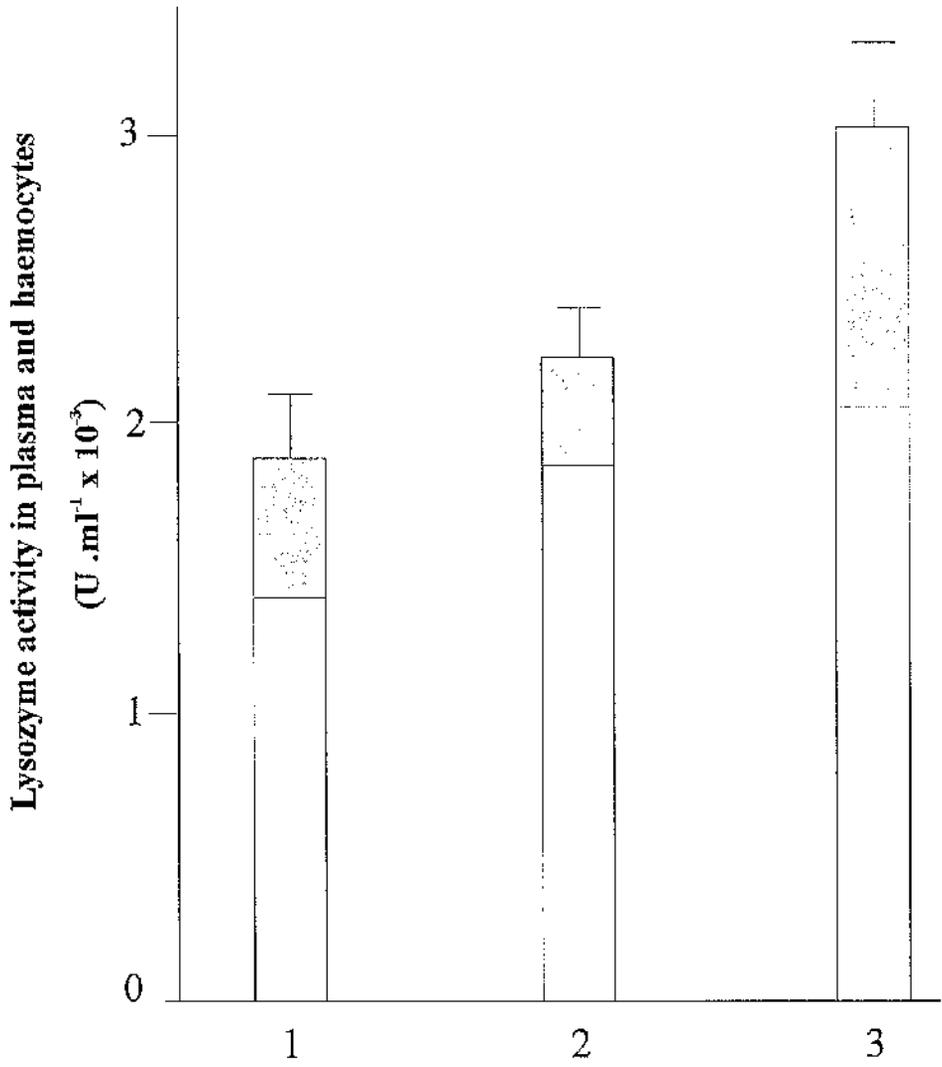


Fig 3.11. The activity of lysozyme in plasma and haemocytes of mature female locusts *S. gregaria*.

Lysozyme activity in cellular fraction was assayed in relation to initial volume of whole haemolymph. Samples were taken after 24 hours of LPS treatment. The values represent the mean (\pm SD) of 4 locusts.

- 1: Basal levels of cellular and plasma lysozyme activities in untreated locusts.
- 2: Fractions of activities in controls received sterile insect saline.
- 3: Lysozyme activities in plasma and haemocytes of LPS-treated locusts.

In each case:  plasma, and  haemocytes



3.3.2.3. Effect of azadirachtin on lysozyme activity

3.3.2.3.1. Effect of azadirachtin on lysozyme distribution in haemolymph

The effect of azadirachtin was investigated in a preliminary experiment on the basal level of lysozyme distribution in haemolymph of *S. gregaria*. As described in materials and methods, 10% (v/v) ethanol was used to dissolve the tetranortriterpenoid and then injected into the body of the locust. The lysozyme activity was decreased by 48% after 40 hours of azadirachtin treatment as compared to controls (Fig 3.12). This reduction in activity was statistically significant ($P < 0.01$) and was even 33% below the basal level of the enzyme activity in untreated controls while in another group cycloheximide caused a reduction of only 21% in the lysozyme activity of haemolymph.

3.3.2.3.2. Effect of azadirachtin on lysozyme-like activity in fat body

The effect of azadirachtin on the basal level of lysozyme activity in fat body of the mature locusts was monitored for the same time period and under same conditions as described for the haemolymph in the above section. There was a 33% reduction in the enzyme activity after azadirachtin treatment, i.e. statistically significant ($P < 0.01$), (Fig 3.13). The decrease in activity due to cycloheximide treatment was 47% suggesting that the fat body is a site of synthesis of lysozyme in this insect.

3.3.2.3.3. Effect of azadirachtin on the LPS-induced lysozyme activity in haemolymph plasma and haemocytes

In order to find out the effect of azadirachtin on the induction of lysozyme activity in haemolymph by LPS, the locusts were injected with a physiologically-effective dose of azadirachtin 16 hours before receiving bacterial lipopolysaccharide. Subsequently, 24 hours after LPS treatment, haemolymph was collected. Plasma and haemocytes were separated to assay the lysozyme activity. Fig 3.14 shows the effect of azadirachtin on

Fig 3.12. The effect of azadirachtin on the distribution of activity of lysozyme in the haemolymph of the desert locust *S. gregaria*.

Each locust received 5µg azadirachtin dissolved in 10% ethanol, haemolymph samples collected after 40 hours of treatment and assayed for the lysozyme activity immediately. Controls received 10% (v/v) aqueous ethanol. In case of cycloheximide, each insect received 25µg of cycloheximide dissolved in sterile insect saline in a volume of 10µl, controls received same volume of insect saline. The values represent the mean ±SD from a group of 4 locusts in each experiment.

1: Basal level of enzyme activity in untreated locusts.

2: Lysozyme activities in azadirachtin-treated insects.

3: Lysozyme activities in cycloheximide-treated locusts.

In 2 and 3: : control and  treated.

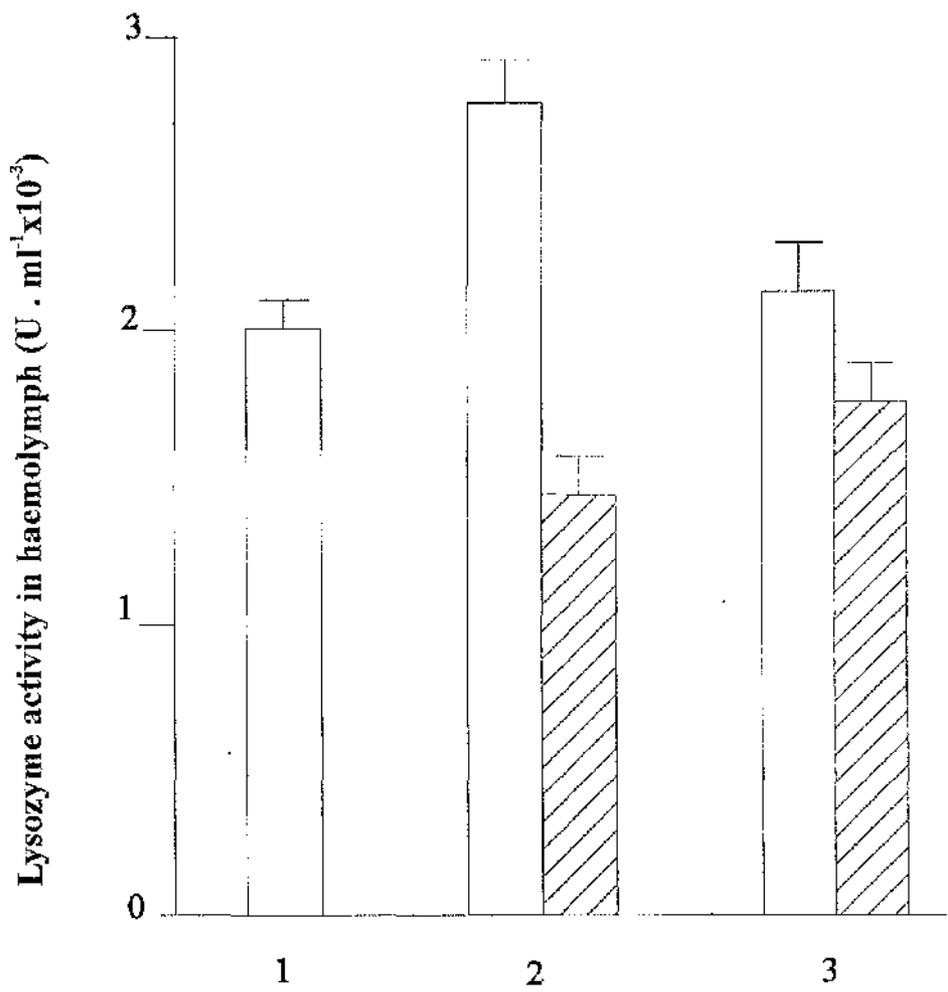


Fig 3.13. The effect of azadirachtin on the activity of lysozyme in the fat body of the desert locust *S. gregaria*.

Each locust received 5µg azadirachtin dissolved in 10% ethanol, fat body samples collected after 40 hours of treatment, prepared and assayed for the lysozyme activity. Controls received 10% (v/v) aqueous ethanol. In case of cycloheximide, each insect received 25µg of cycloheximide dissolved in sterile insect saline in a volume of 10µl, controls received only insect saline. Each result is an averages from 4 locusts and bars indicate the \pm SD.

1: Basal level of enzyme activity in untreated fat body.

2: Lysozyme activities in azadirachtin-treated samples.

3: Lysozyme activities in cycloheximide-treated fat body.

In 2 and 3: : control and  treated.

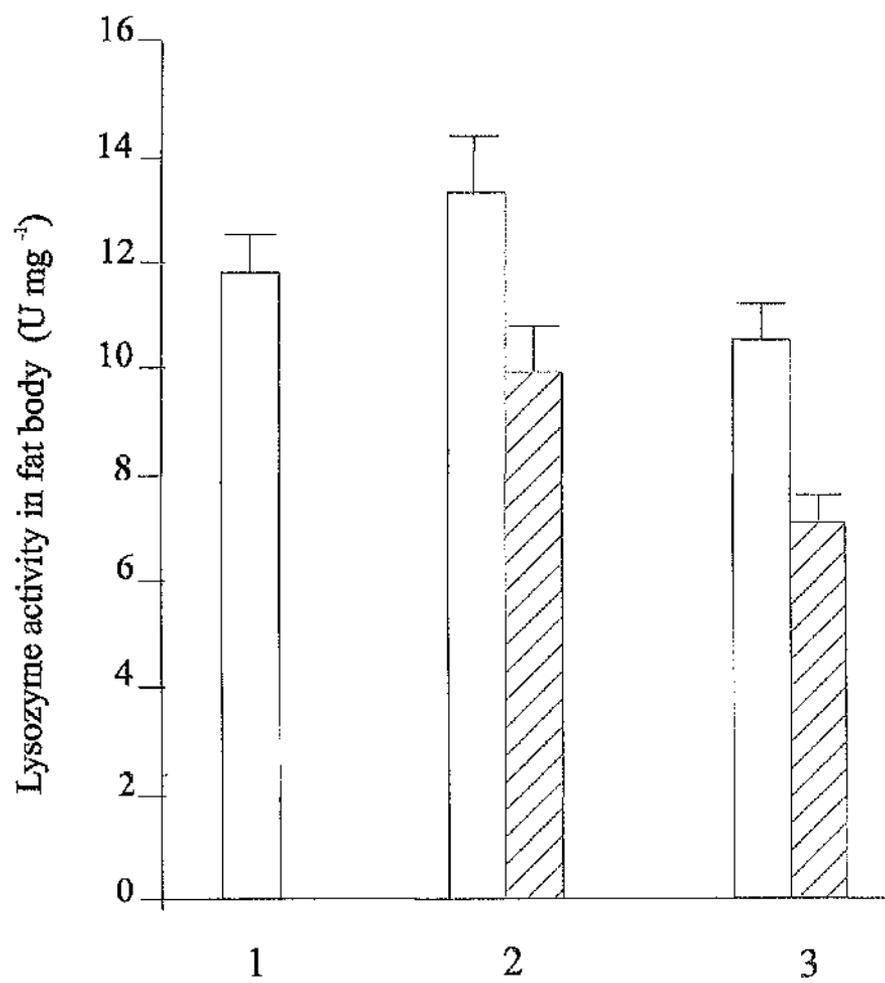


Fig 3.14. The effect of azadirachtin on the LPS-induced activity of lysozyme in the plasma and haemocytes of the desert locust *S. gregaria*.

Each locust received 5µg azadirachtin (10µl volume) dissolved in 10% ethanol 16 hours before the LPS treatment. 100µg of LPS was injected into the body of each locust (a volume of 10µl). Controls received mock injections of 10% (v/v) aqueous ethanol and insect saline. Samples collected after 24 hours of LPS-treatment and assayed for the lysozyme activity. Controls received mock injection of 10% (v/v) aqueous ethanol. The results are means from 4 locusts and bars indicate the ±SD.

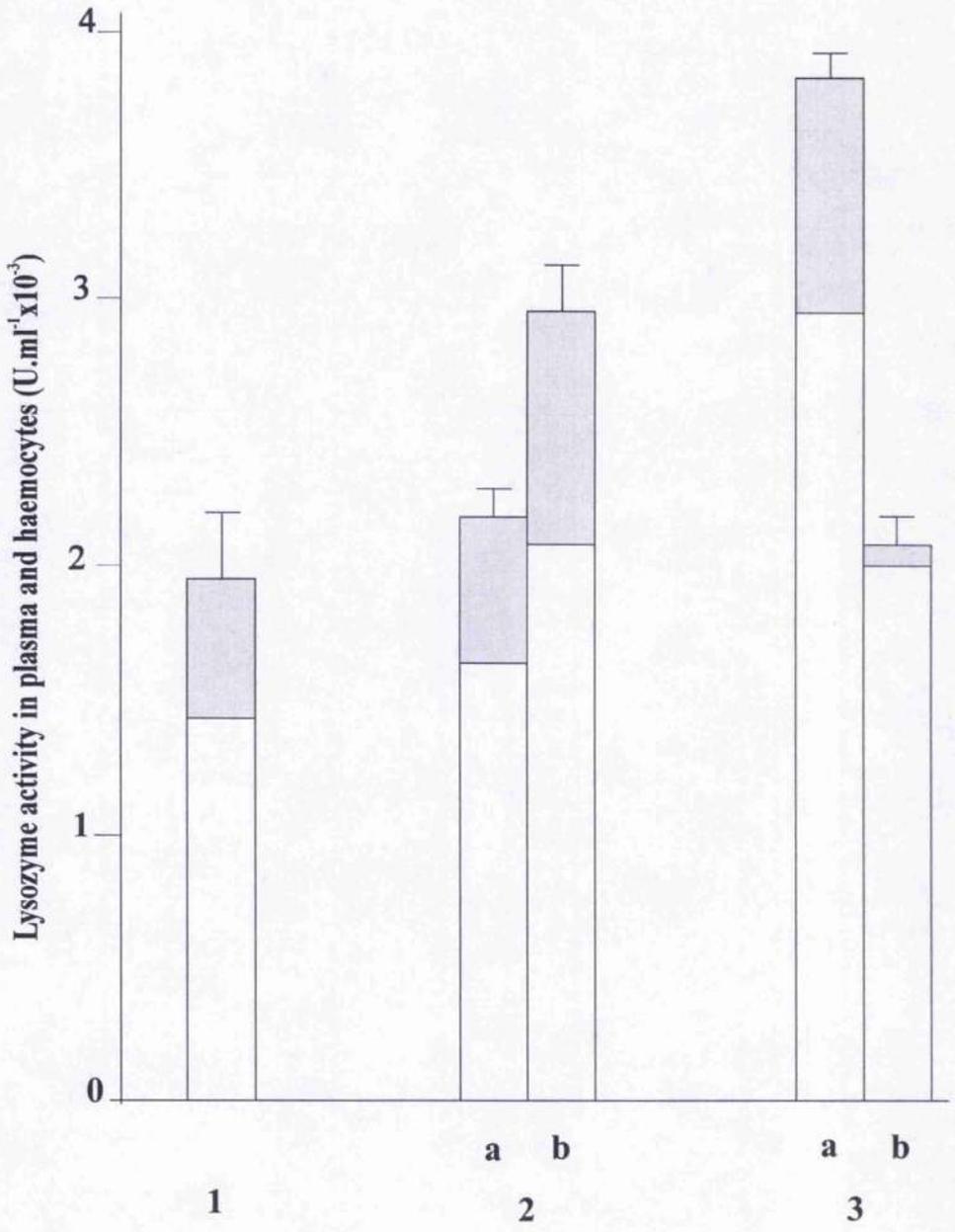
1: Basal level of enzyme activity in untreated insects.

2: Lysozyme activities in LPS-treated insects.

3: Lysozyme activities in azadirachtin-treated locusts.

In each case:  plasma and  haemocytes,

a: control and b: treated



the LPS-induction of antibacterial protein. The lysozyme activity in the whole haemolymph of azadirachtin treated locusts was suppressed by 44%. In plasma the reduction was 32%, while in haemocytes there was a huge decrease of 98% in the activity as compared to controls receiving 10% (v/v) ethanol and LPS. All these differences were statistically significant ($p < 0.05$).

In the haemolymph of azadirachtin-treated insects, it seemed that the cellular fraction of enzyme activity was nearly eliminated due to azadirachtin treatment.

3.3.2.3.4. Effect of azadirachtin on the LPS-induced lysozyme activity in fat body

In *S. gregaria* the effect of azadirachtin on the induction of lysozyme-like activity in fat body in response to bacterial lipopolysaccharide was studied in the same fashion as for haemolymph. There was an increase of 39% in production of lysozyme after LPS-treatment and azadirachtin reduced the induction of enzyme activity by 35% as compared to controls which received LPS and 10% (v/v) ethanol (Fig 3.15). This reduction was statistically significant ($p < 0.01$).

3.3.2.4. Medium of azadirachtin administration

After a series of experiments done on the effects of azadirachtin on lysozyme-like activity, it was noticed that there was a tremendous rise in the activity of lysozyme in controls when 10% ethanol was used as a carrier to deliver azadirachtin in the body of locust. It was observed that ethanol itself was serving as an inducing agent for production of lysozyme-like activity. To verify this observation, azadirachtin was administered in three aqueous dilutions 10%, 5% and 1% (v/v) of ethanol into the body of the locust. These three dilutions of ethanol alone were also injected into the body of locusts in order to find its effect on the enzyme activity induction.

Fig 3.15. The effect of azadirachtin on the LPS-induced lysozyme-like activity in the fat body of the desert locust *S. gregaria*.

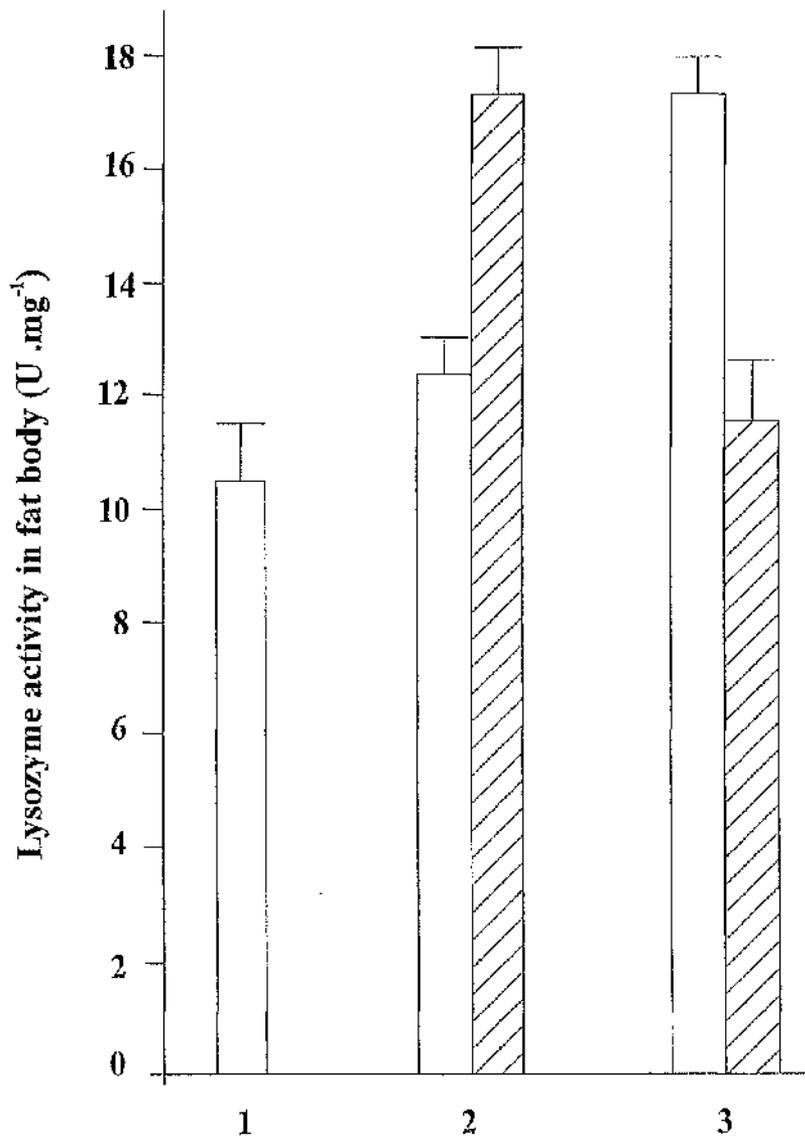
Each locust received 5µg azadirachtin (10µl volume) dissolved in 10% ethanol 16 hours before the LPS treatment. 100µg of LPS was injected into the body of each locust (a volume of 10µl). Controls received 10% (v/v) aqueous ethanol and insect saline. Samples collected after 24 hours of LPS-treatment and assayed for the lysozyme activity. The results are means from 4 locusts and bars indicate the ±SD.

1: Basal level of enzyme activity in untreated insects.

2: Lysozyme activities in LPS-treated insects.

3: Lysozyme activities in azadirachtin-treated locusts.

In 2 and 3: : control and  treated.



3.3.2.4.1. Effect of ethanol as a solvent for azadirachtin on the lysozyme activity in plasma and haemocytes

The effect of azadirachtin administered in ethanol on the circulating lysozyme in haemolymph was studied in two steps.

In first step, the effect of 10% ethanol alone was studied on the haemolymph lysozyme against controls received insect saline for 24 hours. The injected ethanol caused a rise of 46% in the circulating enzyme activity, while the activity increase in response to LPS was only 34%. In the same experiment, in another group of locusts receiving 10% ethanol and bacterial lipopolysaccharide, the rise in enzyme activity was 74% (Fig 3.16) suggesting an additive effect, which was reduced by 42% in azadirachtin-treated insects.

Consequently azadirachtin was injected in either 10%, 5% or 1% ethanol to the locusts. The aim was to choose the appropriate dilution of ethanol to dissolve azadirachtin as an injection vehicle. Fig 3.17 indicates the changes in lysozyme activity in plasma and haemocytes when the same dose of azadirachtin was delivered in above said three dilutions of ethanol. There was a reduction of 27-47% in enzyme activity by azadirachtin treatment.

The effect of azadirachtin is to greatly reduce the combined response to LPS and ethanol, especially in haemocyte fraction (Fig 3.17). The results show very little lysozyme activity in haemocytes of azadirachtin-treated insects compared to the controls in each case.

Only in the case of 5% ethanol, there was some detectable enzyme activity in the cellular fraction of haemolymph.

Fig 3.16. The effect of injection of 10% (v/v) ethanol on the level of activity of lysozyme in the haemolymph of *S. gregaria* and the response to azadirachtin.

The activity of lysozyme was estimated in the haemolymph as whole after 24hours of 10µl of 10% (v/v) ethanol and LPS (100µg/insect in 10µl volume of insect saline) injections in separate groups. Controls received same volume of only insect saline. In another group, control locusts received both ethanol and LPS, while treated insects received azadirachtin (5µg/insect in a volume of 10µl of 10% aqueous ethanol), and 16hours later received the above mentioned amount of LPS. The enzyme activity was estimated after a period of 24hours of LPS injection.

The results are means from 4 locusts and bars indicate the \pm SD.

1: Basal level of enzyme activity in untreated haemolymph.

2:  Controls received insect saline.

 Locusts injected with 10% (v/v) ethanol.

 Insects treated with LPS.

3:  Controls injected with ethanol and LPS.

 Locusts treated with azadirachtin and LPS.

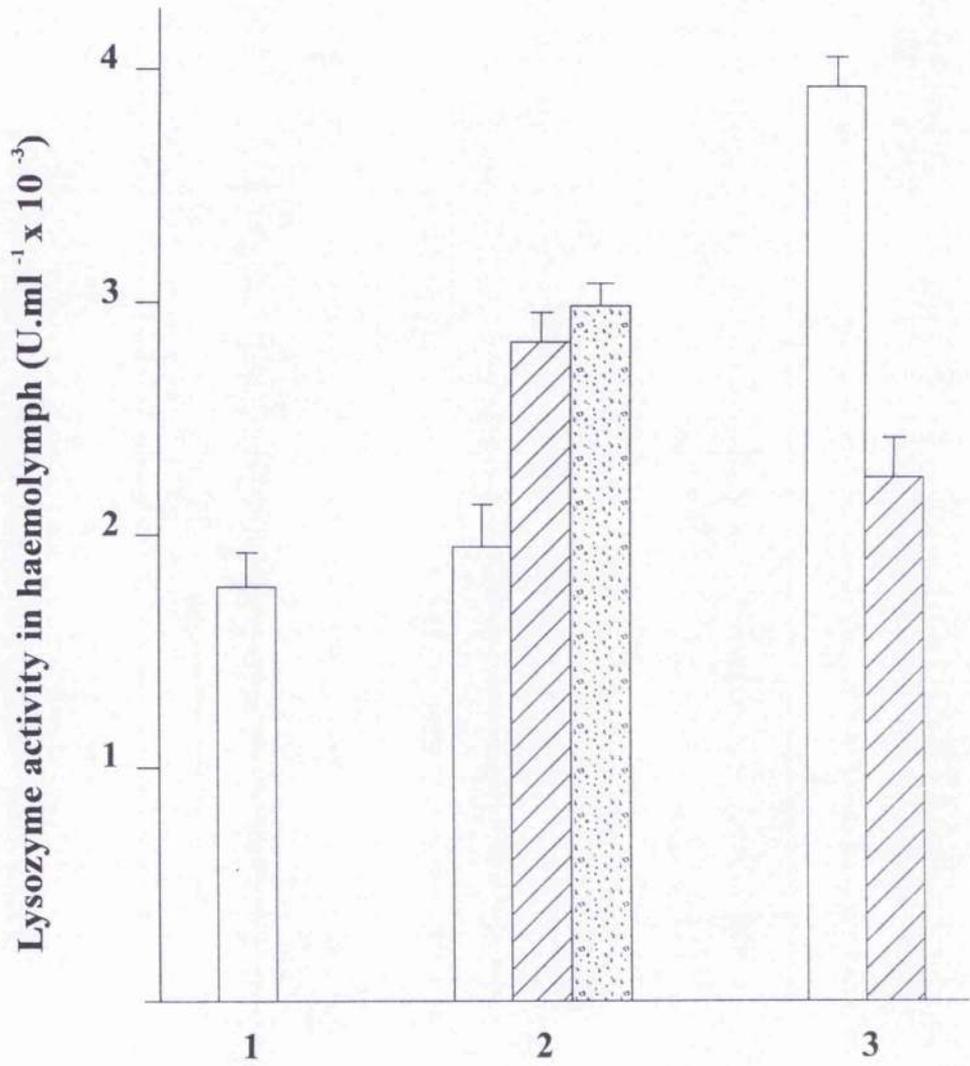


Fig 3.17. The effect of azadirachtin solubilised in 1, 5 and 10% (v/v) of ethanol on the level of activity of lysozyme induced by LPS in the haemolymph plasma and haemocytes.

The activity of lysozyme was estimated in haemolymph plasma and haemocytes 40 hours after the injection of different concentrations of ethanol alone, and as a solvent for azadirachtin (5µg/insect). After 16 hours of azadirachtin injection, each locust received 100µg of LPS (10µl volume). Lysozyme was estimated 24 hours after LPS injection.

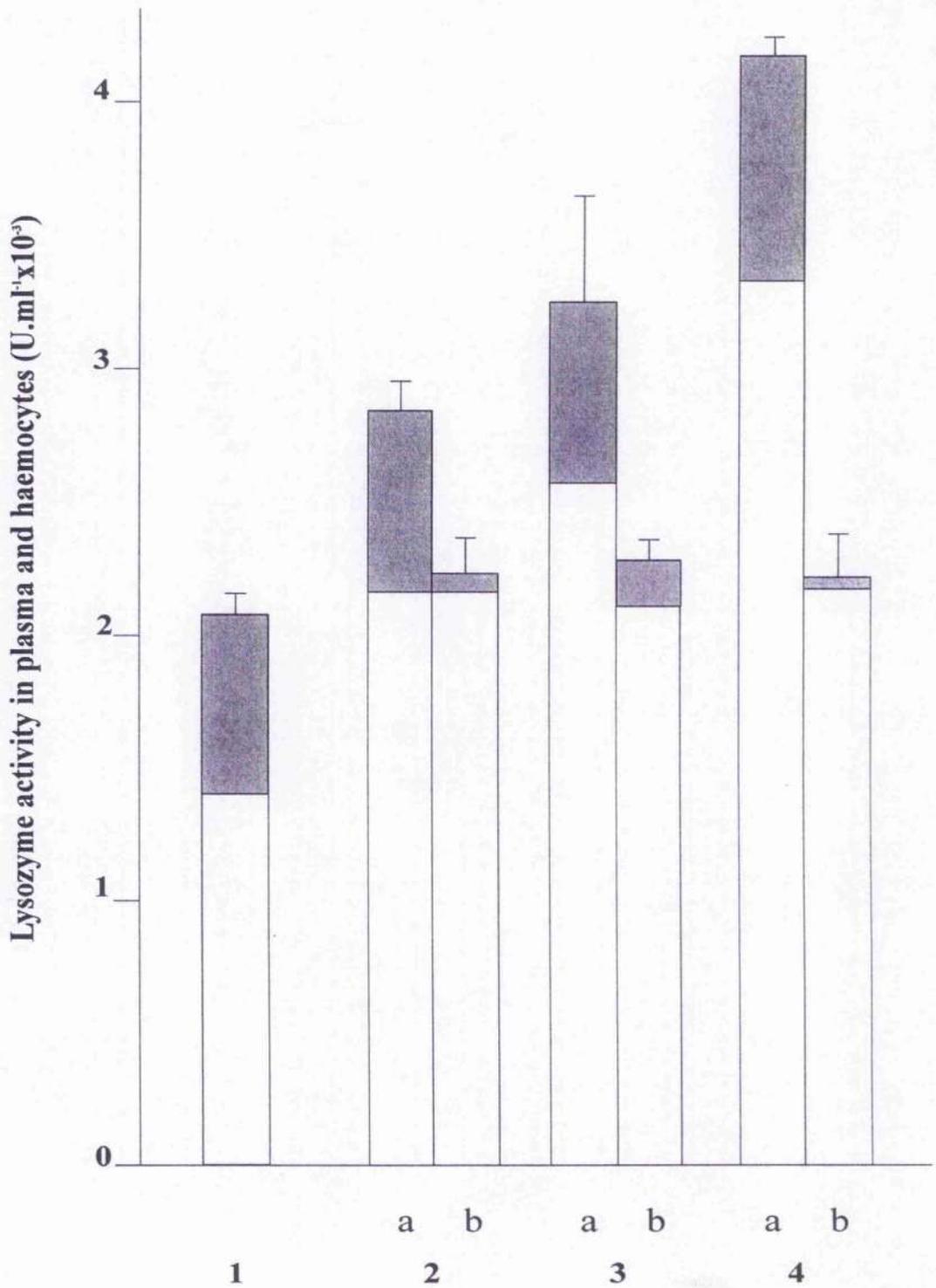
1. Basal level in untreated controls.
2. 1% (v/v) ethanol.
3. 5% (v/v) ethanol.
4. 10% (v/v) ethanol.

In each case: : plasma and  haemocytes;

(a) ethanol only and LPS,

(b) azadirachtin dissolved in ethanol and LPS.

Each result is the average from 4 locusts and vertical bars represent the \pm SD.



3.3.2.4.2. Effect of ethanol as a solvent for azadirachtin on lysozyme-like activity in fat body of *S. gregaria*

The effect of azadirachtin delivered in 10%, 5% and 1% ethanol on the lysozyme-like activity in fat body of locust is indicated in Fig 3.18. In this tissue, there was a decrease of 25-48% in the induced activity of lysozyme in response to ethanol and LPS. However, the rise in enzyme activity in response to LPS was not statistically significant as compared to basal level of activity in untreated controls. There was no clear-cut effect of ethanol in the induction of activity. The effect of azadirachtin was to lower the enzyme activity to well below normal levels suggesting that lysozyme is synthesized in the fat body.

3.3.2.4.3. Effect of azadirachtin solubilised in cyclodextrins on lysozyme-like activity in plasma and haemocytes of *S. gregaria*

It was clear that the use of ethanol at even low concentration caused a rise of lysozyme activity. To lessen the possible rise which was due to the ethanol, cyclodextrins were used as carrier of the terpenoid. Cyclodextrins are often used to "solubilise" hydrophobic compounds for drug delivery, and thus might have a less toxic effect than ethanol. To find out the best vehicle for azadirachtin delivery, the two cyclodextrins, β -cyclodextrin and methyl- β -cyclodextrin, were used as injection vehicles to solubilise azadirachtin.

The total haemolymph lysozyme activity was increased by 34% in response to the combination of β -cyclodextrin and LPS and 90% to methyl- β -cyclodextrin and LPS compared to the basal level. Both of these values were significant ($p < 0.01$) compared to the basal level of untreated locust. The total haemolymph lysozyme activity was reduced by 19% when azadirachtin solubilised in β -cyclodextrin and LPS were injected, compared to cyclodextrin and LPS treatment. Similarly the total enzyme activity was decreased by 21% when methyl- β -cyclodextrin was used to solubilise azadirachtin (Fig 3.19).

Fig 3.18. The effect of azadirachtin solubilised in 1, 5 and 10% (v/v) of ethanol on the level of activity of lysozyme induced by LPS in the fat body of *S. gregaria*.

The activity of lysozyme was estimated in the fat body of *S. gregaria*, 40 hours after the injection of different concentrations of ethanol alone, and as a solvent for azadirachtin (5µg/insect). After 16 hours of azadirachtin injection, each locust received 100µg of LPS (10µl volume). Lysozyme was estimated 24 hours after LPS injection.

1. Basal level in untreated controls.
2. 1% (v/v) ethanol.
3. 5% (v/v) ethanol.
4. 10% (v/v) ethanol.

In each case:

□ ethanol only and 100µg LPS/insect, and

▨ 5µg of azadirachtin dissolved in ethanol and 100µg LPS/insect.

Each result is the average from 4 locusts and vertical bar represents the \pm SD.

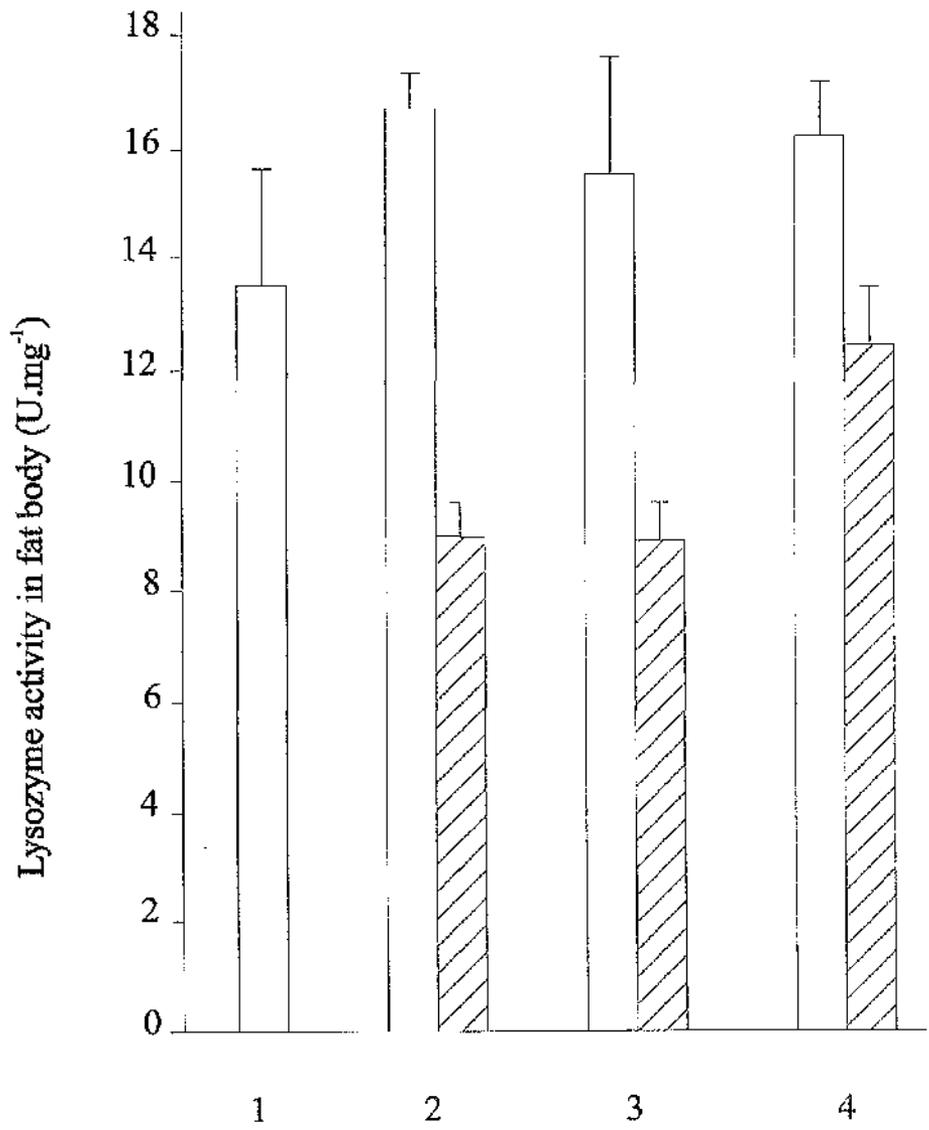


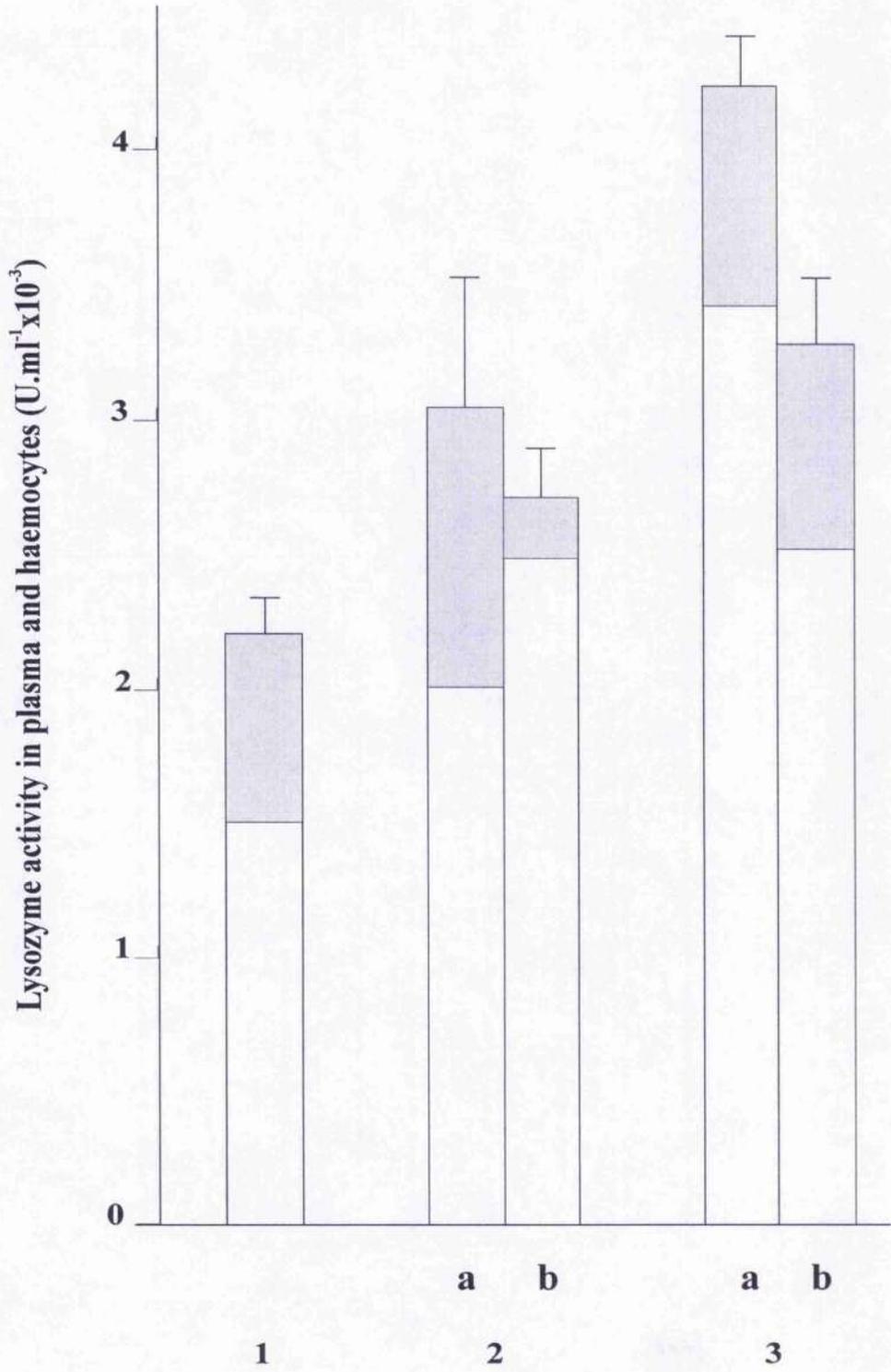
Fig 3.19. The effect of azadirachtin solubilised in cyclodextrins on the LPS-induced level of lysozyme activity in plasma and haemocytes.

The activity of lysozyme was estimated in haemolymph plasma and haemocytes 40 hours after the injection of cyclodextrins alone and as a solvent for azadirachtin (5µg/insect). After 16 hours of azadirachtin injection, each locust received 100µg of LPS (10µl volume). Lysozyme was estimated 24 hours after LPS injection.

1. Basal level in untreated controls.
2. (a) Controls received β -cyclodextrin and LPS.
(b) Insects received azadirachtin solubilised in β -cyclodextrin and LPS.
3. (a) Controls injected with methyl- β -cyclodextrin and LPS.
(b) Insects received azadirachtin dissolved in methyl- β -cyclodextrin and LPS.

In each case:  : plasma and  haemocytes.

Each result is the average from 4 locusts and vertical bars represent the \pm SD.



3.3.2.4.4. Azadirachtin solubilised in cyclodextrins and effect on lysozyme activity in fat body

Fig 3.20 shows the result of the effect of azadirachtin when it was solubilised in β -cyclodextrin and methyl- β -cyclodextrin. There was a 33% reduction of lysozyme-like activity when azadirachtin was solubilised in β -cyclodextrin and 42% in case of methyl- β -cyclodextrin. But these values when compared to the basal level of lysozyme activity showed a lower rise of enzyme, and there was no indication that cyclodextrins induced an increase of lysozyme activity in fat body.

In the light of the results using cyclodextrins to solubilise azadirachtin, finally it was concluded that there is no point in using these compounds as an injection vehicle for azadirachtin delivery to study the effect on lysozyme induction, as fat body is the possible site of lysozyme synthesis.

3.3.2.5. Selection of appropriate drug delivery vehicle

In the end, it was decided to use 5% (v/v) ethanol as the drug delivery vehicle. As shown in Fig 3.18 and Fig 3.19, the use of 5% ethanol seemed to be advantageous for the subsequent future experiments. Although not wholly unambiguous, these results support the assumption for fat body as the likely site of synthesis of lysozyme and to plan the *in vitro* incubation of fat body to find out the actual source of lysozyme synthesis.

Our study suggests that the lysozyme activity has been induced significantly after LPS treatment in the whole haemolymph. However, the assay of enzyme activity for the separated fractions showed that there was a remarkable rise in the cellular proportion. It may be that the lysozyme is equally likely to have come from haemocytes and fat body of female adult *S. gregaria*.

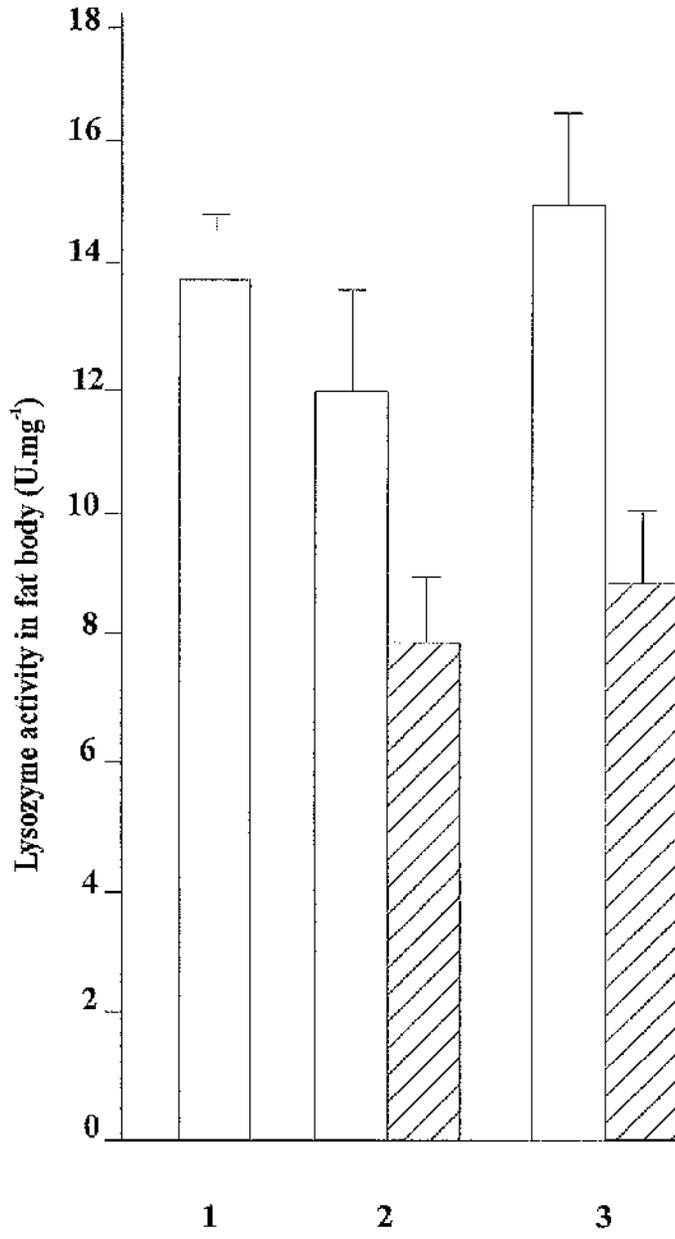
Fig 3.20. The effect of azadirachtin solubilised in cyclodextrins on LPS-induced lysozyme activity in the fat body of *S. gregaria*.

The activity of lysozyme was estimated the fat body of *S. gregaria*, 40 hours after the injection of cyclodextrins alone and as a solvent for azadirachtin (5µg/insect). After 16 hours of azadirachtin injection, each locust received 100µg of LPS (10µl volume). Lysozyme was estimated 24 hours after LPS injection.

1. Basal level in untreated controls.
2. Insects received azadirachtin solubilised in β-cyclodextrin and LPS.
3. Locusts treated azadirachtin solubilised in methyl-β-cyclodextrin and LPS.

In 2 and 3: : control and : treated.

Each result is the average from 4 locusts and vertical bars represent the ±SD.



3.3.2.6. Effect of LPS and azadirachtin on haemolymph protein concentration

Fig 3.21 is a representation of the effects of LPS, azadirachtin and cycloheximide treatments on the concentration of total haemolymph proteins. The LPS treatment caused a three-fold rise of the total concentration of haemolymph protein above the basal level. In control locusts, injected with insect saline, there was nearly a doubling of haemolymph protein concentration above the basal level of untreated insects confirming a large non-specific response to any injury.

The control insects injected with 5% ethanol and LPS showed a four-fold increase in the total protein concentration in haemolymph due to the combined response of these two inducers, but a single haemococlic injection of azadirachtin suppressed the induction of haemolymph protein by 60% ($P < 0.01$), restoring it to the basal level of 7.6mg/ml.

3.3.2.7. Gel electrophoresis of haemolymph and fat body

As described in Materials and Methods, to detect the presence of lysozyme in haemolymph and fat body samples, gel electrophoresis was used under denaturing conditions. In the preliminary experiments, the LPS-treated haemolymph and fat body samples against controls were analysed. Fig 3.22 is representing the resolved protein profile of the above mentioned samples. Due to complexity of the proteins, this technique was not used to study the effect of azadirachtin on the induced lysozyme.

3.3.3. *In vitro* experiments

3.3.3.1. *In vitro* LPS-induction of lysozyme-like activity in fat body of

S. gregaria

The result of a preliminary experiment of fat body incubation in the presence of LPS for a period of 24 hours showed almost a doubling of lysozyme-like activity, Fig 3.23. This significant rise in enzyme activity strongly suggests that fat body is the site of

Fig 3.21. The effect of various treatments on the concentration of total protein in the haemolymph of *S. gregaria*.

The protein concentration in the samples of haemolymph plasma was estimated in locusts which had received injections of possible inducers of lysozyme 24 hours previously. The protein was estimated by means of the Bradford assay.

1. Basal concentration in untreated control insects.
2. Bacterial lipopolysaccharide (LPS) treatment 100µg of LPS/insect.
controls injected with insect saline only.
3. Azadirachtin treatment: 5µg of azadirachtin/insect and LPS
controls received 100µg of LPS and 5% ethanol.
4. Cycloheximide treatment: 25µg of cycloheximide/insect, controls received insect saline.

In each case: : control and : treated.

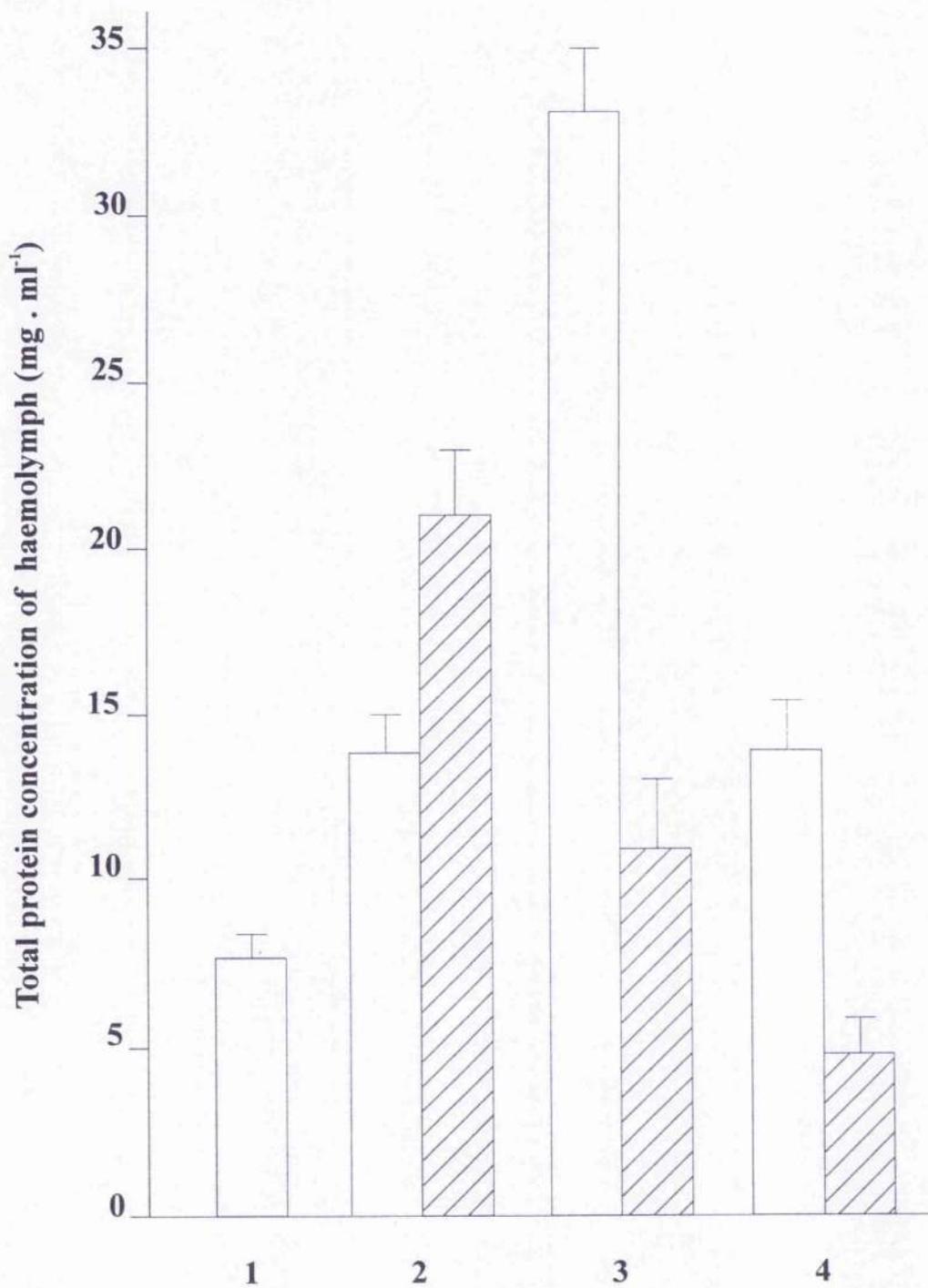


Fig 3.22. Gel electrophoresis of haemolymph and fat body samples of locust after 24 hours of LPS injection.

SDS-PAGE of haemolymph and fat body of adult female locust *S. gregaria*. Insects were treated with 100µg of LPS 24 hours before samples were collected. The amount of protein in each lane was less than 20µg. The gel was 20% resolution with 5% stacking in a discontinuous system. A: Hen egg white lysozyme as a control, B: LPS-treated haemolymph, C & D: Haemolymph and fat body from controls received insect saline, E: Fat body from LPS-treated locust and F: Molecular weight markers (kDa).

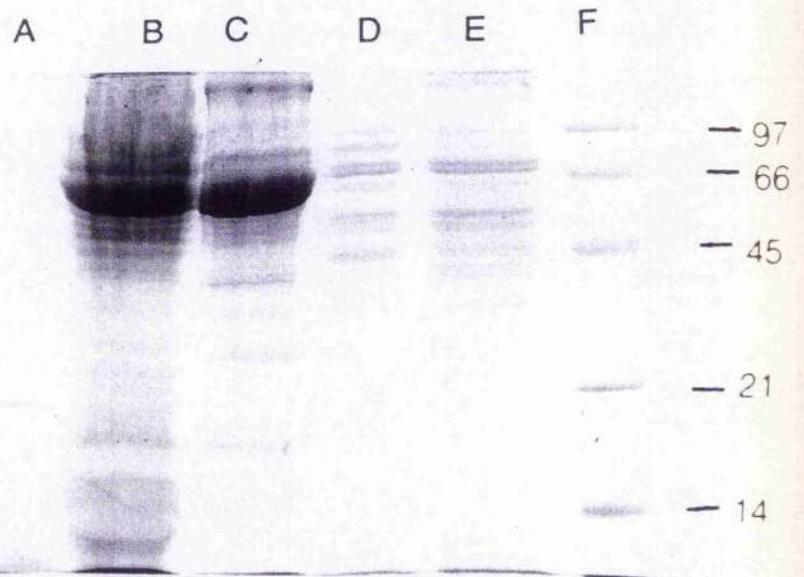


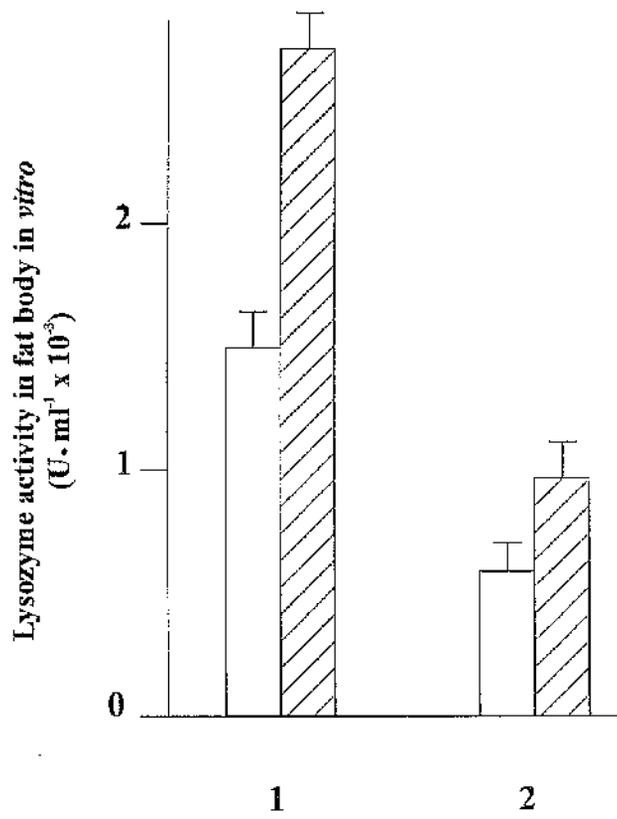
Fig 3.23. *In vitro*, LPS-induction of lysozyme activity by fat body of mature female *S. gregaria*, after 24 hours incubation.

Isolated and pooled fat bodies of locusts incubated in the presence of LPS, showed a statistically significant rise in the activity of lysozyme, compared to controls. The increased enzyme activity in incubation medium suggests *in vivo* the fat body as a site of synthesis of circulating lysozyme of haemolymph.

1: Lysozyme activity in fat body.

2: Lysozyme activity in the incubation medium.

In each case: : control and : treated.



synthesis for lysozyme. The presence of some fraction of enzyme activity in the incubation medium supports the idea that the circulating lysozyme of haemolymph may originate in fat body. In incubation medium, there was also a rise of 74% in the enzyme activity in the presence of bacterial lipopolysaccharide which was synthesized by fat body and then released in the medium during incubation period.

3.3.3.2. Effect of azadirachtin on the LPS induction of lysozyme-like activity in the fat body, *in vitro*

Fig 3.24 indicates the effect of azadirachtin on the *in vitro* synthesis of lysozyme in fat body of the desert locust, *S. gregaria*. There was a statistically significant ($P < 0.05$) reduction of 65% in the enzyme activity by azadirachtin, compared to controls, in which the activity was increased by 58% in the presence of LPS. In case of cycloheximide, the enzyme activity was decreased by 63% in incubated fat body, comparable to the effect of azadirachtin on the *in vitro* lysozyme synthesis in fat body of *S. gregaria*.

3.4. Discussion

The experiments reported in this Chapter were performed to examine the effect of azadirachtin on the constitutive or induced synthesis of lysozyme, a bacteriolytic enzyme in specific tissues of locust *S. gregaria*, using bacterial lipopolysaccharide as an inducer.

To achieve our aim two approaches were used: first to induce the synthesis of lysozyme by specific elicitor *in vivo* and to investigate the suppression of induced enzyme by azadirachtin; and second to verify the *in vivo* results in an *in vitro* system by assuming fat body as a principal site of lysozyme synthesis to ensuring that the effects of azadirachtin were directly upon the chosen tissue.

Fig 3.24. *In vitro*, effect of azadirachtin on LPS-induced lysozyme-like activity by fat body of mature *S. gregaria*, after an incubation period of 24 hours.

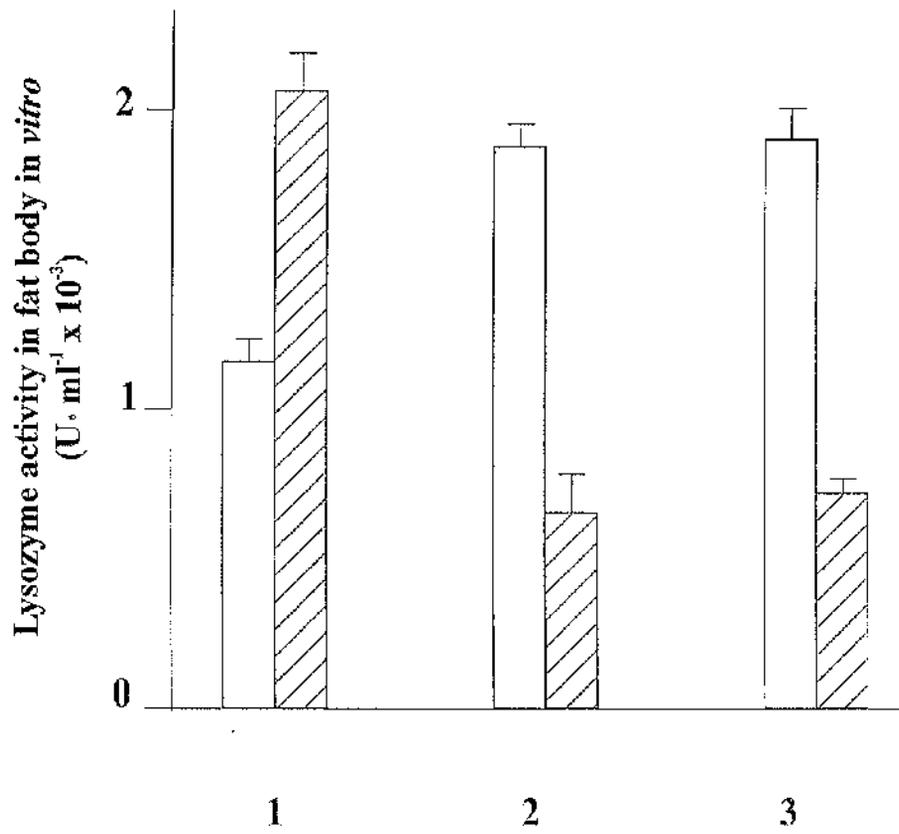
Isolated, pooled fat bodies of locusts incubated in the presence of azadirachtin and LPS showed a statistically significant decrease in the activity of lysozyme compared to that of controls incubated in the presence of LPS alone. The lowered enzyme activity by azadirachtin was comparable to that of the activity decrease by protein synthesis inhibitor cycloheximide.

1: Lysozyme activity induction in fat body by LPS.

2: Effect of azadirachtin on the LPS-induced lysozyme-like activity in fat body.

3: Effect of cycloheximide on the LPS-induced lysozyme activity in fat body.

In each case: : control and : treated.



As described in introduction sections, insects have effective passive defences, although, it is possible for microorganisms to enter the body cavity of insects, multiply and produce disease (Russel and Dunn, 1996). Many insects respond to a bacterial infection with the stimulation of distinct cellular and humoral defence systems, that cooperate in a more or less integrated way to decrease the chance of the microorganism becoming a pathogen. As a defence against bacteria, infected insects synthesize antibacterial proteins.

Antibacterial proteins were originally identified in insects as an important component of their immune response, and their synthesis is a result of combined response of humoral and cellular defence systems. The involvement of blood cells in defence reactions against invading bacteria was first reported in orthopteroid insects by Cuenot in 1896. It was suggested that in insects the major function of haemocytes may be immunologic, involving specialized cells such as lymphocytes and plasmatocytes (Hoffmann *et al.*, 1979).

Once in the hemocoel, the invading bacteria are exposed to a variety of defence mechanisms. They may include naturally occurring and inducible humoral factors like antibacterial proteins. The main types of antibacterial proteins identified in insect haemolymph are: broad spectrum antibiotic peptides such as cecropins, that kill and lyse a majority of bacteria, sepacins or insect defensins also bactericidal peptides, (mainly active against Gram-positive bacteria), attacins, sarcotoxin II, dipteracin, coeloptericin and the bacteriolytic enzyme, lysozyme. These proteins when isolated, affect a limited number of Gram-negative bacteria (Ratcliffe and Rowley, 1979). It is generally accepted that the antibacterial proteins are rapidly synthesized by fat body and certain haemocytes after infection and then secreted into the haemolymph where they eliminate the invading microorganism (Boman and Hultmark, 1987).

In *Drosophila melanogaster*, cecropins, andropin and lysozymes are the main antibacterial peptides (Hultmark, 1994), while Faye and Hultmark (1993) reported lysozyme as one of the major proteins induced after infection in *Hyalophora cecropia* and *Manduca sexta*. Russel and Dunn (1990), observed lysozyme activity in homogenates of pericardial complex in fifth instars of *Manduca sexta* and a remarkable rise in the haemolymph lysozyme activity after injection of peptidoglycans. Previously, in the larvae of same insect, it has been reported that after injection of live or heat-killed bacteria, phagocytosis and nodule formation was followed by the appearance of cecropins, attacins, the bacteriolytic enzyme: and additional proteins of unknown activity in haemolymph (Hughes *et al.*, 1983). In silkworm, *Bombyx mori* and wax moth, *Galleria mellonella*, lysozyme is present in the haemolymph as a bacteriolytic enzyme (Powning and Davidson, 1973).

From the above description it is obvious that among antibacterial proteins, lysozyme is one of the better defined humoral factors identified in many insects and is of great importance in insect immune mechanisms. Lysozyme is a heat-stable low molecular weight protein whose activity can be readily detected. It was used as an indicator to monitor the effect of azadirachtin on humoral defence response in specific tissues of the female adult locust. The age of the locusts used was 7 days after the final ecdysis because at this age the protein synthesis is at its peak. In *S. gregaria*, as a prerequisite to the interaction studies on azadirachtin-LPS-lysozyme a series of experiments were performed to address the LPS-induction of enzyme in haemolymph plasma, haemocytes and fat body.

Our studies with *S. gregaria* have shown that LPS does induce lysozyme-like activity in haemolymph and fat body significantly compared to controls, but the response to LPS was poor compared to other insects like prepupae of *G. mellonella*, and larvae of *B. mori* and *S. eridone* (Powning and Davidson, 1973; Anderson and

Cook, 1979). These findings are in consistent to the one reported by Lackie *et al.* (1985), that *S. gregaria* exhibits a poor cellular response to many types of injected biotic and abiotic particles, compared with *Periplaneta americana*. Another study about the response of *S. gregaria* haemocytes to injected substances of microbial origin suggested that the locust is capable of responding to microorganisms and dissolved substances from the cell walls of microorganisms, injected into the haemocoel (Gunnarsson and Lackie, 1985).

To study the effect of azadirachtin on the synthesis of our selected marker antibacterial protein lysozyme, bacterial lipopolysacchride (LPS) was used as an elicitor and injected into the body of desert locust, *S. gregaria*. The idea of selection of LPS as an effective elicitor to induce lysozyme activity was supported by previous studies of silkworm larvae, *Bombyx mori*. (Morishima *et al.*, 1992), tobacco hornworm larvae, *Manduca sexta* (Kanost *et al.*, 1988), and in beet armyworm, *Spodoptera exigua* (Boucias *et al.*, 1994). In *B. mori*, *in vitro* haemocyte culture with LPS induces gene expression of cecropin, and LPS released by phagocytosis of *Escherichia coli* play an important role in the induction of antibacterial proteins (Taniai *et al.*, 1997).

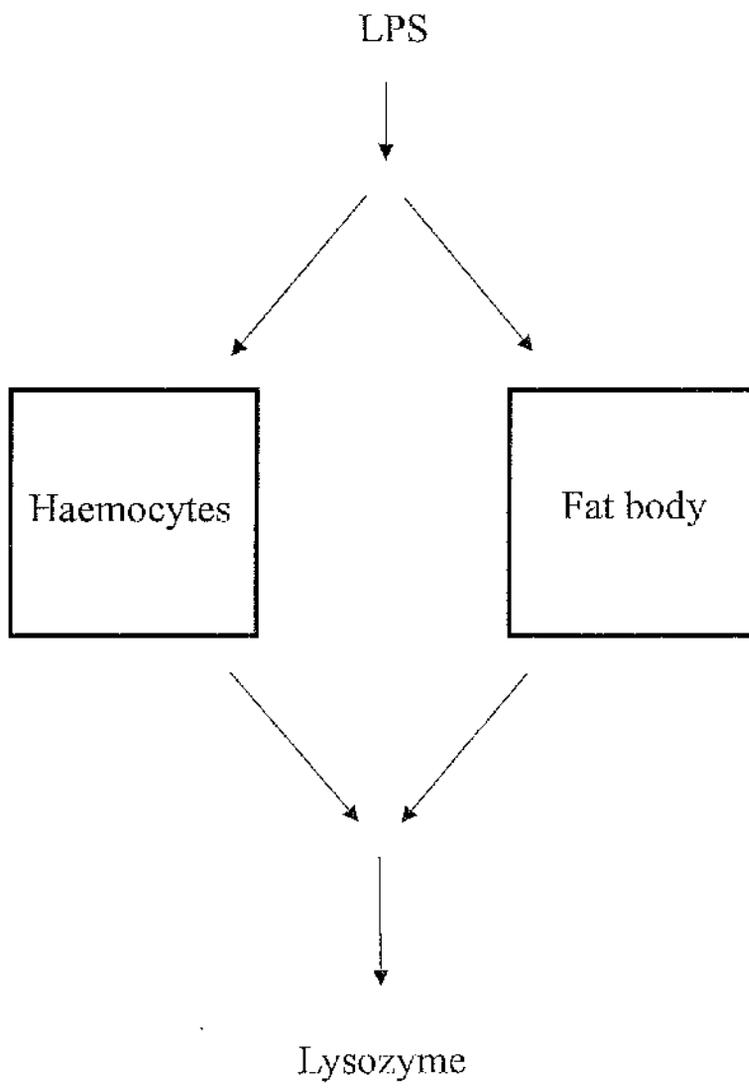
As reported here, in *S. gregaria*, the immune response found was not only specific to LPS. The results showed that even the simple process of injecting sterile saline into the locusts produced a small increase in the activity of circulating lysozyme, and this was doubled or trebled by bacterial lipopolysaccharide. There are reports that lysozyme is not only induced in response to infections like viable or heat-killed bacteria, bacterial lipopolysaccharide or bacterial peptidoglycan but is also present in the haemolymph of a number of nonimmunized insects (Dunn *et al.*, 1987; Knost *et al.*, 1988). Dunn *et al.* (1985) have suggested that one of the important roles of lysozyme present in normal haemolymph would be to release peptidoglycan fragments from invaded bacteria which have been killed by either phagocytosis or direct action of

lysozyme. As a result of this, the released fragments act on receptors of haemocytes and/or fat body and trigger the lysozyme synthesis (Fig 3.25).

Present investigations on humoral immunity in *S. gregaria* showed that the inoculation with bacterial LPS induces lysozyme activities effectively. Initially, 30mg of LPS/locust in 10ml of insect saline was injected into the locust, controls received 10ml volume of insect saline in the same way. But this amount was not sufficient to induce any statistically significant increase in the haemolymph lysozyme activity of locusts. Later the amount of LPS was increased up to 100mg/locust (10 ml volume) to cause a statistically significant rise in the activity of lysozyme locust haemolymph against controls, as indicated in results. In haemolymph of female adult locust *S. gregaria*, LPS injection caused 40-70% increase of lysozyme activity against controls after a period of 24 hours. These findings are nearly same as the one reported in the LPS-infected larvae of *Spodoptera exigua*, (Boucias *et al.*, 1994), and in last instar larvae of *Rhodnius prolixus* challenged with *Enterobacter cloacae* B12 (Azambuja *et al.*, 1991).

The haemolymph lysozyme activity of *S. gregaria* was monitored for 5 days after LPS treatment to select a time period with maximum humoral enzyme activity, so that in subsequent experiments this time period would be used to investigate the depression of activity by azadirachtin. The enzyme activity reached its peak 24 hours after the LPS injection and thereafter the levels of lysozyme activity in both control and experimental insects returned to the resting level by 48 hours. Abu-Hakima and Faye (1981) reported similar findings in *Hyalophora cecropia*. After a peak time, the antibacterial activity declines gradually in about an equal span of time. Similarly in *G. mellonella* the antibacterial activity was at its peak 18-24 hours after the injection and at the third day it returns to its original level (Stephens, 1962; Mohrig and Messener, 1968; Jarosz, 1979)

Fig 3.25. A hypothetical illustration of humoral induction of lysozyme synthesis by haemocytes and fat body of *S. gregaria*, in response to bacterial lipopolysaccharide (LPS).



Initially agar gel lysoplate assay was used to confirm the induction of humoral lysozyme after LPS treatment of locusts, using commercial lysozyme as a standard. Pure commercial egg-white lysozyme gives a linear relation between the area of the cleared zone and \log_{10} of the concentration of lysozyme. When actual haemolymph samples were used to detect the induced enzyme activity by this method, the zone-area versus log-concentration plot was of widely different slope, and same was true for control haemolymph samples. This difference in slopes could be caused by the behaviour of the complex mixture of proteins in haemolymph which alter the diffusional characteristics of lysozyme. The probable reason for this behaviour could be that the insect lysozyme has a different Mr than the avian one. These findings made it inappropriate to make a quantitative estimation of enzyme activity in real samples, so the lysozyme activity was estimated spectrophotometrically using *Micrococcus lysodeikticus* as a substrate, and this method was adopted for all of the reported results.

In *S. gregaria*, there was no evidence of any bacteriostatic agent in the haemolymph of both LPS-treated and control locusts, when tested for growth inhibition of *E. coli*, a Gram-negative bacterium. Absence of any bactericidal activity in haemolymph suggests lysozyme as the only single antibacterial protein in *S. gregaria* compared to other insects. These findings were different from the one reported earlier as the haemolymph of LPS-treated *Spodoptera exigua* larvae was able to inhibit the *in vitro* growth of *E. coli* (Boucias *et al.*, 1994).

As far as the source of circulating lysozyme is concerned, several tissue sources have been identified in other insects. The *in vivo* study of LPS-induction of locust humoral lysozyme showed a remarkable rise in the enzyme activity and the separation of activity in plasma and haemocytes indicated a 25-30% cellular contribution of induced activity, suggesting the involvement of haemocytes in lysozyme synthesis. The results of lysozyme assay for fat body of adult locust indicate the presence of lysozyme-like

activity, which was induced by LPS, but this induction was far less than the total humoral respons. The probable reason for this could be that the induced antibacterial protein is released into the haemolymph after synthesis by fat body cells and fat body is not the only source of lysozyme activity. On the other hand, *in vitro* incubation of fat body of *S. gregaria* with LPS caused a 90% rise in lysozyme activity, strongly supporting the assumption that the fat body is the source of circulating lysozyme activity.

From above arguments, it would be reasonable to say that in *S. gregaria* the synthesis of lysozyme is a combined activity of both fat body and haemocytes. These results suggest interaction of haemocytes and fat body in the regulation of humoral lysozyme, which is consistent with results in other insects. For example, haemocytes of *Locusta migratoria* (Zachary and Hoffmann, 1984) *Spodoptera eridania* (Anderson and Cook, 1979) and *Manduca sexta* (Dunn et al., 1987) have been shown to contain lysozyme, while fat body has been suggested as the major source of haemolymph lysozyme in diapausing pupae *Hyalophora cecropia* (Faye and Wyatt, 1980; Abu-Hakima and Faye, 1981). *In vitro*, synthesis and release of lysozyme by fat body has been demonstrated from both *Galleria mellonella* (De Verna et al., 1984) and *Manduca sexta* (Dunn et al., 1985).

In LPS-treated locust haemolymph, total protein concentration was increased significantly, but gel electrophoresis analysis did not show the same picture. A closer look at the pattern of proteins shown by SDS-PAGE indicated increase in the high Mr proteins. Due to a complex protein profile of haemolymph, it became difficult to identify the induced lysozyme by this method. There is a slight indication of induced proteins but only in fat body, but there is no indication of corresponding protein to standard lysozyme. Similarly, the attempts to detect the lysozyme after gel electrophoresis under non-denaturing conditions completely failed. The reason of failure might be the

technical difficulties of using small amounts of protein to be analysed in one lane i.e not more than 20µg. While the required amount to visualise the enzyme by PAGE was 200µg/lane and it was impossible to use this much protein.

One of the actions of azadirachtin in locust *S. gregaria* is to inhibit the induction of unidentified proteins in various tissues, including the fat body (Paranagama, 1994). Our results show 27-47% reduction of the mature female locust humoral LPS-induction of our marker antibacterial protein: lysozyme, when locusts were injected with a physiologically effective dose of azadirachtin. There was a profound effect on the cellular fraction, when the enzyme activity was assayed separately for plasma and haemocytes. The work presented here demonstrates that azadirachtin does affect the haemocytes of *S. gregaria*, contributing in significant induction of lysozyme in response to LPS injection. This is in accordance with the findings that in *Rhodnius prolixus*, one of the effects of the terpenoid is to reduce the capacity of the insect to respond to bacterial infection (Azambuja *et al.*, 1991). Depression of cellular and humoral immunity in *R. prolixus* by azadirachtin could be due to the inhibition of synthesis of lysozyme and a reduction in number of haemocytes challenged with *E. cloacae* B12. This could be explained as that azadirachtin prevents the production or release of the antibacterial protein, lysozyme, synthesized by haemocytes and fat body of *R. prolixus*. In fat body of *S. gregaria*, *in vivo* suppression of basal level of lysozyme and its LPS-induction by azadirachtin was 33-48%. The *in vitro* experiments were based on the assumption that fat body is a site of synthesis of humoral lysozyme and results supported this. By LPS enzyme activity was increased 99%, but the presence of azadirachtin caused a 70% suppression in the induced activity. *In vitro* a significant reduction of LPS-induced lysozyme-like activity by fat body cells indicates an inhibitory effect of terpenoid on lysozyme synthesis. There is evidence that azadirachtin also has a direct effect on identified proteins in other insects. For example, in *Manduca sexta*, azadirachtin inhibits the production of trypsin by the enzyme-secreting cells of the mid

gut wall (Timmins and Reynolds, 1992), and in *Spodoptera litura* azadirachtin significantly affects the digestive enzymes such as protease, amylase and invertase (Ayyangar and Rao, 1989).

There were some unexpected results such as a tremendous rise in the lysozyme activity in the haemolymph plasma, when 10% (v/v) ethanol was used as an injection vehicle for azadirachtin. There did not seem to be a corresponding increase in the enzyme activity of haemocytes. The explanation would be that the locusts respond to ethanol itself by an increase in soluble lysozyme, which does not seem to originate from the haemocytes. The effect of azadirachtin is to greatly reduce the combined response to LPS and ethanol especially in cellular fraction. The overall effect of azadirachtin is to return lysozyme activity to basal levels in haemolymph plasma and fat body.

In azadirachtin-treated locusts, the LPS-induction of total protein concentration was suppressed by 60%. The effect of azadirachtin was to greatly reduce the humoral response of locusts to LPS. This is in accordance to the one reported by Annadurai and Rembold (1992). According to them number of polypeptides in brain and haemolymph of adult female *S. gregaria* on 2D gels after 48 hours of azadirachtin treatment were reduced. These effects were persistent even 45 days of post-treatment compared to controls. This is also in agreement with the one reported in *Periplaneta americana*. The levels of haemolymph total proteins was decreased gradually by 25-40% when last instars of male and female nymphs of *P. americana* were injected with azadirachtin (Qadri and Narsaiah, 1978).

A closer analysis of the results reported here raises a question about the exact role of haemocytes in lysozyme synthesis. A significant cellular contribution of induction of lysozyme in haemolymph makes it clear that haemocytes play an important role in defence regulation mechanisms. Our findings of profound reduction by azadirachtin on

cellular fraction of lysozyme are likely due to a cytotoxic effect of azadirachtin on haemocytes involved in lysozyme synthesis. This is the mode of action of azadirachtin which is currently being investigated. This would help to explain the fact that the clear-cut reduction of lysozyme synthesis or LPS-induction of lysozyme by azadirachtin is apparent in haemolymph. There was a huge cellular increase in lysozyme activity in response to LPS when plasma and haemocytes were assayed separately for lysozyme activity and this induction was not shown by control insects received a mock injection of insect saline. Azadirachtin induced a similar inhibitory effect on *in vitro* fat body LPS-induction of antibacterial protein when added into the medium. Lysozyme activity assay in the medium alone is a clear indication of the fat body being a source of circulating lysozyme.

The above mentioned conclusions are supported by the reported effects of azadirachtin in locust and other insects. For example, cytological examinations of testes of fifth instars of *S. gregaria* males after 20 days of treatment, exhibit arrested spermatogenic meiosis at Metaphase I (Linton *et al.*, 1997). In another study on the effects of azadirachtin on mid-gut histology of *S. gregaria*, azadirachtin caused a slow necrosis of the epithelial cells and perhaps this effect was associated with a blockage of mitosis (Nasiruddin and Mordue, 1993). Usually morphological studies on dying cells reveal two patterns of cell death, 'apoptosis' and 'necrosis'. Necrosis is caused by the presence of toxic agents in the cell environment. Azadirachtin did induce cytological changes in the fine structure of epithelial cells of the gut of *R. prolixus*, and these changes made the intestinal environment unsuitable for survival of *Trypanosoma cruzi* (Nogueira *et al.*, 1997). Adipocytes and follicular epithelium structures show some typical signs of necrosis after exposure to azadirachtin (Wyllie *et al.*, 1984). Cytopathological findings in adipocytes and follicle cells due to azadirachtin are also reported in *Labidura riparia* young females (Sayah *et al.*, 1996).

Rembold and Annadurai (1993) demonstrated a profound inhibitory effect of azadirachtin on the growth and replication of the insect cell line Sf9, derived from ovarian cells of *Spodoptera frugiperda*. In this study the effects of azadirachtin on Sf9 cells compared with effects of other botanicals on this cell line and a mammalian cell line showed that the effect of azadirachtin was specific to insect cell line. A similar study by Jabbar and Strang (1998) on the same cell line found that Sf9 cells are very sensitive to azadirachtin while mammalian cell lines were little affected.

In summary, the results reported here suggest that azadirachtin interferes with lysozyme synthesis in haemocytes and fat body cells or release of synthesised enzyme in response to LPS in specific tissues of the desert locust. Probably the most striking effect of azadirachtin is on the cellular induction of lysozyme, which is an indication of a cytotoxic effect on these cells. It has yet to be proved that whether azadirachtin blocks the cell division of haemocytes involved in protein synthesis or affects on the fat body cells synthesising lysozyme, thus lowering the "defensive responses" of *S. gregaria* to bacterial infection.

Chapter 4
Effect of azadirachtin on
vitellogenesis in *Schistocerca gregaria*

4.1. Introduction

There are numerous reported effects of azadirachtin treatment on the reduction of fecundity in female insects, inhibiting vitellogenesis and egg production (Karnavar, 1987; Subrahmanyam, 1990). Potentially the action of azadirachtin could be significant in helping to control insect numbers during different stages of development (Ali *et al.*, 1997; Jagannadh and Nair, 1996; Rembold, 1995). The egg is surely one of Nature's most remarkable and versatile inventions. It is a compact self-contained capsule containing everything necessary for the creation of a new life. In an egg, the major source of nutrients for the developing embryo is the egg yolk which constitutes the non-organelle content of the oocyte. In female insects, oocyte growth during the final stages of maturation is a result of massive deposition of yolk proteins by a process called vitellogenesis (Wigglesworth, 1984) and the major protein itself is called vitellogenin. The process of vitellogenesis includes the production of the yolk protein precursors in fat body, their transport to haemolymph and then sequestration by developing oocytes (Ferenz, 1990).

Vitellogenin is the precursor of the major yolk proteins, found in all egg-laying animals. The term "vitellogenin" was first adopted by Pan *et al.* (1969), to denote female-specific blood proteins of insects, that bear common functional characteristics and are closely related in composition to the yolk proteins.

In majority of insects "vitellogenins" conventionally refer to a group of phospholipoglycoproteins. Vitellogenin isolated from a variety of insects consists of usually oligomeric polypeptides which vary in number from one in *Apis mellifera* to four or more in *Periplaneta americana*, *Locusta migratoria* & *Rhodnius prolixus* (Harnish *et al.*, 1982; Kunkel *et al.*, 1985). The native molecular weight is usually in the range of 300-600kDa, but vitellogenins of the higher diptera, with a native molecular weight of 200kDa are an exception (Valle, 1993; Izumi *et al.*, 1994). The

molecular masses of the sub-unit proteins are in the range of 150-200kDa for large ones and 40-65kDa for small subunits, respectively.

4.1.1. Role of fat body in vitellogenesis

Generally, the majority of insect vitellogenins are synthesised by fat body cells of sexually mature females and named as "Vitellin" when sequestered by oocytes (Englemann, 1979). The fat body of an insect generally consists of an arrangement of lobes or sheets, which allows maximum area of contact with the haemolymph. This is admirably suitable for rapid exchange of metabolites. Haemolymph is the only extra cellular fluid which circulates through a poorly defined system of sinuses (haemocoele) and thus bathes all the organs.

Although in all the insect species studied, vitellogenin is synthesised mainly by the fat body, the ovaries of *Drosophila melanogaster* (Bownes, 1986) *Dacus aleae* (Zonga *et al.*, 1988) and *Leptinotarsa decemlineata* (Kanost *et al.*, 1990) also have the capacity to synthesise this protein. In *Stomoxys calcitrans* the ovary is reported to be the sole source of vitellogenin (Chen *et al.*, 1987). Vitellogenins are synthesised in large amounts in the female insects and considered as female-specific proteins, but presence of small amounts of this protein has been reported in the haemolymph of males of *Locusta migratoria* as well (Dhadialla and Wyatt, 1983).

4.1.2. Uptake of vitellogenin by ovary

Of all the haemolymph proteins that are taken up in large amounts by the ovary, vitellogenins the most extensively studied. Once vitellogenin enters the egg, it is referred to as vitellin. Usually vitellogenin and vitellin are very similar in properties and composition (Mundall *et al.*, 1979; Hagedorn *et al.*, 1979; Kunkal *et al.*, 1985), with only minor differences in electrophoretic mobilities under denaturing conditions (Imboden *et al.*, 1983). The vitellogenin and vitellin of *Locusta migratoria* share

properties in common including SDS protein profile & amino acid composition (Chinzei *et al.*, 1981).

Oocyte growth depends on the uptake of vitellogenin. The vitellogenin binds to the specific receptors on the oocyte surface and the external membrane forms oocytotic vesicles. During their formation, the pinocytotic vesicles trap other proteins present in external fluid and latter these proteins are taken up non-specifically depending on their concentration in the excretory fluid.

In *Locusta migratoria*, the vitellogenin changes its subunit pattern during uptake into the ripening oocytes (Chen *et al.*, 1976), but the mechanism of this process is not clear. It might be that the follicle cell layer enveloping the maturing oocyte is implicated in this uptake mechanism and specific proteins of the follicle membrane may be involved in this process (Anderson and Telfer, 1970). It is proposed that vitellogenin binds to specific receptors on the oocytes surface and the extreme membrane oolemma, is invaginated to form oocytotic vesicles (Telfer, 1960).

4.1.3. Vitellogenesis in locusts

The desert locust, *S. gregaria*, has been used in many laboratories as a test organism but unlike *L. migratoria* (Girardie *et al.*, 1998, 1996, 1992; Wyatt, 1988) relatively little is known about many aspects of its reproduction. It has an enormous reproductive potential which enables it to reproduce quickly and efficiently to form migrating swarms. To control these swarms, it is important to understand the physiology of oogenesis. In locusts, oogenesis consists of four periods (Goltzene and Porte, 1978):

- i) pre-vitellogenesis, cells have large lipid inclusions, much glycogen and a contracted nucleus;
- ii) vitellogenesis I, this is characterised by abundant rough endoplasmic reticulum and Golgi apparatus;
- iii) vitellogenesis II, formation of large nucleus and multilobed nucleolus;

iv) choriogenesis, termination phase.

During pre-vitellogenesis, the oocyte-synthesizing structures develop and make the translucent-white oocyte opaque. Vitellogenesis corresponds to the period of deposition of yolk proteins. The first phase of vitellogenesis roughly correlates with the appearance of yellow oocytes and is characterised by slow growth of the oocytes, which at this time are less than 2mm in length. During second phase of vitellogenesis, the oocytes grow rapidly following an active uptake of yolk precursor from the haemolymph and in choriogenesis the oocytes membranes are produced and finally egg-laying occurs.

At the molecular and biochemical levels, the yolk protein of the African migratory locust *L. migratoria*, has been studied in detail (Gellissen *et al.*, 1976; Chinzei *et al.*, 1981). The vitellin is the major yolk protein, > 70%, while its precursor vitellogenin is synthesised in the fat body and circulates in the haemolymph before incorporation into the developing oocytes (Chinzei and Wyatt, 1985; Ferenz, 1995).

Chen *et al.* (1976) have shown that the vitellogenin of *L. migratoria* is synthesised as a 260kDa precursor in the fat body, which gives rise to the five subunits before or during its release into the haemolymph. In many respects the desert locust is similar to the African migratory locust, but for our study more information is required about the vitellogenesis of the desert locust. Although the haemolymph vitellogenin of *S. gregaria* has been purified and characterised (Ferenz *et al.*, 1995; Mahamat *et al.*, 1997), we need to know more details of vitellogenesis to explore the effects of azadirachtin on reproduction physiology of the desert locust.

4.1.3.1 Hormonal regulation of vitellogenesis in locust

In the majority of insects, the process of yolk deposition requires the action of juvenile hormone (JH). This has been known since Wigglesworth's first paper on the hormone in 1936, and is attributed to the dependence of the follicle cells upon JH from

the moment the formation of the ovarian follicles is complete. There are however, many exceptions to this rule. If the JH source is removed at the outset of this period, development of the ovarian follicles ceases just when yolk deposition ought to start. Methoprene, a synthetic analogue can completely replace JH and stimulate vitellogenesis. There are also indications that neurosecretion from some of the brain's neurosecretory cells is also necessary during this period (Thomsen, 1952; Wigglesworth, 1964, 1970).

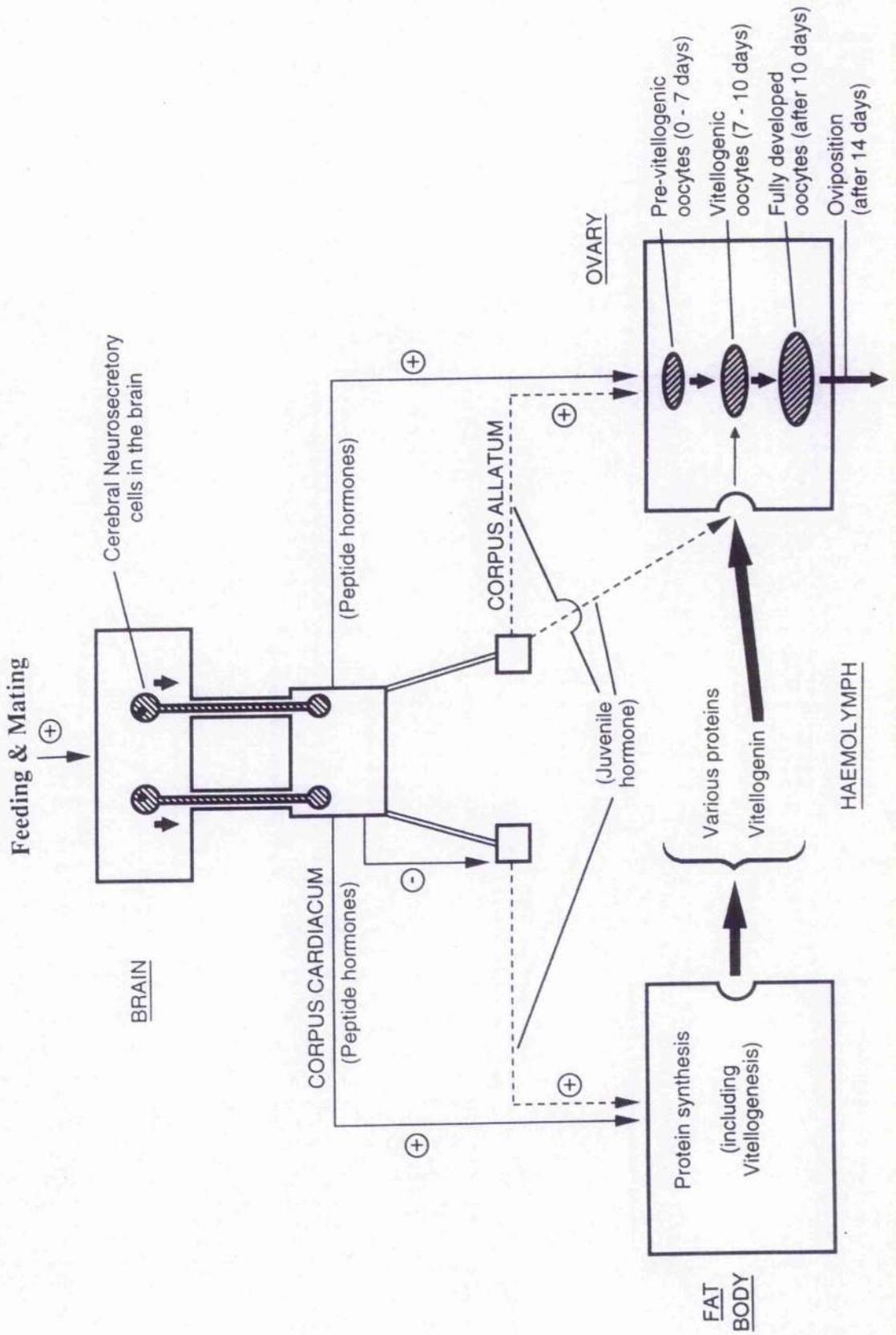
Neurosecretion is required for first phase of vitellogenesis and at the end of the second phase of vitellogenesis when the vitellin membrane and chorion are produced by the follicle cells. Juvenile hormone appears to control transcription of two locust vitellogenin genes in the fat body of *L. migratoria* (Wyatt, 1988). In addition to the fat body, juvenile hormone also has ovarian targets in the locust (Goltzene and Porte, 1978), where it enhances vitellogenin uptake in isolated oocytes (Ferenz *et al.*, 1981). The requirement of the neurohormone during the rapid growth of oocytes suggests that the neurohormone could also control the receptor-mediated uptake of vitellogenin (Girardie *et al.*, 1992). Recently it has been suggested that in *L. migratoria* and *S. gregaria*, in addition to juvenile hormone (JH), there is another gonadotropic neurohormone called, "Ovary Maturing Parsin (OMP)", involved to stimulate vitellogenesis and oocyte growth (Girardie *et al.*, 1998).

Fig 4.1 is an illustration of the neurohormonal regulation of vitellogenesis. Among the regulating hormones, involved in the synthesis of specific proteins in fat body, JH has powerful multiple role in regulating metamorphosis and reproduction (Wyatt, 1985). In insects, to study their morphological and biochemical parameters of development, methoprene is used as a JH mimic. It was designed to confer enhanced stability to a structure closely resembling JH (Wyatt, 1985), and has been used extensively to study vitellogenesis (Wang *et al.*, 1993; Kremen and Nijhout, 1998; Zhang *et al.*, 1993 and Girardie *et al.*, 1998).

Fig 4.1. Schematic illustration of the hormonal regulation of reproduction in female locust.

⊕ : Stimulation

⊖ : Inhibition



In our study, the JH analogue, methoprene, was used for *in vitro* vitellogenesis induction in fat body incubations.

4.1.5. Vitellogenin and vitellin as biomarkers

The vitellogenins and vitellins of insects provide good material for the study of the regulation of protein synthesis, processing, secretion and uptake as well as evolution, and the desert locust *Schistocerca gregaria* is a favourable insect for this type of study. Antibodies raised against vitellins have been shown to react with the precursor vitellogenin synthesized by fat body (Valle, 1993).

4.1.6 Azadirachtin and insect reproduction

It is now well established that azadirachtin is not only a potent antifeedant, but has a strong disrupting effect on growth and development of several insect species (Schmutterer, 1990; Mordue and Blackwell, 1993). It has been proposed that these effects are mainly due to interference with the synthesis and release of regulatory morphogenetic neuropeptides (Sieber and Rembold, 1983; Rembold, 1987). It has been suggested that the effects of azadirachtin on fertility and fecundity are due to alterations in titres and timing of release of neuropeptides and steroid hormones (Subrahmanyam *et al.*, 1983). However, growth-regulatory and sterilizing effects in female insects treated with azadirachtin are thought to result from a blockage of the release of morphogenetic peptides (Mordue *et al.*, 1986; Rembold *et al.*, 1987). The blockage of release of morphogenetic peptides in turn is responsible for altering the ecdysteroid and juvenile hormone titres. Tawfike *et al.* (1997) have reported the relation of haemolymph ecdysteroid peaks with the progress of egg formation in both solitary and gregarious females of *S. gregaria*. In the vast majority of insects, there is a direct relationship between haemolymph vitellogenin levels and ecdysteroid concentration with ovarian development (Adams and Gerst, 1993).

In *L. migratoria* and *S. gregaria*, although 75% of azadirachtin is rapidly excreted after injection, a significant proportion binds to the ovaries, testes and accessory glands (Rembold *et al.*, 1988; Paranagama, 1994 and Nisbet *et al.*, 1995). Nisbet *et al.* (1995) have reported specific time-dependent saturable high-affinity binding of tritiated dihydroazadirachtin to homogenates of testes of adult desert locust *S. gregaria*. There are reports involving cytological changes in ovaries and fat body cells of *Labidura riparia* after azadirachtin treatment (Sayah *et al.*, 1996), and this effect was not linked merely to an inhibition of feeding.

4.1.6.1. Azadirachtin and vitellogenesis

Azadirachtin inhibits vitellogenesis and egg production in many insect species (Subrahmanyam, 1990), for example in the milk weed bug, *Oncopeltus fasciatus* (Dorn *et al.*, 1987), in African migratory locust *Locusta migratoria* (Rembold and Sieber, 1981) and in *Labidura riparia* vitellogenesis inhibition by azadirachtin suggests a direct cytotoxic effect on fat body cells as well as a generalized disruption of endocrine and neuroendocrine functions (Sayah *et al.*, 1998). However, the biochemical effects of azadirachtin at cellular level are still unknown. *In vivo* experiments with mature females of *Locusta migratoria* assume that azadirachtin may interfere with the neuroendocrine control of hormone synthesis, but the inhibitory action of azadirachtin on ecdysteroid *in vitro* by ovaries of female adult cricket *Gryllus bimaculatus* suggest a direct effect (Lorenz *et al.*, 1995).

Similarly, in mature females of the blood-sucking bug *Rhodnius prolixus*, ingestion of azadirachtin induces a dose-dependent reduction of vitellogenin levels both in haemolymph and ovaries, and may interfere its formation by depressing the release of neurohormones both *in vivo* and *in vitro* (Fedcr *et al.*, 1988). In *O. fasciatus*, the females which emerge from azadirachtin-treated larvae exhibit reduction of follicle development (Dorn, 1986).

Reports involving physiological or pharmacological effects of azadirachtin on vitellogenin/vitellin titers in insects are few. The previous work done in our laboratory, suggests that a number of effects of azadirachtin on various tissues of desert locust are on processes ultimately controlled by neurohormones (Paranagama, 1994).

4.1.7. Aims

The aim of this part of my project was to verify whether azadirachtin affects vitellogenesis directly or indirectly via neurohormonal control, resulting in lower fecundity of the female locusts. It is worthwhile to assess the action of this natural substance as a tool to study the reproduction physiology of the desert locust *S. gregaria*. Fig 4.2 indicates the possible target tissues for azadirachtin involved in the developmental physiology of locusts. An *in vitro*, study was aimed to understand the direct effects of azadirachtin on fat body vitellogenesis, eliminating the possible *in vivo* hormonal influences.

4.2. Materials and methods

4.2.1. Insects

Adult *S. gregaria* used in all experiments were fed and maintained under the conditions described in Chapter 2.

4.2.2. Insect saline

The insect saline used was of the same composition as given in Chapter 2.

4.2.3. *In vivo* experiments

4.2.3.1. Tissue collection

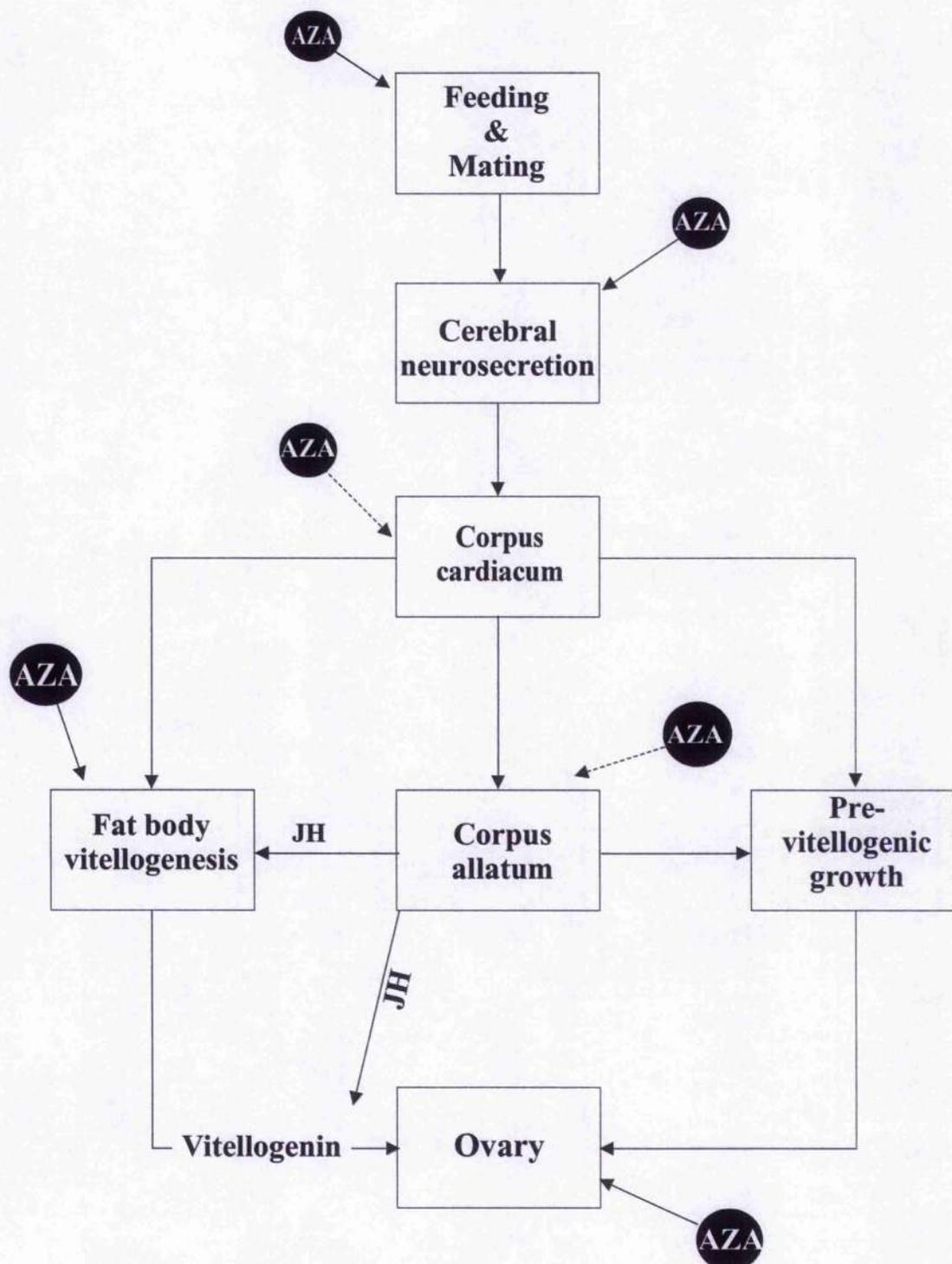
Haemolymph and fat body was collected as detailed in Chapter 2, while ovary was dissected out from the abdomen of the decapitated locusts.

Fig 4.2. The possible target tissues of *S. gregaria*, whose proteins might be affected by azadirachtin.

AZA: Azadirachtin.

▶ Dotted line indicates profused effects.

 —▶ Solid line shows reported effects.



4.2.3.2. Purification of vitellin

After a series of preliminary experiments, a purification scheme previously used for *L. migratoria* vitellin (Chinzei, 1981), finally adopted with little modification was as follows (Fig 4.3):

Ovaries with mature oocytes, were dissected out from adult mature females, rinsed in insect saline and homogenized in 10 vols of cold 0.5M KCl-0.5mM EDTA-0.2M sodium phosphate buffer (pH 7.5), in all-glass Potter homogenizer. The buffer was supplemented with 1mM phenylmethylsulfonyl flouride (PMSF), a protease inhibitor.

The homogenate was centrifuged at 12,000g at 4°C for 15min in a Sorvall® RC-5B Refrigerated Superspeed Centrifuge. The clear supernatant was collected, filtered through glass wool to remove lipids, and diluted six-fold with ice-cold insect saline. The final solution was brought to 70% saturation with ammonium sulphate and centrifuged at 12,000g, at 4°C for 10min. The precipitate was suspended in 10ml of sodium phosphate buffer pH 6.3 diluted 10 fold with chilled water and dialysed against distilled water at 4°C overnight.

The precipitate was then collected by centrifugation at 12,000g 4°C for 15min and dissolved in five times of its volume of 0.2M sodium phosphate buffer pH 6.3. In this solution the water was added until first precipitate appeared, and centrifuged at 12,000g at 4°C for 15min to remove the precipitate. The supernatant was applied to a column of DEAE cellulose, which had been equilibrated with 0.1M sodium phosphate buffer pH 6.3. After washing with the same buffer until the effluent gave $A_{280} \leq 0.01$, vitellin was eluted with 0.25M KCl-0.1M sodium phosphate buffer pH 6.3. Protein was estimated by absorbance at 280nm, using BSA as a standard. The absorbance readings were plotted against the number of fractions. The occurrence of a single peak confirmed the isolation of vitellin (Fig 4.4).

The eluted fractions of main protein peak were analysed separately on SDS-PAGE to resolve the individual sub-units (shown in results section Fig 4.5).

Fig 4.3. Scheme of vitellin purification from mature oocytes of *S. gregaria*.

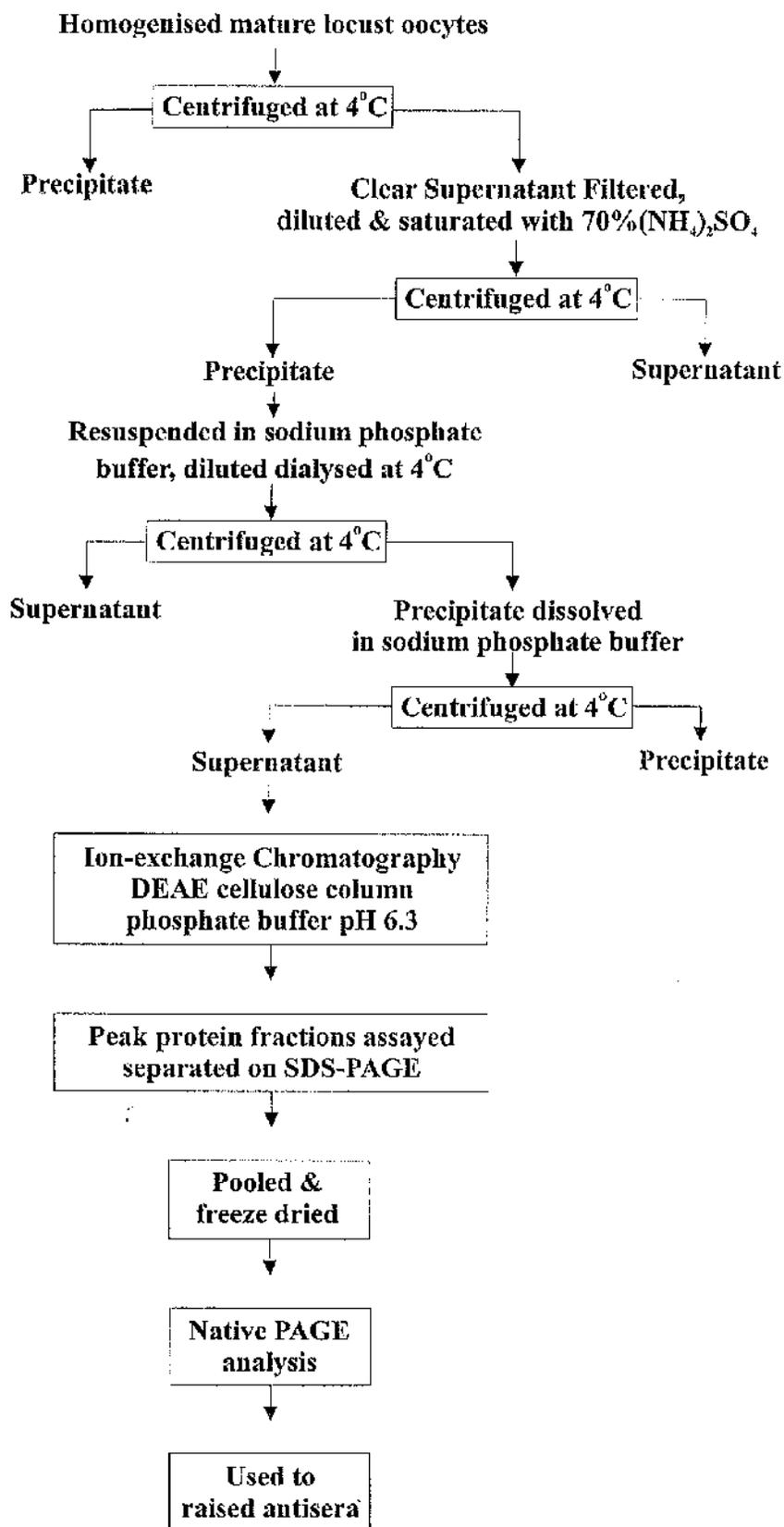
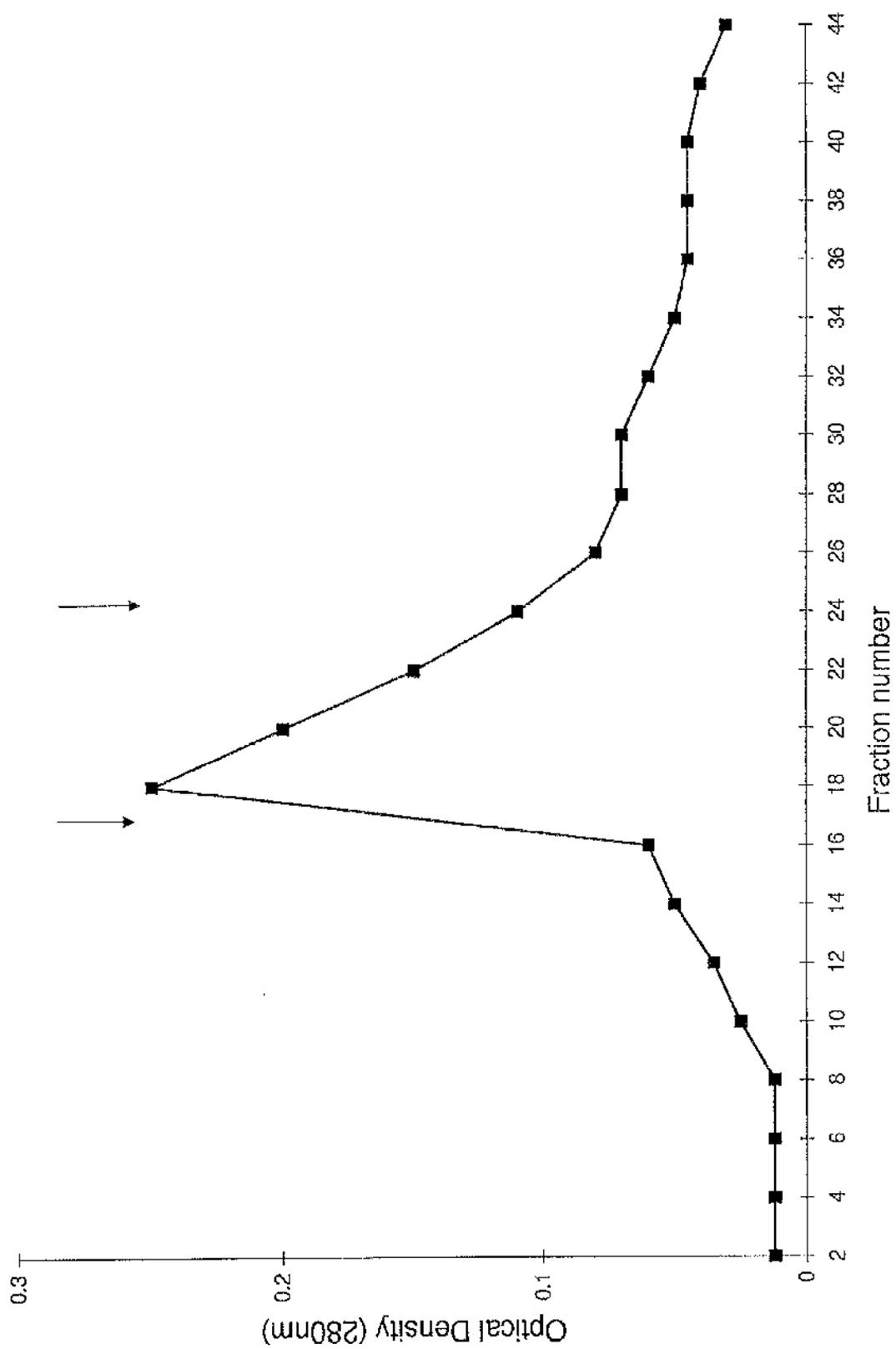


Fig 4.4. Purification of vitellin by ion-exchange chromatography.

The protein was eluted from DEAE cellulose column in phosphate buffer pH6.3. Samples (1ml), were collected and protein content for individual samples was estimated by A_{280} . The arrows indicate fractions shown by electrophoresis to contain vitellin. These fractions were pooled.



Subsequently, the fractions were pooled and concentrated by freeze drying. The PAGE of this purified vitellin gave a single band in native conditions Fig 4.5 (b).

Over 65% of the total protein, extracted from ovaries, was vitellin, and 90% of the pure protein could be recovered from low salt solution by precipitation. It was observed that vitellin and vitellogenin tend to precipitate out when stored in physiological solutions for long periods (Ferenz and Lubzens, 1981). Apparently aggregates are formed, which can be seen in native yolk. Consequently, for each experiment fresh solution from freeze dried vitellin was prepared.

The pure vitellin was used as an antigen to raise antibodies. It was also used as a standard in electrophoretic analysis to detect the presence of vitellogenin in male haemolymph.

4.2.3.3 Protein estimation

The protein concentration of the homogenised tissue samples when and wherever required was estimated by Bradford (1976), described in Chapter 2.

4.2.3.4. SDS-PAGE

Samples to be analysed by SDS-PAGE were added an equal volume of 50mM Tris-HCl pH 6.8, containing 5%(v/v) β -mercaptoethanol, 5% (w/v) SDS, 10% (v/v) glycerol and 0.005%(w/v) bromophenol blue at a final protein concentration of (1mg/ml).

Samples and prestained molecular weight standards (BDH), (Table 4.1) were then boiled for 3min and resolved in 12% polyacrylamide, "minigels" (Bio-Rad) with 5% stacking gel, according to the procedure described by Laemmli (1970) and also given in Chapter 3.

Following electrophoresis, resolved polypeptides were stained in Coomassie Brilliant Blue to visualize them or transferred to nitrocellulose paper for Western blotting.

Table 4.1. Reference proteins of known Molecular^{weight} ratio (Mr)

Name Of Protein	Mr ($\times 10^{-3}$)
Myosin (rabbit muscle)	200
β -Galactosidase (<i>E.coli</i>)	116
Phosphorelase b (rabbit muscle)	97
Ovotransferrin (hen egg)	78
Glutamate dehydrogenase (bovine liver)	55
Ovalbumin (hen egg)	42

4.2.3.5 Native PAGE

The purified sample was resolved using 8% polyacrylamide minigels (Bio-Rad), with 3% stacking according to Laemmli's procedure (1970), with omission of SDS from all the buffers. The gel was aged overnight at 4°C before being run.

4.2.3.6. Antiserum production

Polyclonal antibodies against the purified vitellin were raised in two male NewZealand white rabbits, by the following procedure:

Each of the rabbits was given an interperitoneal injection of 100µg of vitellin in 100µl of phosphate buffer pH 6.3, emulsified with 1:1 (v/v) equal volume of Freund's Adjuvant (Complete) (Sigma BioSciences™). After two weeks, a similar injection of same amount of vitellin emulsified with Freund's Adjuvant (Incomplete) (Sigma Chemical Co.) was given to each rabbit.

After a further two weeks, the rabbits were bled and serum was prepared by centrifugation at 2,000g after clotting for one hour. The sera were tested for the presence of raised antibody by the Ouchterlony procedure and enzyme linked immunosorbant assay (ELISA). Sera taken from the rabbits before injection of antigen were used as controls.

4.2.3.7. Azadirachtin treatment

The experimental locusts were injected with a dose of 3µg azadirachtin/g body weight in 5% aqueous ethanol and the control insects received the same volume of 5% ethanol at the same time and in the same way. Experiments were performed on locusts either 15 or 19 days after imaginal moult. After azadirachtin injection, the insects were left for 48 hours with access only to water.

4.2.3.8. Immunological methods

4.2.3.8.1. Ouchterlony immunodiffusion

Ouchterlony plates were prepared with 1.5% (w/v) agar (DIFCO, 0142-02 DIFCO Laboratories, Detroit 1, Michigan, U.S.A) in PBS pH 6.3. The agar was dissolved and autoclaved as described in Chapter 2. Radial immunodiffusion tests were run at 37°C for a period of 24 hours. The plates were washed for 48 hours in 0.4M NaCl before being photographed. The haemolymph and fat body samples of both male and female locusts were used in these tests, while ovarian samples of mature female locusts were also tested. The purified antigen, vitellin, was used as a positive control.

4.2.3.8.2. Enzyme linked immunosorbant assay (ELISA) for vitellogenin and vitellin

ELISA microtitre plates (Nunc Immunoplates) were coated with the purified vitellin as standard antigen. Each well received 1µg of antigen dissolved in phosphate buffer saline (PBS) pH 7.2 (in a volume of 100µl), except the first line as a control received only the buffer. Plates were covered and kept overnight at room temperature.

Next morning the contents of the plate were discarded and plates washed twice times with PBS and residual liquid was removed by knocking vigorously on paper towels. In each well 100µl of 10% BSA in PBS was added and left for 10 minutes at room temperature. The plates were again washed twice and dried. The sample of antiserum was diluted 1:1000 in PBS containing 1% BSA. With the exception of first line of control wells containing water, all the others received 100µl of PBS. Then a 100µl of diluted antisera was added into line 2 and serial dilutions were made by pipetting 100µl into the subsequent wells, diluting x 2 each time. The plates were left for 1 hour.

At the end of one hour, the plates were washed, dried, and 100µl of donkey anti-rabbit antiserum conjugated to horseradish peroxidase (HRP), (Scottish Antibody

Production Unit, Law Hospital, Carlisle Lanarkshire, Scotland), 1:500 diluted in PBS was added into each well. The plates were incubated for 1 hour.

After washing with PBS and drying, each well received 100µl of substrate, orthophenylenediamine (OPD) (Sigma Chemical Co.) 4mg dissolved in 10ml of McIlvaine buffer (18ml 0.1 M citric acid was added into 32ml of 0.2M Na₂HPO₄ and this was made up to 100ml with water) with 0.1% (v/v) H₂O₂. The plates were placed in the dark for 15 minutes at room temperature. Lastly, 50µl of 2M H₂SO₄ was added into each well to stop the reaction. Absorbance of the supernatant was read at 492nm with a Multiskan spectrophotometer. The presence of antibody raised against locust vitellin was confirmed by comparing the result of the sera collected before the injections of antigen.

4.2.3.8.3. Generation of the titre curve by ELISA

An ELISA estimate of the antigen, as described in the above section was carried out to generate a standard curve on the basis of the known amounts of vitellin, as shown in results section. This curve was used to quantify the level of vitellin in the ovarian samples of female adult locusts, and its precursor vitellogenin in the haemolymph and fat body samples of both male and female insects.

The amounts of antigen used were ranged 0.1-1.0µg/well. The experiment was repeated four times and average absorbance reading (A_{492}) used to generate the standard curve. The 1:1000 dilutions of polyclonal antiserum and 1:500 of donkey HRP anti-rabbit were used throughout, for subsequent experiments.

4.2.3.8.4. ELISA estimation of the concentration of vitellogenin in haemolymph and fat body

ELISA was repeated for the samples of haemolymph and fat body of female adult locusts aged up to 28 days, in order to find out the age of the locusts when the

vitellogenin is at its maximum level in these tissues and compared to the total protein concentration.

4.2.3.8.5. ELISA estimation of the concentration of vitellin in ovarian tissues of the locust

The vitellin of locusts ovaries age 4-28 days, was estimated by ELISA. The age of the insects were counted from the final instar. The results of these preliminary experiments were used to check the appropriate age of insects for azadirachtin treatment in later experiments.

4.2.3.8.6. Effect of azadirachtin on vitellogenin level in haemolymph of female locusts.

A group of 12 locusts, aged 15 days were treated with azadirachtin, as described in 4.2.3.6, and monitored for two days. By the end of this time, half of the insects were dead, and tissue samples were collected from the remaining living azadirachtin-treated and control locusts as described in 2.2.3.1. Haemolymph samples were taken by procedure given in 3.2.3.3, immediately after azadirachtin injection and after 48 hours. These samples provided a mean of measuring changes caused by azadirachtin treatment. The vitellogenin level of haemolymph was assayed by ELISA. In second experiment, the locusts age 19 days were chosen to study the effect of azadirachtin on the vitellogenin levels in specific tissues of *S. gregaria*, exactly in the same way as for the first experiment of locusts aged 15 days.

4.2.3.8.7. Effect of azadirachtin on the fat body vitellogenin of locusts.

At the end of three days, the controls and surviving azadirachtin-treated locusts were decapitated, and haemolymph, fat body and ovaries were collected from each locust as previously described 4.2.3.1. The fat body samples were used to study the effect of

azadirachtin on the synthesis of vitellogenin by ELISA. The vitellogenin content was expressed as a percentage of total soluble protein of fat body.

4.2.3.8.8. Effect of azadirachtin on vitellin of locusts.

The ovarian samples of control and azadirachtin-treated insects were used to find out the effect of azadirachtin on the level of vitellin by ELISA, and standard curve was used to quantify the vitellin in these samples. Finally a comparative percentage was calculated on the basis of total protein concentration of each sample.

4.2.3.9. Western Blotting

Proteins were transferred from SDS gels to nitrocellulose paper using constant current of 250mA for a period of 90 minutes in a Bio-Rad transblot apparatus, with blotting buffer (0.2M Glycine, 25mM Tris, and 20% Methanol). Non-specific binding to filters was blocked by incubation in 5% non-fat dried milk powder in tris buffer saline (TBS) (20mM Tris, 500mM NaCl, pH 7.4), at room temperature for 90 minutes or left overnight. The blot was washed briefly with distilled water before washing twice with TBS containing 0.2% (v/v) Tween-80 and twice with TBS only for 5 minutes each.

The nitrocellulose was then immunoblotted with a 1:1000 dilution of the antisera with TBS containing 1% dried milk powder for 90 minutes at room temperature with vigorous shaking (Rotatest Shaker, Luckham, England). The blot was then washed as above and incubated for 90 minutes at room temperature with 1:1000 dilution of donkey anti-rabbit antibodies (horse radish peroxide, HRP) in TBS buffer containing 1% non-fat dried milk powder with gentle shake.

After final washing with TBS, the protein bands of vitellogenin were detected by incubating the blot in 30% (w/v) of 3,3 diaminobenzidine (Sigma Chemical Co.) in PBS pH 7.2 with 0.01% (v/v) H₂O₂ for a time period of 5-10 minutes until bands

appeared. Lastly the blot was washed with distilled water several times and scanned to keep the record or was stored in dark to avoid the bleaching of visible bands, until scanned. The experiment was repeated for various samples of haemolymph, fat body and ovary of both control and azadirachtin-treated ones. The antigen was used as a positive control. For every experiment protein estimation was done by Bradford (1976), described in Chapter 2.

4.2.4. *In vitro* experiments

In order to find out the direct effect of azadirachtin on vitellogenesis, an *in vitro* experiment with the fat body of female adult locust was carried out.

4.2.4.1. Incubation medium

The tissue culture medium (GIBCOBRL, Life Technologies) described in Chapter 3 was used as the incubation medium in all the *in vitro* experiments.

4.2.4.2. Chemicals

4.2.4.2.1. Azadirachtin

Azadirachtin stored dry at -20°C and prepared for use as described in Chapter 2, was used in these experiments.

4.2.4.2.2. Methoprene

Methoprene was a gift from Novartis (Novartis, Produkta AG animal health Sector AH4 CH-4002 Basel Switzerland.).

4.2.4.3. Fat body isolations

A group of 20 female adult mature locusts precooled at -20°C , and each one was surface sterilized by 70% ethanol before dissection. Fat bodies dissected from 20 locusts were pooled in a 35x10mm plastic tissue culture dish under sterile conditions in

a, "Microflow Laminar Hood" rinsed and washed by sterilized insect saline, to remove any traces of haemolymph. The pooled fat bodies were divided approximately equally into 8 wells of a sixteen well cell culture cluster dish (Coaster Corporation Cambridge MA, U.S.A).

4.2.4.4. Incubation of fat body

The *in vitro* incubations of fat bodies were done according to the plan indicated in Table 4.2. The experiment was repeated four times.

The tissue culture plate was covered with parafilm and incubated at 30^oC with vigorous shaking, (Eberbach shaker, 200rpm) for a period of 24 hours (Verno *et al.*, 1984), at which time the samples were transferred to individual sterile 1.5ml polypropylene centrifuge tubes to prepare the tissue for assay of vitellogenin by ELISA.

4.2.4.5. ELISA estimation of vitellogenin in fat body material of female locust incubated *in vitro*

The fat body samples from each well and for each treatment were homogenized by a teflon homogeniser, sonicated for 30sec by an ultrasonic microprobe, and then centrifuged for 6min at 10,000g. The samples from the middle layer were used for ELISA as described in 4.2.3.8.2. The protein estimation was done by Bradford (1976), for samples from each well and each treatment separately before assayed for vitellogenin by ELISA.

4.3. Results

The preliminary investigation using SDS-PAGE of haemolymph, fat body and ovarian samples of female adult locust, S. gregaria (age 4-21 day), was aimed to detect the vitellogenin and vitellin. Due to the complexity of protein mixtures in the tissues, it was not possible to identify the proteins in question, so attempts were made to isolate

Table 4.2. The effect of azadirachtin on the *in vitro* fat body vitellogenesis.

No of well	Medium (1ml)	Fat body	Ampicillin (100 μ g)	AZA (50 μ g)	Methoprene (60 μ g)
1	+	+	+	-	-
2	+	+	+	-	-
3	+	+	+	+	-
4	+	+	+	+	-
5	+	+	+	-	+
6	+	+	+	-	+
7	+	+	+	+	+
8	+	+	+	+	+

AZA: Azadirachtin

and characterise the vitellin for further analytical studies to identify vitellogenin or vitellin of *S. gregaria*.

4.3.1. Vitellin purification

With little modification, the technique previously used successfully for the isolation of vitellin of *Locusta migratoria* by Chinzei *et al.* (1981) was applied to isolate and purify the vitellin of the desert locust, *S. gregaria*. The purity of the vitellin was confirmed by the presence of only a single peak in the eluted fractions from DEAE cellulose column, as shown in materials and methods section. The isolated fractions were analysed by PAGE to a further confirmation of purity.

4.3.1.1. Polyacrylamide gel electrophoresis of purified vitellin

The individual fractions (17-24) of the main peak eluted by DEAE cellulose column were analysed separately on polyacrylamide gel electrophoresis under denaturing conditions in 12% resolving gel. The protein profile was exactly same for all the eight fractions analysed, Fig 4.5 (a). The molecular weights of the six peptides were estimated to be 110, 97, 82, 68, 60 and 50 kDa respectively. The 68, 60 and 50kDa subunits occurred as weak bands. As described in materials and methods, the individual fractions were pooled, freeze-dried and then analysed by native and reducing polyacrylamide-gel electrophoresis. There was a single band in native gel, indicating the purified vitellin Fig 4.5 (b). The protein ran only a short distance in the 8% gel confirming its high Mr, i.e $> 5 \times 10^5$.

4.3.2. SDS-PAGE for vitellin or vitellogenin in various tissues of female adult locusts of different age groups

4.3.2.1. SDS-PAGE for ovarian vitellin

Fig. 4.6 shows the appearance of vitellin-like protein in the ovarian sample of 7-28 days old female locusts, analysed by SDS-PAGE. The results suggest that the protein concentration is its peak in the ovarian tissue of 21 days old after imaginal moult, and

Fig. 4.5. Polyacrylamide gel electrophoresis of main peak fractions eluted by DEAE cellulose column.

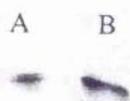
(a) SDS-PAGE of the eight fractions of main peak of vitellin purification as described in text. A-H: peak fractions and I: Mr standards. The gel was 12% resolution with 5% stacking.

(b) PAGE analysis of purified and concentrated vitellin of *S. gregaria* under non-denaturing conditions. A & B: vitellin.

The resolving gel was 8% with 3% stacking, and was aged overnight before run.



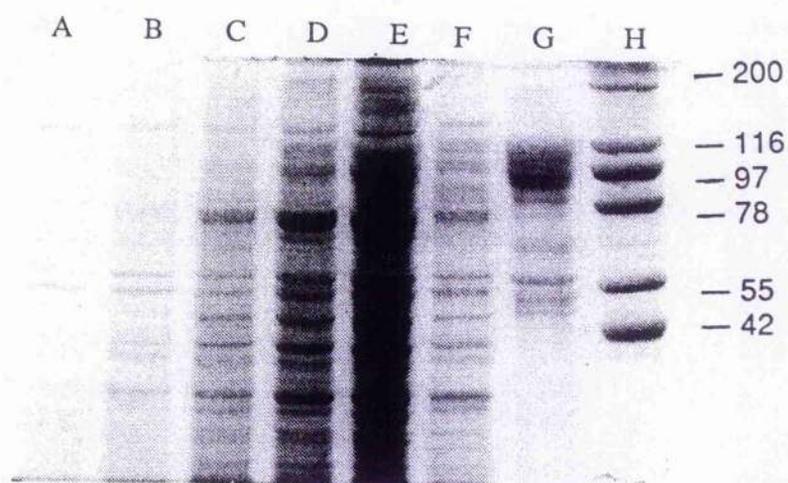
(a)



(b)

Fig. 4.6 SDS-PAGE of ovary of adult locust *S.gregaria*.

The ovarian samples from different aged 7-28 day, insects were collected and analysed to identify the corresponding vitellin protein sub-units, using pure vitellin as a standard. Lane A: 7, B: 10, C: 14, D: 17, E: 21 & F: 28 days old insects respectively, G: vitellin & H: standards. The gel was 12% resolution with 5% stacking, in a discontinuous system.



the 97kDa protein is present in the samples of 14 & 21 days old insects while the 60kD band is common in all samples. But a clear indication of peptides with corresponding mobilities to isolated vitellin subunits is difficult due to the number of protein bands.

4.3.2.2. SDS-PAGE of fat body for vitellogenin

The protein profile of fat body of 7, 10, 14, 17, 21, & 28 days old female adult locusts by 12% SDS-PAGES indicates the presence of female-specific proteins, (Fig. 4.7, lanes B-G). The proteins bands are likely to be subunits of vitellogenin, the precursor of vitellin. The result shows that in this insect by simple SDS-PAGE there seems no similarity in vitellin and its precursor vitellogenin, synthesised by fat body. These proteins are specific for female insects because these are absent in the fat body samples of 14 days old male adults analysed in the same gel, lanes A & J.

4.3.2.3. SDS-PAGE of haemolymph for vitellogenin

The polyacrylamide gel electrophoresis of haemolymph of mature female locust aged 7, 10, 14, 17 & 21 days shows the presence of circulating vitellogenin which is completely absent in the haemolymph of 10 and 14 days old male adult locusts, lane D and I, Fig 4.8. It is clear from this analysis that in mature locusts, the total haemolymph protein concentration is at its peak at the age of 17 days. But it is not easy to conclude the occurrence of any protein subunit with same mobility in haemolymph samples and pure vitellin used as a positive control.

4.3.2.4. SDS-PAGE for vitellogenin or vitellin in fat body and ovarian samples of locusts

Fig 4.9 represents the presence of some common protein bands of vitellogenin or vitellin in the fat body and ovarian samples of locusts from different age groups as explained in caption when analysed on PAGE under denaturing conditions. The protein band of 60kDa seems to be common in all the samples of fat body and ovarian tissue.

Fig 4.7. SDS-PAGE of fat body of adult locust *S. gregaria*.

The fat body samples of insects from different aged 7-28 day. Tissues were analysed to identify the vitellogenin sub-units, using male fat body samples as a negative control, and pure vitellin as a positive control. Fat body samples from lane B: 7, C: 10, D: 14, E: 17, F: 21 & G: 28 days old insects respectively, H: vitellin, A & J: fat body samples from 10 and 14 days old male locusts respectively and I: standards. The gel was 12% resolution with 5% stacking, in a discontinuous system.

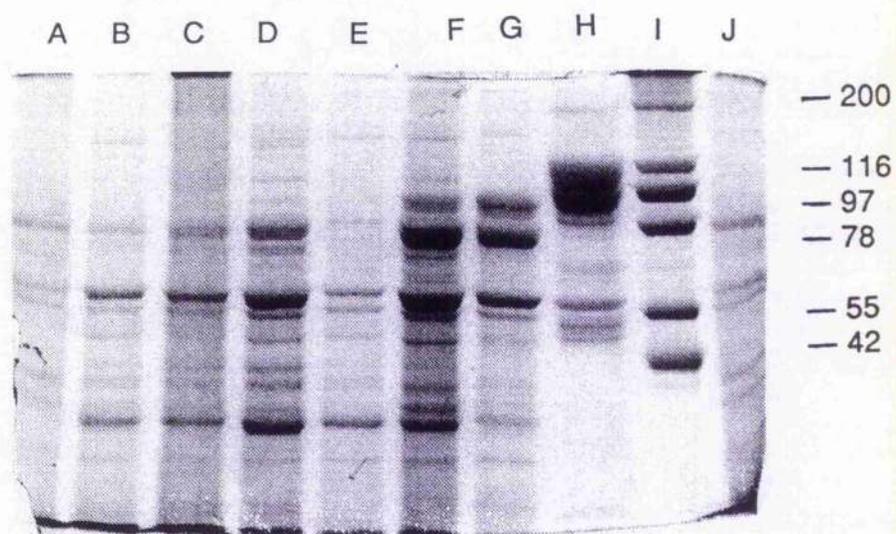


Fig 4.8. SDS-PAGE of haemolymph of adult locust *S. gregaria*.

The haemolymph samples from 7-21 days old locusts were collected and analysed to identify the vitellogenin sub-units, using male haemolymph samples as a negative control. Lane A: 7, B: 10, C: 14, D: 10 days old male haemolymph, E: 17 & F: 21 days old insect haemolymph respectively, G: vitellin, H: standards and I: haemolymph from 10 days old male locust. The gel was 12% resolution with 5% stacking, in a discontinuous system.

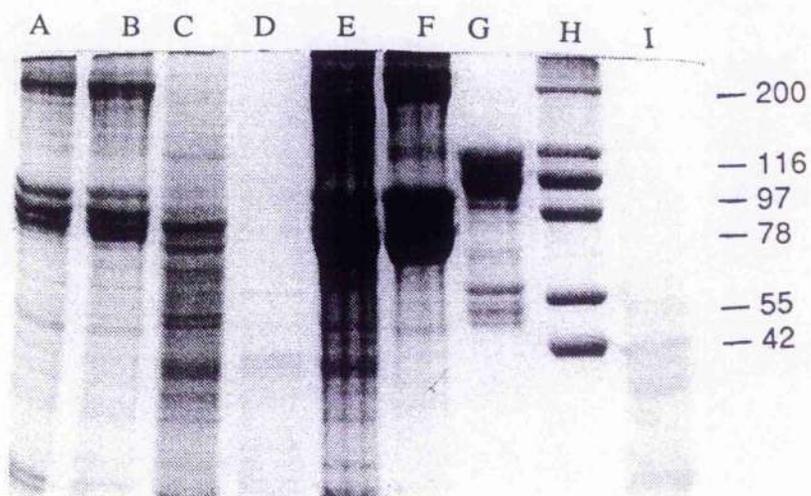
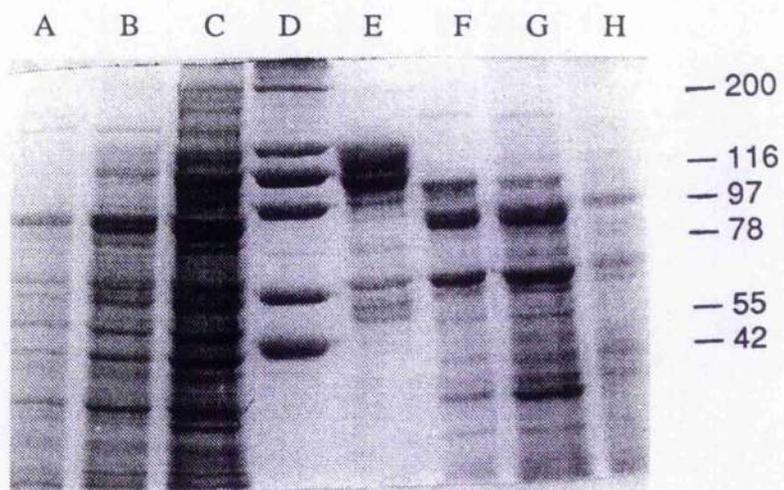


Fig 4.9. SDS-PAGE of fat body and ovarian samples of adult locust *S. gregaria*.

The fat body and ovarian samples of locusts from different age groups were analysed to identify the common vitellogenin and vitellin sub-units, using pure vitellin as a standard. Lane A, B and C: 10, 14 and 21 days old ovarian samples respectively. D: standards E: pure vitellin, and lanes F, G and H: fat body samples of 28, 21 and 10 days old locusts respectively.

The gel was 12% resolution with 5% stacking, in a discontinuous system.



4.3.3. Immunological techniques

The results of SDS-PAGE for all the tissue samples of female adult locusts were too ambiguous and not clear enough to identify the exact peptides of vitellin or vitellogenin in real samples. So the pure vitellin was used to raise antibodies to use in subsequent experiments to study the effect of azadirachtin on vitellogenesis by immunological methods.

4.3.3.1 Ouchterlony immunodiffusion

Ouchterlony double diffusion tests were carried out with male and female haemolymph fat body and ovarian proteins against antisera from rabbit immunized against pure vitellin. They demonstrate that purified vitellin crossreacts with female haemolymph and fat body, but not with the male haemolymph and fat body. The absence of any precipitation line for male samples supports the assumption that these proteins are strictly female-specific in *S. gregaria* (Fig 4.10).

4.3.3.2. Quantification by ELISA

In initial experiments, presence of anti-vitellin antibodies in the antisera raised was detected by ELISA. Various dilutions of the antisera from two rabbits (a and b), with different amounts of antigen were used to optimise conditions. The peroxidase activity of antisera was compared to that of pre-bled sera for the confirmation of raised antibody. There was an increase in the Absorbance in the ELISA tests between pre-injection sera and the antiserum raised.

The absorbance for the antisera of two rabbits was also compared to select the antiserum with the highest titre of raised antibody. Fig 4.11 shows the differences in the values of absorbance obtained with the antisera of the two rabbits by ELISA. This experiment was repeated four times and finally the antiserum of rabbit b was chosen for further experiments as it gives maximum absorbance. The optimal concentrations

Fig 4.10. Ouchterlony immunodiffusion of vitellogenin and vitellin.

Centre well contained anti-vitellin antibodies and well 1 had pure antigen as a standard. The precipitation line is showing identity of haemolymph and fat body vitellogenin and ovarian vitellin from mature female locusts (wells 2, 3 & 4). There is no line of precipitation for haemolymph and fat body samples of mature male insects (wells 5 & 6).

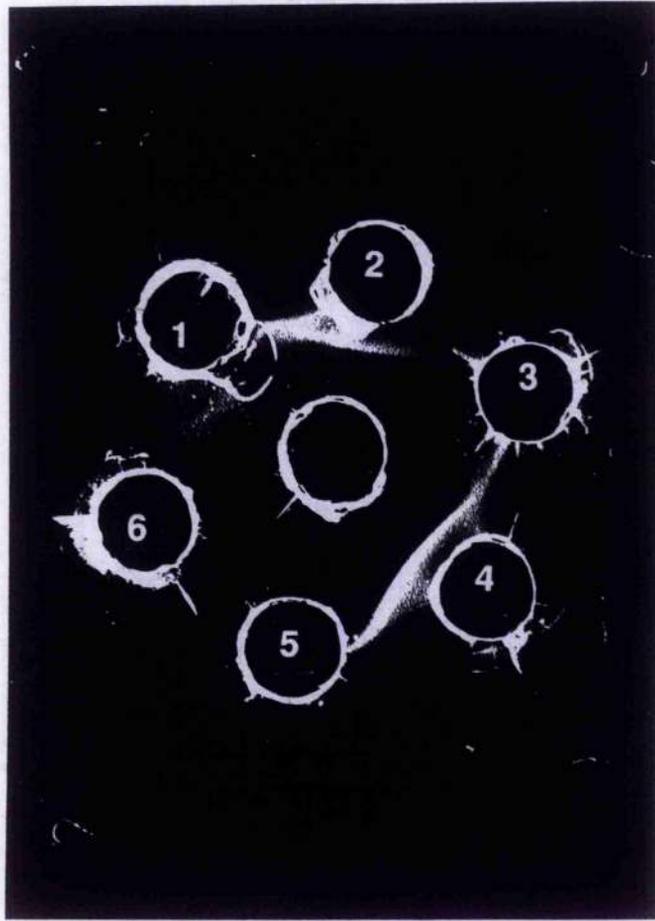
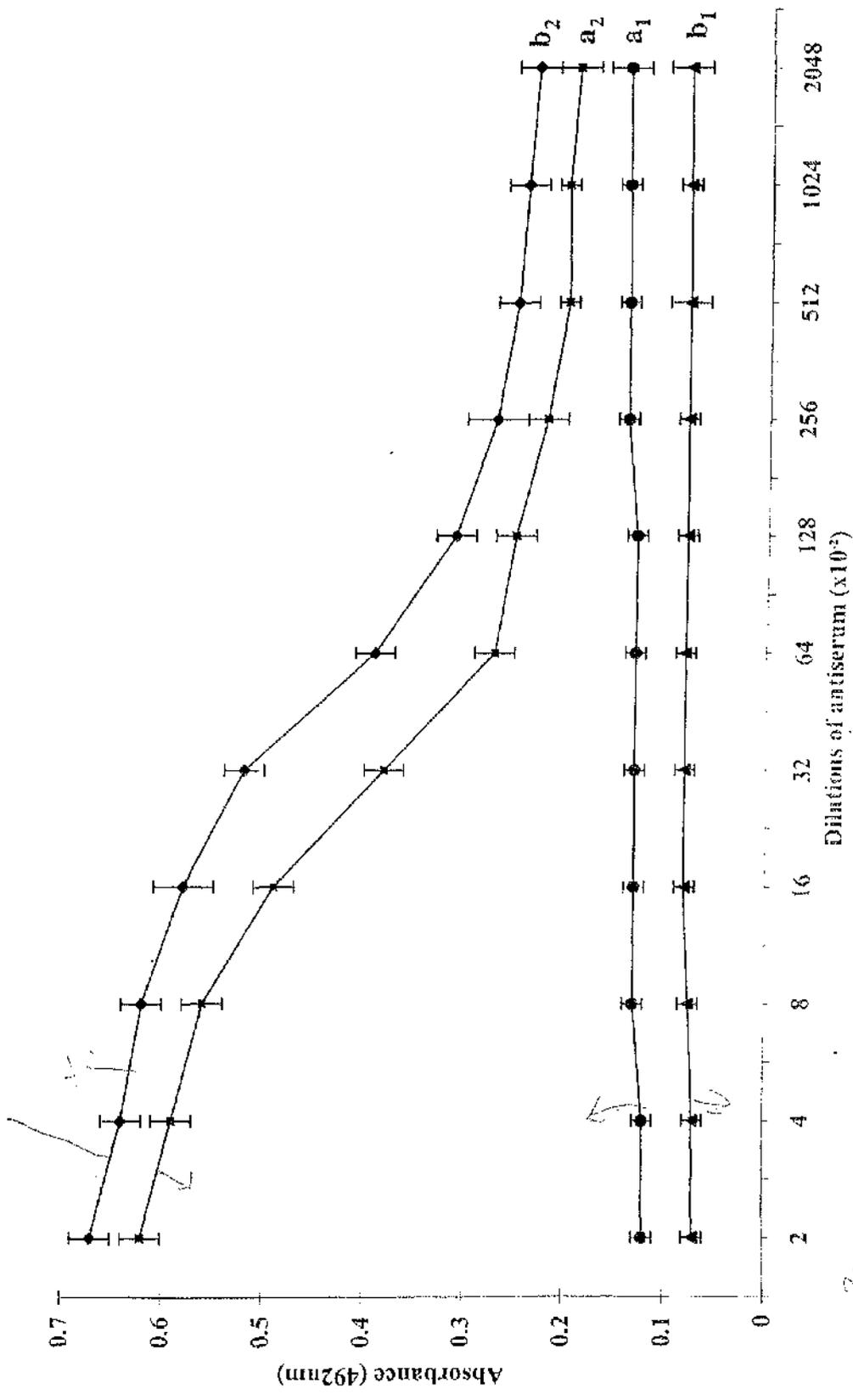


Fig 4.11. Characterisation of antisera to vitellin by ELISA.

This figure shows the difference in the absorbance produced by different dilutions of the antisera of rabbits a and b. a_1 and b_1 represent the absorbance for pre-bled sera while a_2 and b_2 is for post-bled sera. Each well contains $1\mu\text{g}$ of vitellin antigen.

Experiment was repeated four times. Each point is an average of 4 observations and vertical bar indicates $\pm\text{SD}$.



2
3/5

4.3.3.2.1. Generation of standard titre curve using increasing amounts of antigen subjected to ELISA

Fig 4.12 indicates the results of routine ELISA with increasing amounts of vitellin (0.1-1 μ g), used to generate the standard curve. The anti-vitellin antibody was used at 1:1000 (v/v) dilution. Background values have been subtracted.

4.3.3.2.2. Vitellogenin estimation in haemolymph of female adult locusts from different age groups by ELISA

The level of vitellogenin in the haemolymph over a period of one month of maturity was estimated by ELISA. The main aim of this experiment was to find out the age of insects when haemolymph vitellogenin is at its maximum level. The vitellogenin level was then compared to the total amount of protein concentration for each age group. Fig 4.13 represents the vitellogenin level of haemolymph of 7, 10, 14, 17, 21 and 28 days old insects compared to that of the total protein concentration of haemolymph from the same age groups respectively.

Vitellogenin represented 33-70% of the total protein concentration in the haemolymph of female adult insects over the period of 7-28 days. It was observed that the vitellogenin content increases along with the total protein concentration of the haemolymph as locusts mature. Vitellogenin content is 21 μ g/ μ l of haemolymph when locusts are 17 days old and then rose sharply to 71 μ g/ μ l in locusts at the 21 day, and decreased to 38 μ g/ μ l of haemolymph at 28 days.

4.3.3.2.3. Vitellogenin estimation in fat body of female adult locusts by ELISA

The result of estimation of vitellogenin of fat body from different age groups by ELISA is shown in Table 4.3. In 4 and 7 days old insects it was not detectable but as the insects grow and mature, the percentage of vitellogenin increased gradually.

Fig 4.12. Generation of standard titre curve of vitellin by ELISA.

Typical standard curve results generated using increasing amounts of *S. gregaria* vitellin, subjected to ELISA with 1:1000 (v/v) dilution of antiserum. Antigen was 0.1-1 μ g.

Each point is an average of 6 values and vertical bar represents \pm SD. The experiment was repeated four times.

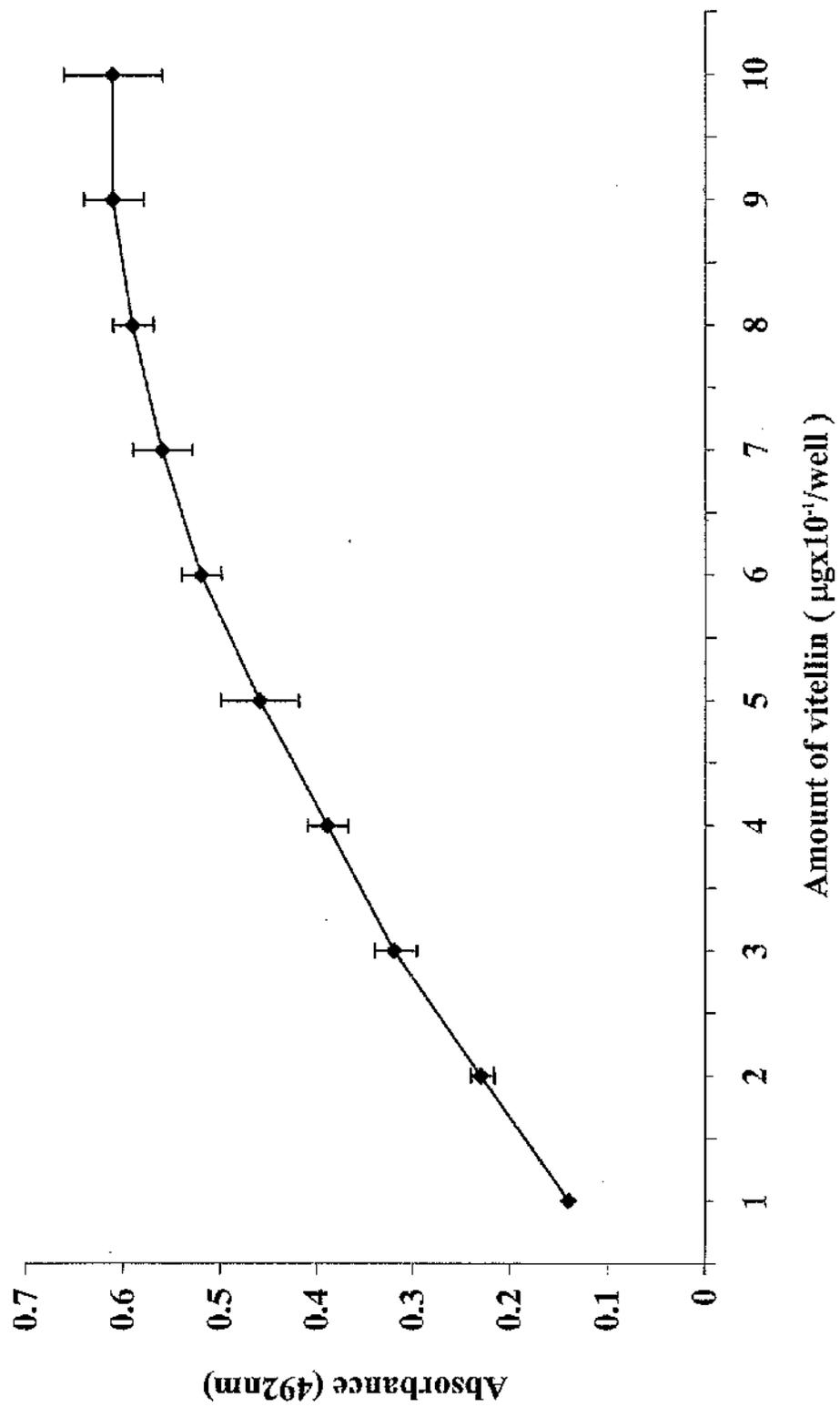


Fig 4.13. ELISA estimation of vitellogenin in haemolymph of female adult locust.

The total protein concentration was estimated by Bradford (1976) for haemolymph of each age group 7-28 days old, and vitellogenin in haemolymph was detected by ELISA. Each estimate was an average of 4 values and vertical bar is for \pm SD.

In each case:

□: total haemolymph protein concentration, and ▒: vitellogenin.

The age of the insects were as:

A: 7 days, B: 10 days, C: 14 days, D: 17 days, E: 21 days and F: 28 days.

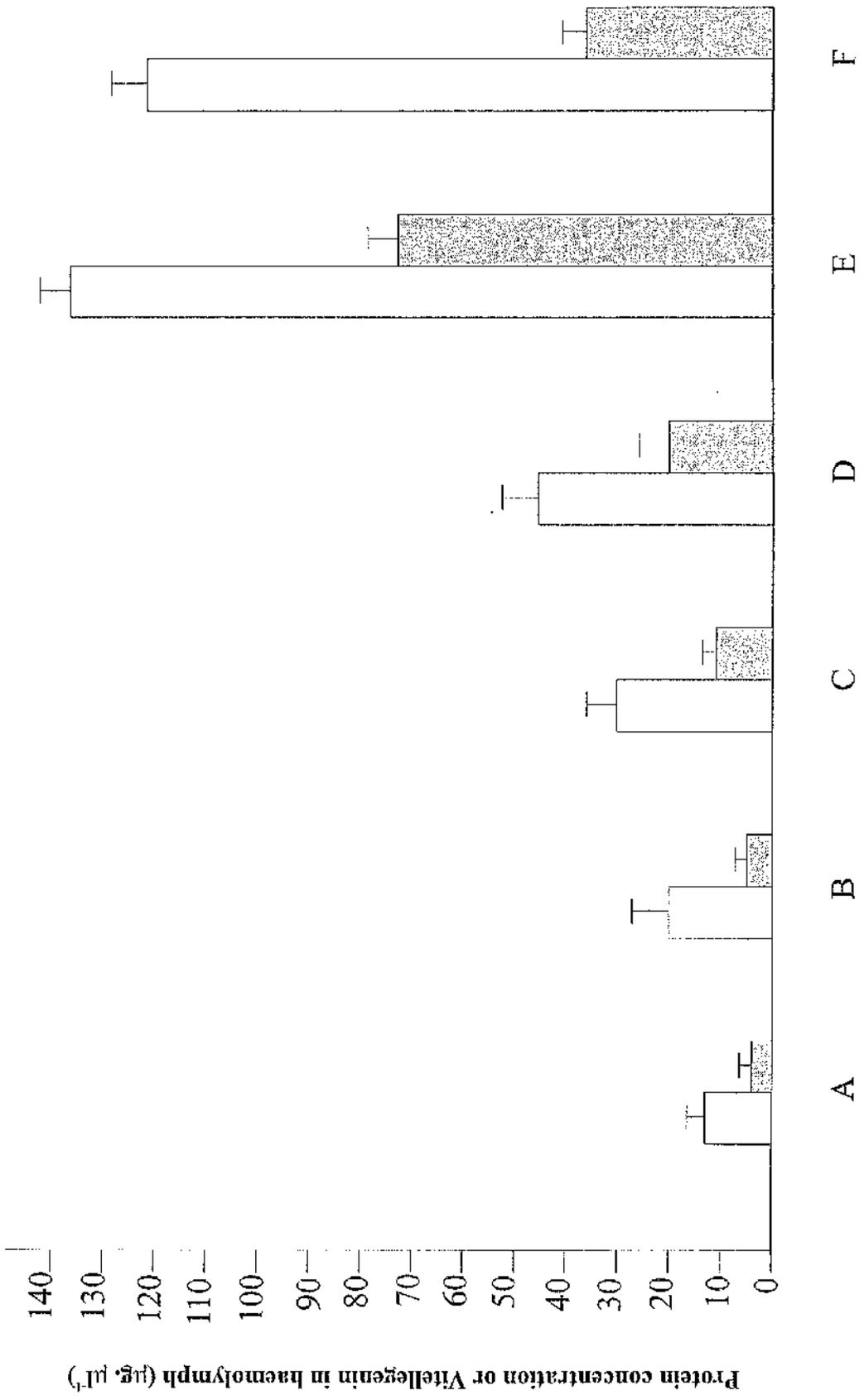


Table 4.3. Vitellogenin in the fat body of female adult locusts of different age groups

Age of locust (days)	Vitellogenin % of total protein
4	-
7	-
10	11±2
14	24±1
17	34±3
21	39±3
28	55±2

Each value is a mean of four observations ±SD.

Locust age is in days after final moult.

4.3.3.2.4 Vitellin estimation in ovaries of female adult locusts by ELISA

The proportion of total ovarian proteins due to vitellin varied with age. In 4 and 7 day old insects, it was low (corresponding with pre-vitellogenic stage), but as insects grow and mature, the proportion of vitellin increased until it made up almost all the ovary protein by 28 days (Table 4.4). The percentage of vitellin was calculated on the basis of 1µg of total protein concentration by using the standard curve (Fig 4.12).

4.3.3.3. Effect of azadirachtin on vitellogenin and vitellin

The results of above experiments of ELISA were used to study the effect of azadirachtin on the level of vitellogenin or vitellin in specific tissues of the desert locust *S. gregaria*.

4.3.3.3.1. Effect of azadirachtin on locust haemolymph vitellogenin

The effect of azadirachtin on the level of haemolymph vitellogenin of female locusts of two age groups was monitored by ELISA, in two different experiments over a period of 48 hours

Fig 4.14 indicates a comparison of the levels of vitellogenin in both controls and azadirachtin-treated insects for the first experiment, where the locusts were 15 days old. At this age vitellogenin made up 55% of the total haemolymph proteins. The total protein concentration has been also plotted parallel to the vitellogenin to show the proportion of vitellogenin. There was a decrease of 68% (statistically significant at $p < 0.01$) in vitellogenin of haemolymph of locusts after 48 hours of azadirachtin treatment against controls.

Similarly azadirachtin treatment in locusts caused 45% reduction in the total protein concentration compared to controls.

In a second experiment the selected insects were 19 days old at which time vitellogenin made up 63% of the total haemolymph protein. After 48 hours of azadirachtin treatment, vitellogenin was reduced to 40% of total protein and the total protein concentration itself fall to half control values i.e 65µg/µl (Fig 4.15).

Table 4.4. Vitellin in the ovarian samples of locusts from different age groups

Age of locust (days)	Vitellin % of total protein
4	20±2
7	32±3
10	42±4
14	50±3
17	55±2
21	78±2
28	89±4

Each value is a mean of four observations ±SD.

Fig 4.14. Effect of azadirachtin on vitellogenin content of haemolymph of 15 day-old locusts.

The haemolymph samples were collected and assayed after azadirachtin treatment over a period of 48 hours. The total protein concentration was estimated by Bradford (1976).

In each case

□; total haemolymph protein concentration, and ▨; vitellogenin,

a: control and b : azadirachtin treated.

Locusts were 15 days old at the start of the experiment and kept under standard rearing conditions, described in Chapter 2

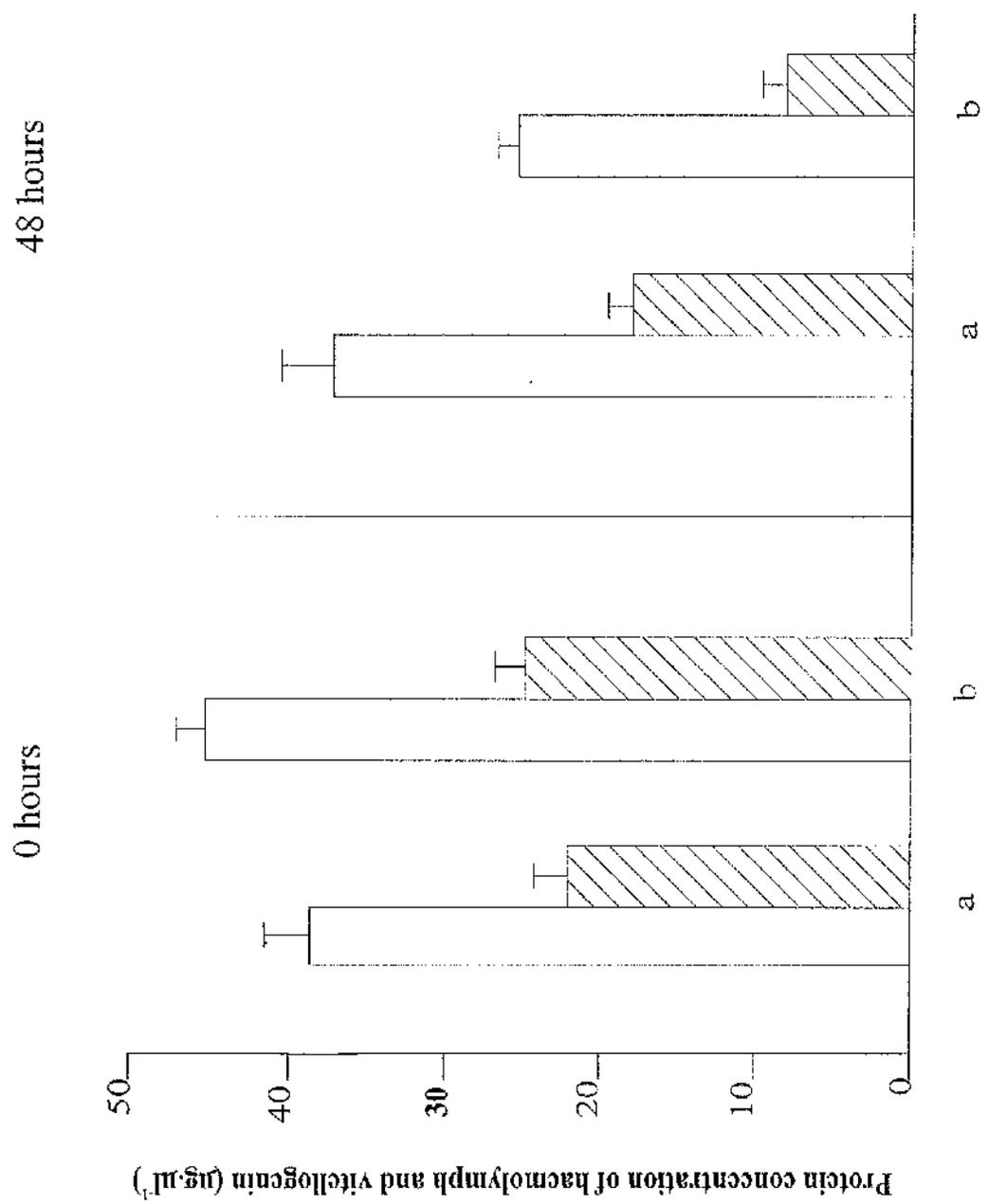


Fig 4.15. Effect of azadirachtin on vitellogenin level of haemolymph of 19 days old locusts assayed by ELISA.

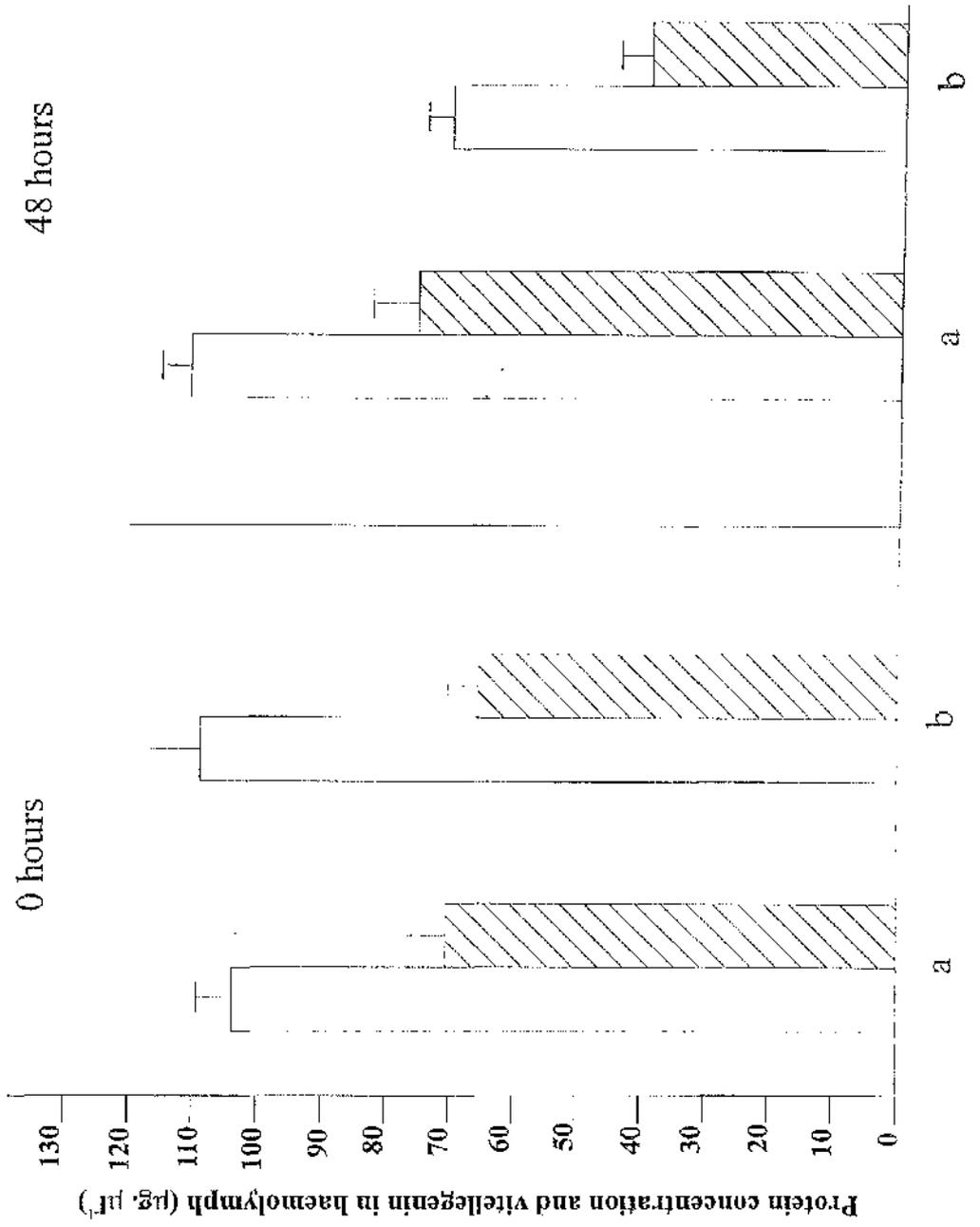
The treated haemolymph samples were collected and assayed for azadirachtin treatment after 48 hours. The total protein concentration was estimated by Bradford (1976).

In each case

□: total haemolymph protein concentration, and ▨: vitellogenin,

a: control and b: azadirachtin treated.

Locusts were 19 days old at the start of experiment and kept under standard rearing conditions described in Chapter 2



4.3.3.3.2. Effect of azadirachtin on vitellogenin level of female adult locust fat body

The effect of azadirachtin on vitellogenin in fat body of the female adult locusts was examined in 15 and 19 day old insects for a period of 48 hours. Table 4.5 represents the percentage of vitellogenin estimated by ELISA after 48 hours of azadirachtin treatment. The results are based on the observations of two different experiments. The azadirachtin-treatment of locusts at the age of 19 days caused a considerable reduction in vitellogenesis compared to the one in 15 days old insects. In both experiments, the fat body vitellogenin level of azadirachtin treated insects was reduced significantly ($p < 0.01$).

4.3.3.3.3. Effect of azadirachtin on locust vitellin of ovarian samples

The percentage of vitellin in the developing oocytes of azadirachtin treated locusts was estimated by ELISA after 48 hours of treatment (Table 4.6). It is clear that the percentage of vitellin has been reduced tremendously (by 73%), after 48 hours of azadirachtin treatment in 19 day old locusts, although the reduction at both 15 & 19 day old insects was statistically significant, ($p < 0.05$).

4.3.3.4. Western blotting the effects of azadirachtin on vitellogenin in haemolymph and fat body and ovarian vitellin

Although vitellin of the desert locust was used as an antigen to raise antiserum, its precursor vitellogenin showed cross-reactivity to antiserum as well. Western blotting after SDS-PAGE was used to specifically detect the precise effect of azadirachtin on the subunits of this high Mr protein and its precursor vitellogenin. As shown in Fig 4.16 (b), only four subunits of 110, 97, 82 and 50 kDa are visible in pure vitellin and the common subunit with same mobility is of 97 Mr in haemolymph, fat body and ovary of control locusts. In azadirachtin-treated tissues there are no visible subunits of

Table 4.5. Effect of azadirachtin on vitellogenin of fat body of female locust *S. gregaria* after 48 hours of treatment.

Age of locust (days)	Vitellogenin % of total protein concentration		
	Controls	Treated	Reduction (%)
15	49±5	35±4	30
19	26±2	11±2	62

Table 4.6. Effect of azadirachtin on ovarian vitellin of female locust *S. gregaria* after 48 hours of treatment.

Age of locust (days)	Vitellogenin % of total protein concentration		
	Controls	Treated	Reduction (%)
15	53±3	28±2	48
19	62±4	17±2	73

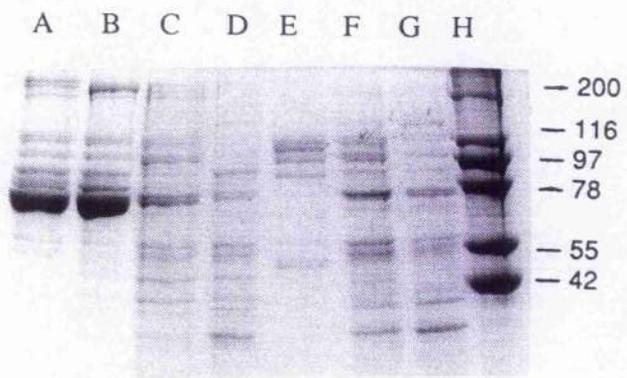
Fig 4.16. Immunodetection of the effect of azadirachtin on vitellogenesis in *S. gregaria*, as revealed by SDS-PAGE blotting to nitrocellulose membrane, and reaction with anti-vitellin antisera.

15 days old female locusts were treated with azadirachtin (5µg/insect). Tissue samples were collected after 48 hours of azadirachtin treatment and analysed by SDS-PAGE following the probing with rabbit polyclonal anti-vitellin serum.

(a) SDS-PAGE of samples from azadirachtin treated locusts and controls injected 5% ethanol (v/v) alone. Lane A: haemolymph from control, B: haemolymph from azadirachtin treated locust, C: fat body sample from control, D: fat body from azadirachtin treated insect E: pure vitellin, F: ovarian sample of control, G: azadirachtin treated ovarian sample and H: standards

For each lane samples from 4 or 5 insects were pooled.

(b) Following electrophoresis, proteins were blotted to nitrocellulose and vitellogenin or vitellin was immunolocalized by probing with rabbit polyclonal anti-vitellin serum (1:1000 dilution). The antigen-antibody complex was visualized with donkey anti-rabbit horse-radish peroxidase (1:500 dilution).



(a)



(b)

vitellogenin in fat body, while in ovarian vitellin only one subunit is visible and there is no difference in control and azadirachtin-treated locust haemolymph vitellogenin. The fat body electrophoretic analysis under denaturing conditions is a clear indication of the effect of azadirachtin on fat body vitellogenesis after 48 hours of treatment in 15 days old locusts Fig 4.16 (a). Gels (b) was a duplicate of (a), and used to transfer proteins to nitrocellulose for Western blotting.

4.3.4. *In vitro* experiments

In vitro analysis of vitellogenin synthesis by fat body can give insights into the possible mode of action of azadirachtin on mature females of *S. gregaria*. The vitellogenin synthesis by fat body of 19 days old insects was studied by incubation of isolated tissues in the presence of azadirachtin for a period of 24 hours. Table 4.7 shows the effect of azadirachtin on the level of vitellogenin synthesis in this *in vitro* study. The vitellogenin level of fat body incubated in the presence of azadirachtin was reduced to 33% ($p < 0.05$), compared to control. As described in materials and methods the juvenile hormone analogue, methoprene was used to study hormonal regulation of vitellogenesis and effect of azadirachtin. The vitellogenin content was increased by 40% in the presence of methoprene alone. The effect of azadirachtin was rescued by methoprene and the vitellogenin level rose back to normal level, Table 4.7.

4.4. Discussion

The present results clearly indicate that in *S. gregaria* azadirachtin inhibits vitellogenesis. Such an effect of azadirachtin has been reported in several insect species with some variations due to the differences in the experimental methods. Enzyme-linked immunosorbant assay showed reduced levels of vitellogenin in haemolymph and fat body and vitellin in ovarian samples, pointing to an inhibition of vitellogenesis caused by azadirachtin. The findings are in agreement with those

Table 4.7. The effect of azadirachtin on the *in vitro* fat body vitellogenesis of *S. gregaria* after 24 hours of incubation.

Type of treatment	Vitellogenin % of total protein
Control	39±1
Azadirachtin	26±2
Methoprene	65±2
Azadirachtin and methoprene	40±3

Each value is a mean of four observations ±SD.

reported by Sayah *et al.*, (1998) for the earwig *Labidura riparia*. In azadirachtin-injected females *L. riparia*, no vitellogenin was detected in fat body nor vitellin in ovaries compared with the same tissues of normal females at the same age. When *L. riparia* females were simultaneously treated with azadirachtin and methoprene, juvenile hormone analogue, both vitellogenin and vitellins were detected. In *L. riparia* azadirachtin also induced follicle degeneration.

In our study, the experiments to identify the vitellogenin and vitellin in locusts tissues using simple SDS-PAGE failed due to the presence of a complex protein profile, as shown in results. Although SDS-PAGE is a powerful analytical tool, it was not capable of distinguishing clearly these female-specific proteins in this insect. However, in a number of reports of other insects, vitellogenin or vitellin was determined by polyacrylamide gel electrophoresis under denaturing conditions. In the armyworm moth, *Pseudaletia unipuncta* (Cusson *et al.*, 1994), the earwig *Labidura riparia* (Sayah *et al.*, 1996), *Locusta migratoria* (Wyatt, 1988; Girardie *et al.*, 1996), *Blatella germanica* (Martin *et al.*, 1996) and in the gypsy moth *Lymantria dispar*, studies of vitellogenin demonstrated that vitellogenin was easily identified by simple polyacrylamide gel electrophoresis under reducing conditions.

Due to failure of SDS-PAGE, it became necessary to try some other technique like ELISA or Western blot for a quantitative study of the effects of azadirachtin on vitellogenesis in *S. gregaria*. For immunological studies there were possibilities to use pure vitellogenin or pure vitellin as an antigen to raise antibody. In the haemolymph of normal locusts, the level of vitellogenin is low at the start of adult life (Mahamat *et al.*, 1997), so vitellin was chosen to isolate and use as an antigen to raise antibodies for immunological studies of effects of azadirachtin on vitellogenesis. The advantages of purifying vitellin for our study were:

a) it constitutes a high percentage of oocyte proteins, (>70%),

- b) it is easily isolated due to insolubility in water and low salt solutions,
- c) the raised antibody also cross-reacts with the precursor, vitellogenin.

Since it is accepted that the vitellin contributes approximately 90% of the total soluble egg protein, it was assumed that the major protein isolated was in fact vitellin by a purifying scheme successfully used previously to isolate vitellin of *L. migratoria* (Chen *et al.*, 1981). This eluted single peak, when analysed by PAGE in native conditions gave only a single band. It has been found that in a number of holometabolous insects, these proteins when purified by the previously described methods were contaminated by lipoproteins (Chinzei *et al.*, 1981), which could interfere in the study of their chemistry and biology. So there was a need to adopt such a procedure which could yield pure vitellin free of contaminants. Much of the time was spent on a series of trial experiments to achieve this goal. The purified vitellin was used to raise the antibodies to study the effect of azadirachtin on the synthesis and uptake of our selected marker vitellogenin by immunological methods.

In insects, a multitude of approaches have been developed as direct and indirect indicators of vitellogenin levels. Vitellogenin-specific quantitative immunoassay is one of the techniques used to estimate the level of vitellogenin. Many investigators have employed the enzyme-linked immunosorbant assay (ELISA), as a sensitive rapid and convenient method to quantify vitellogenin (Voller *et al.*, 1979; Mahamat *et al.*, 1995).

The purified vitellin from mature oocytes has been shown to be homogeneous by Ouchterlony immunodiffusion and PAGE. The ouchterlony results showed that vitellogenin and vitellin appeared to be immunochemically identical. The lack of cross-reactivity with male haemolymph and fat body demonstrated the female-specificity of these proteins in *S. gregaria*.

In this study, as a logical preliminary investigation, the haemolymph vitellogenin was estimated by ELISA from 7-28 days old locusts. The haemolymph proteins have been shown to contain a number of polypeptides and these appear to change over the course of development of locusts. The decline of vitellogenin in haemolymph protein level suggests uptake of these proteins by some tissue i.e ovary. The vitellogenin content of haemolymph was in the range of 33-70%. However, the 14-20 days old insects indicate an intense synthesis of all proteins including vitellogenin, as revealed by SDS-PAGE and ELISA. The protein pattern is almost similar for all ages observed, however the levels of haemolymph vitellogenin vary, but this is at least in part a reflection of the fat body protein biosynthesis.

ELISA-estimated haemolymph vitellogenin percentage of *S. gregaria* was high compared to other insects, for example, 4% in *Periplaneta americana* (Bell, 1969), 5-6% in *Locusta maderae* (Englemann, 1978) and 18-19% in *B. germanica* (Martin *et al.*, 1995).

As it was intended to study the effect of azadirachtin on vitellogenesis, the fat body was actually the main target tissue in planning all the experiments reported in this Chapter. The vitellogenin content of fat body was in the range of 11-55% of total proteins. The low level of fat body vitellogenin compared to the ovary's vitellin (20-89%) indicates its immediate transport to haemolymph after synthesis. One of the effects of azadirachtin is to lower the fecundity of insects. It is thought likely that one possible cause of lower fecundity by azadirachtin is a lack of vitellogenin, by inhibiting the synthesis or its uptake by the developing oocytes. Usually, it is believed that vitellogenesis is controlled by juvenile hormone secreted by *corpus allatum*. The study was aimed to find out if the reduced fecundity by azadirachtin is linked to suppression of vitellogenin or the juvenile hormone in the mature females of the desert locust. The majority of investigations into the reduced fecundity of insects by

azadirachtin have been focused on females, but the effects have also been reported in males. In male azadirachtin-induced overaged nymphs of *Oncopeltus fasciatus*, maturation of the spermatozoa is comparable to controls of the same age suggesting that this process is largely independent of the presence of morphogenetic hormones (Dorn *et al.*, 1987). In fifth instars of azadirachtin-treated male *S. gregaria*, after 20 days of treatment cytological examinations of testes showed arrested spermatogenic meiosis at Metaphase I (Linton *et al.*, 1997).

Although, in a majority of adult insects, fat body is considered as a major source of yolk proteins, studies have shown that in some insects substantial quantities of these proteins are synthesised in ovaries as well (Zhai *et al.*, 1984; Isaac and Bownes, 1982; Chen *et al.*, 1987). The vitellogenin production begins during the last larval stadium in the gypsy moth, *Lymantria dispar* (Davis *et al.*, 1990), in the prepupal stage of the silkworm, *Hyalophora cecropia* (Pan, 1977), during early pupal development in *Bombyx mori* (Tsuchida *et al.*, 1987) and in late pharate adult stages of the indian meal moth, *Plodia interpunctella* and tobacco hawkmoth, *Manduca sexta* (Shirk *et al.*, 1990; Imboden and Law, 1983). In other species such as the Monarch butterfly *Danaus plexippus* (Pan and Wyatt, 1976), and the corn earworm, *Helicoverpa zea* (Satyanaryane *et al.*, 1992), vitellogenesis does not begin until after adult emergence.

The insect haemolymph vitellogenin is normally restricted to the reproductively competent females and this was also true for *S. gregaria*. The synthesis of vitellogenin by fat body cells has been assumed due to an ability to respond to its normal inducing factor which in most species is Juvenile Hormone. The haemolymph and fat body of male adult *S. gregaria* were analysed by Ouchterlony immunodiffusion, SDS-PAGE and ELISA to identify or estimate the vitellogenin but no corresponding protein was found in male tissues by any of the employed techniques. However, in *Haylophora*

cecropia, (Telfer, 1954), *Bombyx mori* (Lamy, 1979), *Drosophila melanogaster* (Bownes and Hames, 1977), and *Oncopeltus fasciatus* (Kelly and Telfer, 1977) male haemolymph contains traces of vitellogenin. The haemolymph of male *Rhodnius prolixus* (Mundall, 1976) and *Galleria mellonella* (Lamy, 1979) contain larger quantities of vitellogenin.

As far as hormonal regulation is concerned, in *S. gregaria*, *in vitro* incubation of fat bodies isolated from mature insects in the presence of Juvenile Hormone analogue methoprene supported the hormonal stimulation of vitellogenesis. It is believed that in adult insects, Juvenile Hormone is produced by *corpus allatum* and is necessary for the deposition of yolk in oocytes. This has been shown in *Rhodnius prolixus*, in grasshoppers, in *Dytiscus* and other beetles and in Diptera. It seems not to be necessary in some lepidoptera such as the silkmoths *Bombyx mori* and *Haylophora cecropia* which develop their eggs during the pupal stage. In orthoptera JH has another effect: it stimulates the fat body cells to synthesize the yolk protein precursor, vitellogenin and to liberate it into the haemolymph. In some insects, such as Diptera, ecdysteroids stimulate vitellogenesis after exposure to JH (Hedgedorne, 1985). Other researchers have shown that ecdysteroid inhibit vitellogenesis, in some cockroaches, beetles and flies (Englemann, 1970; Wright *et al.*, 1971; Stay *et al.*, 1980). Neurohormones from the brain may also be involved in regulating vitellogenesis directly (Girardie *et al.*, 1987). Recently, Girardie *et al.*, (1998), have characterized another hormone called Ovary Maturing Parsin (OMP) in locusts involved in vitellogenesis and ovarian development. One anomaly is that our results seem to show that vitellin was present in ovaries of adult locusts before the time when fat body started synthesising its own proteins. The recent findings of Girardie *et al.* (1998), can give a possible explanation of this as, they suggest a protein hormone OMP can have a direct effect on ovary to stimulate vitellogenin synthesis.

The *in vitro* analysis of the effect of azadirachtin on the synthesis of vitellogenin by fat body has given an insight into the mode of action of azadirachtin eliminating the other general hormonal effects of *in vivo* vitellogenesis. In the *in vitro* study, the vitellogenin level was reduced by azadirachtin significantly, but when fat bodies were incubated in the presence of azadirachtin and methoprene, the Juvenile Hormone analogue, vitellogenin level was same as to the controls. It was obvious from the results that JH analogue did rescue the effect of azadirachtin on vitellogenesis suggesting that azadirachtin has an inhibitory effect on the JH stimulated increase in fat body vitellogenesis *in vitro*. This agrees with earlier reported findings (Rembold *et al.*, 1987; Schluter *et al.*, 1984 Malczewske *et al.*, 1988; Paranagama, 1994). Nevertheless, after azadirachtin treatment, JH does not restore vitellogenesis in some insects (Mordue and Blackwell, 1993). Our findings are also in accordance with the one recently reported by Sayah *et al.*, (1998) for the *Labidura riparia*, where the effect of azadirachtin was rescuable by methoprene, and not linked to feeding inhibition. It has been reported that NeemAzal F (5% azadirachtin) reduced haemolymph vitellogenin and sex pheromone production in the Colorado potato beetle, *Leptinotarsa decemlineata* (Otto, 1997).

Polyacrylamide gel electrophoresis of fat body and ovarian proteins following Western blotting, of control and azadirachtin-treated insects demonstrated a significant reduction of vitellogenin and vitellin, while for haemolymph proteins, there was not any apparent difference in vitellogenin after azadirachtin treatment. By Western blot analysis, the female locusts treated with azadirachtin failed to show any of the subunits of vitellogenin in fat body or vitellin in ovaries, suggesting a significant effect on the synthesis of vitellogenin by fat body and its uptake from haemolymph by the developing follicles.

The possibility can not be excluded that vitellogenin might be processed during incorporation into oocytes. In *L. migratoria*, vitellogenin changes its subunit pattern during uptake into the ripening oocytes as there are no common corresponding bands in haemolymph and fat body samples and in isolated vitellin (Gellissen *et al.*, 1976). There are reports indicating similarities of vitellogenin and vitellin in some insects. For example in Gypsy moth, *Lymantria dispar*, there has been a sequence relatedness between subunits of vitellogenin and its counterparts in vitellin. Despite the fact that we were not able to identify corresponding vitellogenin and vitellin subunits in locust tissues by standard SDS-PAGE, Ouchterlony and the Western blot results suggest that much of the 3 dimensional structure of vitellogenin is retained during transformation into vitellin.

In locusts vitellogenesis is a heterosynthetic process and requires a number of steps. These includes: hormonally stimulated vitellogenin gene activity, vitellogenin secretion into the haemolymph, accumulation of vitellin in oocytes and packaging into yolk granules. The study of these steps offers a basis for exiting development in the field of insect physiology, biochemistry and cellular biology. These findings ultimately would help us to understand the effects of azadirachtin on vitellogenesis and ultimately the cause of reduced fecundity. In recent years, the effect of azadirachtin has been studied at cellular levels, but many questions have yet to be answered and require further investigation. The cytopathological studies of growing or developing cells suggest the presence of binding sites at membrane proteins (Nisbet *et al.*, 1996, 1997). During vitellogenesis, spaces are developed in the follicular epithelium to allow the passage of vitellogenin to the oocyte surface and then it binds to specific receptors on the oocyte surface. In the result of this binding, oolemma is produced into oocytotic vesicles. It might be possible to suggest that the azadirachtin is affecting on the specific receptors of vitellogenin and binding proteins are affected. However, in the light of reports of multiple effects of azadirachtin, it is very difficult to be certain that any

particular effect is due to the direct action of it and not to be a secondary one. There are possible direct effects of azadirachtin on tissues such as muscle, gut epithelia and cells undergoing mitosis (Nasiruddin and Mordue, 1994). The work presented here clearly demonstrates that physiological effective amount of azadirachtin administered into mature females of *S. gregaria* is capable of inducing inhibition of vitellogenesis and it could be the result of reduction of overall proteins including vitellogenin or of only some selective ones.

Chapter 5
Final discussion

Despite world-wide research into the insecticidal effects of azadirachtin over the past 30 years, the primary site of action of azadirachtin in insect tissues remains elusive. Unlike many natural toxins, such as pyrethrin, no single specific site of action is recognised. The results presented here, like those of many previous authors, indicate that one possible fundamental action of azadirachtin is to interfere with protein biosynthesis in various insect tissues. Reports on effects of azadirachtin on levels of individual proteins are few. The basic aim of the work reported here was to study the effect of azadirachtin on the synthesis of two individual proteins, lysozyme and vitellogenin/vitellin. Previous reports had suggested that the synthesis of lysozyme and vitellogenin was affected by azadirachtin in other insects (Azambuja *et al.*, 1991; UHL and Rembold, 1987; Sayah *et al.*, 1996; Otto, 1996). Another advantage was that our chosen proteins were easy to quantify. In insects it is believed that both of the proteins are largely produced by the fat-body, a tissue easy to isolate and to maintain for a reasonable length of time *in vitro*. It was hoped that both could be induced by simple single stimuli in a controlled fashion and in general, the results fulfilled most of the original hopes.

It was found that the response of the locust to bacterial antigens was a very simple one. The synthesis of bacteriolytic enzyme lysozyme appeared to be the only detectable antibacterial response of *S. gregaria.*, and that this response, by the increase of the circulating concentration of the enzyme, was rather weak compared to other insects. Moreover, it was not specifically dependent on the use of purified LPS, but appeared to be a general result of mechanical and chemical insult to the insects. Nor was it wholly clear if the increased humoral activity was as a result of synthesis in the fat body or the haemocytes. However it was clear, that injection of azadirachtin, in amounts known to produce clear physiological and biological results, sharply reduced the antibacterial response in both fat-body and haemocytes, and thus in the haemolymph. There seems

no doubt that this would lower the biological "fitness" of the insect, by reducing its capacity to withstand infection by gram-positive organisms.

The relative ease of purification of vitellin, the major protein of the developing ovary, and the fact that a polyclonal antiserum raised against vitellin cross-reacts to vitellogenin makes this an excellent protein for investigation by various immune methods. The problem with these proteins is that they are hetero-oligomers of very high Mr values which makes it difficult to use polyacrylamide gel electrophoresis. The results presented here by Ouchterlony immunodiffusion and Western blotting confirmed that only vitellin and its precursor vitellogenin reacted with the antiserum. Estimation of the two proteins, vitellogenin and vitellin by the ELISA method show that azadirachtin had a rapid effect on the amounts of vitellogenin in fat body, and vitellin in the ovaries. Induction of vitellogenin in the isolated fat body by methoprene, was also reduced to control levels by azadirachtin, in a clearly direct effect. There seems little doubt that this effect can account for the female sterility often observed as one of the effects of azadirachtin administration.

Does this get us any nearer to identify the actual cellular site of action of azadirachtin? The hope underlying the questions posed in this thesis, was that a clear stimulus could be found to induce a specific protein, which would then allow the study of, for instance, second-messenger systems. Although the results with methoprene and vitellogenin might suggest a useful model system, there is no proof that second-messenger systems are involved. One obvious question is whether or not the effect on these specific proteins is unique to them or much more general to a whole range of proteins. The results of electrophoresis presented here as well as those obtained by much more elaborate methods by Rembold and Annadurai (1993) suggest that the synthesis of the majority of proteins is reduced, and that it is only a few which are maintained or even increased in amount. This might suggest a mode of action similar

to, for instance, cycloheximide, which interferes with protein synthesis at the level of the ribosome, and not with the mechanisms of induction.

One obvious approach to solve the question of the primary site of action in the cell is to use binding studies with a radio-labelled derivative of azadirachtin. This work has only just started and biochemical studies, (Nisbet *et al.*, 1996, 1997) have revealed specific binding of [^3H] dihydroazadirachtin in two insect tissues; testis from mature *S. gregaria* and cultured Sf9 cells. Chemical characterisation suggested that the putative common binding site bears close similarities in both tissues and may be associated with cellular RNA (Nisbet *et al.*, 1995, 1996, 1997). Linton *et al.*, (1998) have reported arrested spermatogenic meiosis at Metaphase I, in a cytological examination of testes after 20 days of azadirachtin treatment of fifth instar of the desert locust. Mordue *et al* (1998), suggest that the competitive binding of different analogues of azadirachtin occurs in a similar order of potency to that found with antifeedant and IGR bioassays, an indication of a causal link between specific binding to membrane proteins and the ability of the molecule to exert biological effects.

A nuclear site for azadirachtin binding would be also consistent with observed effects on dividing insect cells. An incidental observation reported in this thesis relates to the blood cells. Insect haemocytes contribute to the synthesis antibacterial proteins and may divide mitotically when challenged by invading bacteria. Although the resting humoral lysozyme level of female adult locusts was low, there was a significant rise in the enzyme activity in response to bacterial lipopolysaccharide (LPS). The cellular contribution of azadirachtin-treated insects was reduced tremendously, supporting the possible direct effect of azadirachtin on cells involved in protein synthesis. Azambuja *et al.*, (1991) reported a similar finding of reduced immunity in *Rhodnius prolixus*, when treated with azadirachtin. Unfortunately no attempt was made to estimate the numbers of haemocytes in azadirachtin treated locusts in this work. In future it will be

reasonable to study the cell-cycle specific interference and evaluate the molecular target of azadirachtin.

Evidence is accumulating that there is a specific binding protein in the cells for azadirachtin, and that this in turn may be associated with the DNA or the RNA of the insect cell. It may thus inhibit polymerisation of DNA, RNA or protein. It is not yet clear where the main effect is exerted. Studies such as this reported here, support the idea that it is possible to study clear direct effects of azadirachtin on specific protein synthesis in isolated tissues, thus helping to establish protein synthesis as one of the fundamental if not necessarily the primary site of action of azadirachtin.

References

Abramovitz, J.N., (1997) Valuing Nature's Services. in: State of the World, Earthscan London. pp. 95-114.

Abu-Hakima R. and Wyatt G.R. (1979) Juvenile hormone controlled vitellogenin synthesis in *Locusta migratoria* fat body, *Devl. Biol.* 69, pp. 59-72.

Abu-Hakima, R. and Davey, K.G. (1977) The action of JH on follicle cells of *Rhodnius prolixus*: the importance of volume changes. *J. Exp. Biol.* 69 33-44.

Abu-Hakima, R. and Faye, I. (1981) An ultrastructural and autoradiographic study of the immune response in *Hyalophora cecropia* pupae. *Cell Tissue Res.* 217, pp. 311-320

Adams, T.S. and Gerst, J.W. (1993) Effect of diet on vitellogenin, vitellin and ecdysteroid levels during the second cycle of oogenesis in the house fly *Musca domestica*. *J. Insect. Physiol.* 39(10) pp. 835-843.

Ahmed, S., Bamofleh, S., Munshi, M. (1989) Cultivation of Neem in Saudi Arabia. *Econ. Bot.* 43, pp. 35-38.

Ahn, Y.J., Funaki, E. and Motoyama, N. (1991) Mechanism of resistance to Pyrethroids and DDT in a Japanese strain of the Housefly. in *Neurotox'91: Molecular Basis of Drug and Pesticide Action, An International Symposium on Pesticide Science*, pp.257-269. Elsevier Applied Science London (Duce, I. R. (ed)

Akhter, S., Pirzada, M., Siddiqui, M.H., Baloch, U.K. (1998) Effect of Neem cake on persistence of Diazinon and Endosulfan in Paddy soil. *Pesticide. Sci.* 52, pp.218-222.

Ali-niaze, M.T., Alhumeyri, A., Saeed, M. (1997) Laboratory and field evaluation of a neem insecticide against *Archips rosanus* L. (Lepidoptera: Tortricidae). *Can. Ent.* 129(1) pp. 27-33.

Anderson, I.M., Telfer, W.H. (1970) Trypan blue inhibition of yolk deposition- a clue to follicle cell function in the cecropia moth. *J. Embry. Exp. Morph.* 23 pp. 35-52.

- Anderson, R.S. and Cook M.L. (1979) Induction of lysozyme like activity in the haemolymph and haemocytes of an insect *Spodoptera eridania*. *J. Invert Pathol.* 33. pp. 197-203.
- Annadurai, R. S., Oberthur, W., and Rembold, H (1992) High resolution two dimensional mapping of tissue-specific polypeptides in the desert locust *Schistocerca gregaria*, *Electrophoresis* 13, pp. 456-458.
- Annadurai, R.S. and Rembold, H. (1993), Azadirachtin A Modulates the Tissue-Specific 2D Polypeptides Patterns of the Desert Locust, *Schistocerca gregaria*, *Naturwissenschaften*, 80, pp. 127-130.
- Ayyangar, G.S.G. and Rao, P.J. (1989) Azadirachtin effects on consumption and utilization of food and midgut enzymes of *Spodoptera litura*, *Indian J. Ent.* 51, pp. 373-376.
- Azambuja, P Garcia, E. S, Ratcliffe, N. A and Warthen Jr. J. D. (1991). Immune-depression in *Rhodnius prolixus* induced by the growth inhibitor Azadirachtin. *J. Insect Physiol.* 37, 771-777.
- Banerjee, S., and Rembold, H. (1992) Azadirachtin A interferes with control of serotonin pools in the neuroendocrine system of locusts. *Naturwissenschaften* 79 pp. 81-84.
- Basedow, T. and Peters, A. (1997) Control of Colorado potato beetle (*Leptinotarsa decemlineata* Say) by an azadirachtin-formulation (NeemAzal-T/S), by *Bacillus thuringiensis tenebrionis* (Novodor), and by combination of both: short-term and long-term effects. Proc. 5th Internat. Workshop "Practice Oriented Results on Use and Production of Neem ingredients and Pheromones" 1996 (Eds.: H. Kleeborg & C. P. W. Zebitz 1997), pp. 59-65.
- Benge, M.D.(1988) Cultivation and propagation of the neem tree. In: Focus on Phytochemical Pesticides, Vol. I, M. Jacobson (Ed). 1988 .
- Blaney, W. M. and Simmonds, M. S. J. (1995) Effects of azadirachtin on Feeding Behaviour of insects. In: Schmutterer, H. 1995 (Ed). *The Neem Tree Azadirachta indica* A.Juss. and other Meliaceous Plants. VCH Weinheim, New York, Cambridge,

Tokyo 1995. pp. 171-76.

Bohm, G. A., Richter, K., Kleeberg, K. (1996) Effects of NeemAzal-containing diet on the cockroach *Periplaneta americana* (L.). Proc. 5th Internat. Workshop "Practice Oriented Results on Use and Production of Neem ingredients and Pheromones" 1996 (Eds.: H. Kleeberg & C. P. W. Zebitz 1997), pp. 165-74.

Boman, H. G. and Hultmark. D. (1987) Cell-Free Immunity in insects. Ann. Rev. Microbiol 41, pp. 103-126.

Boucias, D.G., Hung, S. Y., Mazet, I. and Azbell. J, (1994) Effects of the Fungal Pathogen *Beauveria bassiana* on the Lysozyme Activity in *Spodoptera exigua* Larvae. J. Insect Physiol. 40(5), pp. 385-391

Bownes, M. (1986) Expression of the genes coding for vitellogenin (yolk protein) Annu. Rev. Entomol. 32 pp.507-530.

Bradford, M.M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analt. Biochem. 72 pp. 248-254.

Butterworth, J. H. and Morgan, E. D. (1971) Investigation of the locust feeding inhibition of the seeds of the neem tree *Azadirachta indica*. J. Insect Physiol. 17 pp. 969-977.

Butterworth, J.H. and Morgan, E.D. (1968) Isolation of a substance that suppresses feeding in locust, J. Chem. Soc. Chem. Comm., pp. 23-24.

Chapman, R. F. (1976) A Biology of Locust. Edward Arnold Ltd. London, pp. 4-6.

Chen, A.C., Kim, H.R.; Mayer, R.T. and Norman, J.O. (1987) Vitellogenin in the stablefly *Stomoxys calcitrans*, Comp. Biochem. Physiol., 88B, 897-903.

Chen, T. T., Couble, P., de Lucca, F. L. and Wyatt, G. R. (1976) Juvenile hormone control of vitellogenin synthesis in *Locusta migratoria*. In: The Juvenile Hormones , Eds: Gilbert, L.I., pp. 505-529, Plenum Press, New york.

Chinzei, Y. and Wyatt, G.R. (1985) Vitellogenin titre in haemolymph of *Locusta migratoria* in normal adults after ovariectomy, and in response to methoprene. *J. Insect. Physiol.* 31 pp. 441-445.

Chinzei, Y., Azumi, C., Miura, K., Matsuoka, H., Ando, K. (1994) Changes in haemolymph proteins and their synthetic activities during development and engorgement and after Juvenile hormone treatment in the kissing bug *Rhodnius prolixus*. *J. Insect. Physiol.* 40(6) pp. 491-499.

Chinzei, Y., White, B.N. and Wyatt, G.R. (1982) Vitellogenin mRNA in locust fat body; identification, isolation, and quantitative change induced by juvenile hormone, *Can. J. Biochem.* 60, pp. 243-251.

Clemants, A.N. (1959) Studies on the metabolism of locust fat body. *J. Exp. Biol.*, 36, pp. 665-675.

Cuenot, L. (1896) Etudes physiologiques sur les orthopteres. *Arch. Biol.* 14 pp. 293-341.

Davis, R.E., Kelley, T.J., Masler, E.P., Thyagaraja, B.S., Paramasivan, V., Fescemeyer, H.W., Bell, R.A. and Borkovec, A.B. (1990) Vitellogenesis in Gypsy moth *Lymantria dispar* (L.): Characterisation of haemolymph vitellogenin, ovarian vitellin, follicle growth and vitellin content. *Invert. Reprod. Dev.* 18 pp. 137-145.

De Verno, P.J., Chadwick J.S., Peter Aston W, and Dunphy Gray. B, (1984) The *in vitro* Generation of an antibacterial activity from fat body and haemolymph of non immunised larvae of *Galleria mellonella*. *Dev. Comp. Immunol.*, 8, pp. 537-546.

Dhadialla, T.S. and Wyatt, G.R. (1983) Juvenile hormone dependent vitellogenin synthesis in *Locusta migratoria* fat body: inducibility related to sex and stage. *Dev. Biol.* 96 436-444

Dinham, B. (1996) Getting off the Pesticide Treadmill. *Our planet* 8(4) pp. 27-28 United Nations Environmental Program, Kenya.

Dorn, A., Rademacher, J.M. and Sehn, E. (1986), Effects of azadirachtin on the moulting cycle, endocrine system and ovaries in last-instar larvae of the milkweed bug,

Oncopeltus fasciatus, J. Insect Physiol., 32, pp. 321-328.

Dorn, A., Ramecher, J.M. and Sehn, E. (1987) Effects of azadirachtin on reproductive organs and fertility in the large milkweed bug *Oncopeltus fasciatus*, Proc. Third Int. Neem Conf. H. Schmutterer and K.R.S. Ascher (Eds.) GTZ, Eschborn, Germany.

Dunn P. E., Dai, W., Kanost, M. R. and Geng, C. (1985) Soluble peptidoglycan fragments stimulate antibacterial protein synthesis by fat body from larvae of *Manduca sexta*. Develop and Comp Immun, 9 pp. 559-568.

Dunn, P.E. (1986) Biochemical aspects of insect immunology. A. Rev. Ent. 31 pp. 321-339.

Dunn, P.E. and Dai, W. (1990) Bacterial peptidoglycan: a signal for initiation of the bacterial-regulated synthesis and secretion for lysozyme in *Manduca sexta*. In Defence Molecules pp. 33-46 Liss. New york

Dunn, P.E. and Drake, D.R. (1983) Fate of bacteria injected into naïve and immunized larvae of the tobacco hornworm *Manduca sexta*. J. Invert. Pathol. 41 pp. 77-85.

Dunn, P.E. Kanost, M.R. and Drake, D.R. (1987) Increase in serum lysozyme following injection of bacteria into larvae of *Manduca sexta*. In Molecular Entomology (Ed. Law J.H.) pp. 381-390. Alan R. Liss. New york.

Englemann, F. (1979) Insect vitellogenin: Identification biosynthesis and role in vitellogenesis. Adv. Insect. Physiol. 14 pp. 49-109.

Faye, I. and Wyatt, G.R. (1980) The synthesis of antibacterial proteins in isolated fat body from *Cecropia* silkworm pupae. Experientia 36 pp. 1325-1326.

Faye, I., Hultmark, D., (1993) The insect immune proteins and the regulation of their genes. In: Beckage NE, Thompson SN, Federici BA (eds) Parasites and Pathogens of insects Vol 2: Pathogens. Academic Press, San Diego, CA, pp. 25-53.

Feder, D., Mello, C.B., Garcia, E.S., Azambuja, P., (1995) Immune responses in *Rhodnius prolixus*: influence of nutrition and ecdysone. J. of Insect Physiol 43(6) pp. 513-519.

Feder, D., Valle, D., Rembold, H. and Garcia, E.S. (1988) Azadirachtin-induced sterilization in mature females of *Rhodnius prolixus*, Z. Naturforsch, 43c, pp. 908-913.

Feinstein, L. (1952) Insecticides from plants. In Insects: Yearbook of Agriculture, USDA, Washington, DC pp. 222-229.

Ferenz, H.J., (1990) Locust pheromones basic and applied aspects. Proceedings of the 5th International Meeting of the Orthopterist Society, 17-20 July 1989. Valsain (Spain) pp. 29-37.

Ferenz, H.J., Lubzenes, E. and Glass, H. (1981) Vitellin and vitellogenin incorporation by isolated oocytes of *Locusta migratoria*. J. of Insect Physiol., 12, pp. 869-875.

Kennedy, D., (1997) World Rivers Review 12(5) pp.13 International Rivers Network

Frear, D. E. H. (1943) Chemistry of Insecticides and Fungicides. Van Nostrand, New York, NY.

Frear, D., (1979) Multiplication of haemocytes. In insect haemocytes (Ed: Gupta A.P) Cambridge University press, Cambridge.

Frenz, H.J. Heije, D., Abd-el-Hadi, F. and Applebaum. S.W. (1995) Desert locust vitellogenin : characterisation, properties and uptake by terminal oocytes.

Gellissen, G., and Wyatt, G.R. (1981) Production of lipophorin in the fat body of adult *Locusta migratoria* : Comparison with vitellogenin, Can. J. Biochem., 59, pp. 648-654.

Gellissen, G., Waje, E., Cohen, E., Emmerich, H., Applebaum, S.W. Flossdorf, J. (1976) Purification and properties of oocyte vitellin from the migratory locust. J. Comp. Physiol. 108 pp. 287-301.

Gill, J.S. and Lewis, C.T. (1971), Systemic action of an insect feeding deterrent. Nature, 232, pp. 402-403.

Girardie, J., Geoffre, S., Delbecque, JP. Girardie, A. (1998) Arguments for two distinct gonadotropic activities triggered by different domains of the ovary maturing parsin of *Locusta migratoria*. J. of Insect Physiol. 44(11) pp. 1063-1071.

Girardie, J., Heut, JC., Ataykadiri, Z., Ettaouil, S., Delbecque, JP. Fournier, B., Pernollet, JC., Girardie, A. (1998) Isolation, sequence determination, physical and physiological characterization of the neuroparsins and ovary maturing parsins of *Schistocerca gregaria*. Insect Biochem and Molecular Biology, 28(9) pp. 641-650.

Girardie, J., Richard, O. and Girardie, A. (1992). Time-dependent variation in the activity of a novel ovary maturing neurohormone from the nervous corpora cardiaca during oogenesis in the locust, *Locusta migratoria migratoroids*. J. Insect. Physiol, 38, pp. 215-221.

Girardie, J., Richard, O. and Girardie, A. (1996) Detection of vitellogenin in the haemolymph of larval female locust (*Locusta migratoria*) treated with the neurohormone, Lom OMP. J. Insect. Physiol. 42 pp. 107-113.

Girardie, J., Richard, O., Huet, J.C. Nespoulous, C. Van Dorsselaer, A. and Pernollet, J.C. (1991). Physical characterization and sequence identification of the ovary maturing parsin. Eur. J. Bioch. 202, pp. 1121-1126.

Goltzene, F., and Porte, A. (1978) Endocrine control by neurosecretory cells of the pars intercerebralis and the corpora allata during the earlier phases of vitellogenesis in *Locusta migratoria*. Gen. Comp. Endocrin. 35 pp 35-45.

Grainge, M. , Ahmed, S. (1988) Handbook of Plants with Pest-Control properties. Wiley. Interscience, New York NY.

Gunnarsson, S.G.S. and Lackie, A.M. (1985) Haemocytic aggregation in *Schistocerca gregaria* and *Periplaneta americana* as a response to injected substances of microbial origin. J. Inver. Pathol. 46 pp. 312-319.

Gupta, AP. (1979) (Ed) Insect haemocytes. Cambridge University Press, Cambridge.

Hagedorn, H.H. and Kunkel, J.G. (1979) Vitellogenin and vitellin in insects. Annu.

Rev. Entomol., 24, pp. 475-505.

Harnish, G.D. and White, B.N. (1982) Insect vitellins: Identification, Purification and characterization from eight orders, *J. Exp. Zool.*, 220, pp. 1-10.

Harnish, G.D. Wyatt, G.R. and White, B.N. (1982) Insect vitellins: Identification of primary products of translation, *J. Exp., Zool.*, 220, pp. 11-19.

Hill, L. (1965), The incorporation of C¹⁴-glycine into the proteins of the fat body of the desert locust during ovarian development, *J. Insect Physiol.*, 11, pp. 1605-1615.

Hultmark, D., Steiner, H., Boman, H.G. (1980) Insect immunity: Purification and properties of three inducible proteins from haemolymph of immunized pupae of *Hyalophora cecropia*. *Eur. J. Biochem.* 106 pp.7-16.

Ignatowicz, S. and Wesolowska, B. (1996) Repellency of powdered plant material of the Indian neem tree, the *Labrador tea* and the sweet-flag, to some stored product pests. *Polskie-Pism-Entomol.* 65(1-2) pp.61-67.

Imboden, H. and Low, J.H. (1983) Heterogeneity of vitellins and vitellogenins of the tobacco hornworm, *Manduca sexta* L., Time course of vitellogenins appearance in the haemolymph of the adult female, *Insect Biochem.*, 13, pp. 151-162.

Injeyan, H.S. and Tobe, S.S. (1981) Phase polymorphism in *Schistocerca gregaria*: Reproductive parameters *J. Insect. Physiol.* 27 pp. 97-102.

Isaac, P.G and Bownes, M. (1982) Ovarian and fat body vitellogenin synthesis in *Drosophila melanogaster*. *Eur. J. Biochem.* 123 pp. 527-534.

Islam, N., (1996) Securely Sustainable. *Our Planet* 8(4). pp. 33-34. United Nations Environmental Program, Kenya.

Ismail, S.M., Satyanarayana, K., Bradfield, J.Y., Dahm, K.H., Bhaskaren, G (1998) Juvenile hormone acid: Evidence for a hormonal function in induction of vitellogenin in larvae of *Manduca sexta*. *Archives of Insect Biochem & Physiol.* 37(4) pp. 305-314.

Isman, M.B., Koul, O., Luczynski, A. & Kaminski, J., (1990) Insecticidal and

antifeedant bioactivities of neem oils and their relationship to azadirachtin content, J. Agric. Food Chem., 38, pp. 1406-1411.

Izumi, S., Yano, K., Yamamoto, Y. Takahashi, S.Y. (1994) Yolk proteins from insect eggs: Structure, Biosynthesis and programmed degradation during Embryogenesis. J. Insect. Physiol. 35 pp. 735-746.

Jabbar, A. and Strang, R.H.C. (1998) A comparison of the effects of azadirachtin A on cultured insect and mammalian cells. In: Pract. Oriented results on use and production of Neem-Ingredients and pheromones VII. Kleeberge, H. (ed) pp. 5-8.

Jacobson, M. (1986) Neem research and cultivation in the Western Hemisphere, In Proc. 3rd Int. Neem Conf., Nairobi, Kenya, 1986, Schmutterer, H. and Ascher, K. R. S., Eds., GTZ Press, Eschborn, Germany, 1987.

Jacobson, M. (1988) Pharmacology and toxicology of neem. In: Jacobson, M. (ed) 1988 Focus on Phytochemical Pesticides. Vol I, The Neem Tree. CRC Press, Boca Raton, Florida.

Jakob, G. and Dickler, E. (1994) Untersuchungen zur Wirksamkeit von Niempräparaten bei der Bekämpfung des Apfelschalenwicklers *Adoxophyes orana* F. V.R. (Lepidoptera: Tortricidae) Mitt. Dtsh. Ges. Allg. Angew. Ent. 9, pp. 51-56.

Jagannadh, V. and Nair, V. S. K. (1992) Azadirachtin-induced effects on larval-pupal transformation of *Spodoptera mauritia*. *Physiol. Entomol.* 17 pp. 56-61.

Jagannadh, V. and Nair, V. S. K. (1996) Azadirachtin: Its effects on eggs and postembryonic development in *Spodoptera mauritia*, Boisduval (Lepidoptera: Noctuidae). *Uttar-Pardesh. J. of Zool.* 16(2) pp. 109-113.

Jarvis, AP., Johnson, S., Morgan, ED. Simmonds, MSJ. Blaney, WM. (1997) Photooxidation of nimbin and salanin, tetraterpenoids from the neem tree (*Azadirachta indica*). *J. of Chem. Ecol.* 23(12), pp. 2841-2860.

Johnson, S. and Morgan, ED. (1997) Supercritical fluid extraction of oil and triterpenoids from neem seeds. *Phytochemical Analysis* 8(5) pp. 228-232.

Jones, P.S., Ley, S.V., Morgan, E.D. and Santafianos, D., (1988) The Chemistry of the neem tree, In: M. Jacobson (ed), 1988 Focus on Phytochemical Pesticides Vol.1 The neem tree, CRC Press Inc., Boca Raton, Florida.

Jonsson, H., (1996) Greening the fields. Our Planet. 8(4) pp.16-17. United Nations Environmental Program, Kenya.

Kanost, M.R., Kawooya, J.K., Law, J.H., Ryan, R.O., Van Heusden, M.C. and Ziegler, R. (1990) Insect Haemolymph Proteins, In: Advances in Insect Physiology, Eds., Evans, P.D. and Wigglesworth, V.B., Academic Press, New York, pp. 22, 323.

Kanost, M.R., Dai, W., Dunn, P.E. (1993) Peptidoglycan fragments elicit antibacterial protein synthesis in larvae of *Manduca sexta*. Arch. Insect Biochem & Physiol. 8 pp.147-164.

Karanavar, G.K., (1987) Influence of azadirachtin on insect nutrition and reproduction. proceedings of the Indian Academy of Science (Animal Sciences) 96 pp. 341-347.

Kollien, A.H. Goncalves, T.C.M., Azambuja, P., Garcia, E.S. Schaub, G.A. (1998) The effect of azadirachtin on fresh isolates of *Trypanosoma cruzi* in different species of triatomines. Parasit. Research. 84(4) pp. 286-290.

Koul, O. (1984) Azadirachtin II. Interaction with the reproductive behaviour of red bug *Z. angew. Ent.*, 98, pp. 221-223.

Koul, O., Amanai, K. and Ohtaki, T. (1987) Effect of azadirachtin on the endocrine events of *Bombyx mori*, J. Insect Physiol., 33, pp. 103-108.

Koul, O., and Isman, M.B. (1991) Effects of azadirachtin on the dietary utilization and development of the variegated cutworm *Peridroma saucia*. J. Insect Physiol., 37, pp. 591-598.

Kremen, C. and Nijhout, H.F. (1998) Control of pupal commitment in the imaginal disks of *Precis coenia* (Lepidoptera: Nymphalidae). J. Insect Physiol. 44(3-4) pp. 287-296.

Kunkel, J.G., and Nordin, J.H. (1985) Yolk proteins, In "Comprehensive Insect Physiology, Biochemistry and Pharmacology", Eds: Kerkut, G.A. and Gilbert, L.I., 1, pp. 83-111, Pergamon Press, Oxford.

Lamy, M. (1984) Vitellogenesis, vitellogenin and vitellin in the males of insects: A reiview, Int. J. Invert. Reprod. and Develop. 7, pp. 311-321.

Law, J.H. and Wells, M.A. (1989) Insects as Biochemical Models. J. Biol. Chem. 264 pp. 16335-16338.

Lemos, F.J.A., Ribeiro, A.F. and Tersa, W.R. (1993) A bacteria-digesting midgut lysozyme from *Musca domestica* (Diptera) larvae. Purification properties and secretory mechanism. Insect. Biochem. Molec. Biol. 23(4) pp. 33-541.

Levinson, Z. H., Levinson, A.R. (1985). Storage and insect species of stored grains and tombs in ancient Egypt. Z. Angew. Entmol. 100, pp. 321-339.

Linton, Y.M., Nisbet, A.J., Mordue, A.J. (1997) The effects of azadirachtin on the testes of the desert locust, *Schistocerca gregaria* (Forsk.) J. Insect Physiol. 43(11) pp.1077-1084.

Lorenz, J. , Lenz, M and Hoffmann, K.H. (1995) Effects of pharmacological agents on ecdysteroid synthesis in vitro in ovaries and abdominal integument from female adult crickets *Gryllus bimaculatus* de Geer (Ensifera Gryllidae) Z. Naturforsch. 50c pp. 286-293.

Lowery, D.T. and Isman, M.B. (1994) Insect growth regulating effects of neem extracts and azadirachtin on aphids. Entomol. Exp. Appli. 72 pp. 77-84.

Mahamat, H., Hassanali, A., Ferenz, H.J (1997) Vitellogenin titres in normal and accelerated maturation of gregarious-phase *Schistocerca gregaria*. Comp. Biochem. Physiol. 116(4) pp. 447-451.

Marco, M.-P., Pascual, N., Belles, X., Camps, F. and Messeguer, A. (1990), Ecdysteroid depletion by azadirachtin in *Tenebrio molitor* pupae, Pest. Biochem. Physiol., 38, pp. 60-65.

Martin, D., Piulachs, M.D., Belles, X. (1995) Patterns of haemolymph vitellogenin and ovarian vitellin in the german cockroach and the role of Juvenile hormone. *Physiol. Entomol.* 20 pp. 59-65.

McGregor, D.A. and Laughton, B.G. (1977) Amino acid composition, degradation and utilization of locust vitellogenin during embryogenesis, *Wilhelm Roux Arch. Entw. Mech. Org.*, 181, pp. 113-122.

McMillian, W.W., Bowman, M.C., Burton R.L., Stark, K.J. and Wiseman, B.R. (1969), Extracts of Chinaberry leaf as feeding deterrent and growth retardant for larvae of corn earworm and fall armyworm, *J. Econ. Entomol.*, 62, pp. 708-710.

Mironova, M. K. and Khorkhordin, F. G. (1996) Effect of NeemAzal-T/S on *Tetranychus urticae* Koch. Proc. 5th Internat. Workshop "Practice Oriented Results on Use and Production of Neem ingredients and Pheromones" 1996 (Eds.: H. Kleeberg & C. P. W. Zebitz 1997), pp. 129-137.

Mordue (Luntz), A.J., Nisbet, A.J., Nasiruddin, M. and Walker, E., (1996) Differential thresholds of azadirachtin for feeding deterrence and toxicity in locusts and an aphid. *Entomologia. Exp. Appl.* 80 pp.69-72.

Mordue (Luntz), A.J.; Cottee, P.K. and Evans K.A. (1985) Azadirachtin: its effect on gut mortality, growth and moulting in *Locusta*, *Physiol. Entomol.*, 10, pp. 431-437.

Mordue, A.J. and Highnum, K.C. (1973). Incorporation of cysteine into the cerebral neurosecretory system of adult desert locust. *Gen. and Com. Endocrinol*, 20, pp. 351-357.

Mordue, A.J. and Blackwell, A. (1993), Azadirachtin: an update, *J. Insect Physiol.*, 39, pp. 903-924.

Mordue, A.J., Evans, K.A. and Charlet, M. (1986) Azadirachtin, ecdysteroids and ecdysis in *Locusta migratoria*, *Comp. Biochem. Physiol.*, 85C, pp. 297-301.

Mordue, A.J., Simmonds, M.S.J., Ley, S.V., Blaney, W.M., Mordue, W., Nasiruddin, M., Nisbet, A.J., (1998) Actions of azadirachtin, a plant allelochemical against insects.

Pesticide Science 54(3) pp. 277-284.

Morishima, I., Yamada, K. and Ueno, T. (1992) Bacterial peptidoglycan as elicitor of antibacterial protein synthesis in larvae of silkworm, *Bombyx mori*. Insect Biochem. Mol Biol. 4, pp. 363-367.

Morishima, Y. (1994). Lysozyme activity in immunised and nonimmunised haemolymph during the development of the silkworm, *Bombyx mori*. Comp. Biochem. Physiol, 108 A, 23, pp. 311-314.

Munakata, K. (1975) Insect antifeeding substances in plant leaves. Pure and Applied Chem. 42 pp.57-66.

Mundall, E.C. and Law, J.H. (1979) Physical and chemical characterization of vitellogenin from the haemolymph and eggs of the tobacco hornworm, *Manduca sexta*, Comp. Biochem. Physiol., 63B, pp. 459-468.

Nasiruddin, M. and Mordue (Luntz), A.J. (1994) The effect of azadirachtin on the midgut histology of the locusts, *Schistocerca gregaria* and *Locusta migratoria*. Tissue and Cell 25, pp. 875-884.

Natori, S., (1994) Function of antimicrobial proteins in insects. In: Antimicrobial Peptides, Wiley, Chichester (Ciba Foundation Symposium 186) pp. 123-134.

Nisbet, A. J., Mordue, A.J., Grossman, R. B., Mordue, W. (1997) Characterization of azadirachtin binding to Sf9 nuclei *in vitro*. Archives of Insect Biochem & Physiol. 34(4) pp. 461-173.

Nisbet, A.J. (1992), The effects of azadirachtin on the feeding behaviour and virus transmission of the Green peach aphid *Myzus persicae* (Sulzer), Ph.D. Thesis, University of Glasgow, U.k.

Nisbet, A.J., Mordue (Luntz) A.J. and Mordue, W. (1995) Detection of [22, 23-³H₂] dihydroazadirachtin binding sites on *Schistocerca gregaria* (Forsk.) testes membranes. Insec. Biochem. and Mol. Biol. 25 pp. 551-557.

Nisbet, A.J., Woodford, J.A.T., Strang, R.H.C., and Connolly, J.D. (1993),

Systemic antifeedant effects of azadirachtin on the peach-potato aphid *Myzus persicae*, *Entomologia exp. appl.*, 68, 87-98.

Nogueira, NFS., Gonzales, M., Garcia, EM., DeSouza, W. (1997) Effects of azadirachtin A on the fine structure of the midgut of *Rhodnius prolixus*. *J. Inver Pathol.* 69(1) pp. 58-63.

Otto, D. (1996) Inhibition of vitellogenesis and sex pheromone production in females of the Colorado potato beetle, sterilized by Neem ingredients (Azadirachtins). *Proc. 5th Internat. Workshop "Practice Oriented Results on Use and Production of Neem ingredients and Pheromones" 1996* (Eds.: H. Kleeberg & C. P. W. Zebitz 1997), pp. 263-68.

Ouchterlony, O. (1949) Antigen-antibody reaction in gels *Acta. Pathol. Microbiol. Scand.*, 26 pp. 507-515.

Pan, H.L., Bell, W.J., Telfer, W.H. (1969) Vitellogenic blood protein synthesis by the insect fat body. *Science* 165 pp. 393-394.

Pan, M.L. (1979) Juvenile hormone and vitellogenin synthesis in the cecropia silkworm. *Biol. Bull.* 153 pp.336-345.

Paranagama, P. (1994). A Study of Effects of Azadirachtin in specific tissues of Locust *Schistocerca gregaria*. Ph.D Thesis, University of Glasgow.

Parmer, B.S. and Ketkar, C.M. (1993) Commercialization, Neem Research and Development, Eds. Randhawa, N.S. & Parmar, B.S., No.3, Society of Pesticide Science, India, pp. 270-283.

Pathak, P.H. and Krishna, S.S. (1985) Neem seed oil, a capable ingredient to check rice moth reproduction (Lepid. Galleriidae), *Z. angew. Ent.*, 100, pp. 33-35.

Patton, R.L. (1963) *Introductory Insect Physiology*. W.B. Saunders Company Philadelphia and London p. 51.

Perrow personal communication

Powning, R.F., and Davidson W.J. (1973) Studies on insect bacteriolytic enzymes-I. Lysozyme in haemolymph of *Galleria mellonella* and *Bombyx mori*. *Comp. Biochem. Physiol.* 45 B pp 669-681.

Powning, R.F., and Davidson W.J. (1976) Studies on insect bacteriolytic enzymes-II. Some physical and enzymatic properties of lysozyme in haemolymph of *Galleria mellonella*. *Comp. Biochem. Physiol.* 55 B pp. 221-228.

Pradhan, S., Jotwani, M. Rai, B. K. (1962) The neem seed deterrent to locusts. *Indian Farming*, 12 pp. 7-11.

Price, C. D. and Ratcliff, N. A. (1974) A reappraisal of insect haemocyte classification by the examination of blood from 15 insect orders. *Z. Zellforsch. Mikrosk. Anat.* 147(3) pp. 537-541.

Prior, C. and Streett, D.A. (1997) Strategies for the use of entomopathogens in the control of the desert locust and other acridoid pests. *Memoirs of the Entomological Society of Canada*. 171 pp. 5-25.

Qadri, S. S. H. and Narsaiah, J. (1978) Effects of azadirachtin on the moulting processes of last instar nymphs of *Periplaneta americana* (Linn.) *Indian. J. Exp. Biol.* 16 pp. 1141-43.

Rembold, H. (1988) Isomeric azadirachtins and their mode of action. In: M. Jacobson (ed), 1998 *Focus on Phytochemical Pesticides Vol I The Neem Tree* CRC Press Inc., Boca Raton, Florida.

Rembold, H. (1995) (Growth and Metamorphosis) In: Schmutterer, H. 1995 (Ed). *The Neem Tree *Azadirachta indica* A.Juss. and other Meliaceous Plants*. VCH Weinheim, New York, Cambridge, Tokyo 1995. pp. 177-194.

Rembold, H. (1995) Biological effects of Neem and their modes of action, Growth and metamorphosis. In: Schmutterer, H.(ed). *The Neem Tree* Weinheim, New york etc (VCH)

Rembold, H. and Annadurrai, R.S. (1993) Azadirachtin inhibits proliferation of Sf9 cells in monolayer culture. *Naturforsch.* 48c pp. 495-499.

Rembold, H. and Sieber, K.P. (1981) Inhibition of oogenesis and ovarian ecdysteroid synthesis by azadirachtin in *Locusta migratoria migratooides* (R&F), Z. Naturforsch., 36c, pp. 466-469.

Rembold, H., Sharma, G.K. and Czoppelt, Ch. (1981), Growth regulating activity of azadirachtin in two holometabolous insects, In: Natural pesticides from the Neem Tree (*Azadirachta indica* A. Juss), Eds, Schumutterer, H., Ascher, K.R.S., and Rembold, H., GTZ Press, Eschborn, West Germany, pp.121.

Rembold, H.; UHL, M. and Muller, T. (1987) Effect of azadirachtin A on hormone titers during the gonadotrophic cycle of *Locusta migratoria* In Natural Pesticides from the Neem Tree and Other Tropical Plants, Eds: Schmutterer, H. and Ascher, K.R.S., GTZ, Eschborn, pp. 289-298.

Rembold,H.; Forster, H.; Czoppelt, Ch., Rao, P.J. and Sieber, K.P. (1983) The azadirachtins a group of insect growth regulators from the neem tree, 2nd Int. Neem Conf., Rauischholzhausen, pp. 153 - 162.

Richards, J. M., Boyter, A. C., Nathwani, D. (1996) A jaundiced Farmer. Scot. Med. Jn. 41 pp. 176-77.

Rossner, J. and Zebitz, C.P.W. (1987), Effect of neem products on nematodes and growth of tomato plants, In: Natural pesticides from the neem tree (*Azadirachta indica* A. Juss.) and other tropical plants, Eds. Shumutterer, Hl and Ascher, K.R.S., GTZ Press Eschborn, West Germany, pp. 611-621.

Ruscoe, C. N. E. (1972) Growth disruption effects of an insect antifeedant, Nature New Biol., 236, pp. 159-160.

Ruskin, F. R. (Ed) (1992) "Neem" a tree for solving global problems, National Academy Press, Washington DC.

Russel, V. W. and Dunn, P. E. (1990). Lysozyme in the pericardial complex of *Manduca sexta*. Insect Biochem. 20(5) pp. 501-509.

Satyanarayana, K., Yu, J.H., Bhaskaran, G., Dahm, K.H. and Meola, B. (1992)

Regulation of vitellogenin synthesis by juvenile hormone in the corn earworm *Helicoverpa zea*. *Invert. Rep. Devel.* 21 pp. 169-178.

Saxena, R. C., Jilani, G., Abdul Kareem, A. (1989) Effects of neem on stored grain insects . In: Jacobson, M. (ed) 1988 Focus on Phytochemical Pesticides. Vol I, The Neem Tree. CRC Press, Boca Raton, Florida pp. 97-111.

Saxena, R.C. Liquido, N.J. and Justo, Jr.H.D. (1981) Neem seed oil, a potential antifeedant for the control of rice brown planthopper, *Nilaparvata lugens*, In: Natural pesticides from neem tree, *Azadirachta indica* A. Juss., Eds. Schmutterer, H., Ascher, K.R.S. & Rembold, H., Proc. 1st Intl. Neem conf., Rottach Egern, GTZ Press, Eschborn, West Germany, pp. 171-188.

Sayah, F., Fayet, C., Idaomer, M., Karlinsky, A. (1996) Effect of azadirachtin on vitellogenesis of *Labidura riparia* (Insect Dermaptera). *Tissue and Cell* 28(6) pp. 741-749.

Sayah, F., Idaomer, M., Soranzo, L., Karlinsky, A. (1998) Endocrine and neuroendocrine effects of Azadirachtin in adult females of the earwig *Labidura riparia* . *Tissue and Cell* 30(1) pp.86-94.

Schauer, M. (1984) Effects of variously formulated neem seed extracts on *Acyrtosiphon pisum* and *Aphis fabae*. Proc. 2nd Int. Neem conf. (Rauischholzhausen, Germany, 1983) pp. 141-149.

Schmutterer, H. (1990) Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*, A. Rev. Ent., 35, pp. 271-297.

Schmutterer, H. and Rembold, H. (1995) (Reproduction) In: Schmutterer, H. 1995 (Ed). The Neem Tree *Azadirachta indica* A.Juss. and other Meliaceae Plants. VCH Weinheim, New York, Cambridge, Tokyo 1995. pp. 195-204.

Schroder, P. and Kahrt, M. (1996) Mosquito control by NeemAzal- a pilot study in the Seychelles. Proc. 5th Internat. Workshop "Practice Oriented Results on Use and Production of Neem ingredients and Pheromones" 1996 (Eds.: H. Kleeberg & C. P. W. Zebitz 1997), pp. 155-160.

Schulz, C., Kienzle, J., Zebitz, C. P. W. (1996) Effects of different NeemAzal-formulations on apple aphids and *Aphis fabae* Scop. Proc. 5th Internat. Workshop "Practice Oriented Results on Use and Production of Neem ingredients and Pheromones" 1996 (Eds.: H. Kleeberg & C. P. W. Zebitz 1997), pp. 81-92.

Schumutterer, H. (1992), Einfluß von azadirachtin, einer azadirachtin-freien fraktion eines alkoholischen niemsamenextraktes und von formulierten extrakten auf verpuppung, schlupf und imagines der kohlweißlingsbrackwespe *Apanteles glomeratus* (L) (Hym., Braconidae), J. App. Ent., pp. 113, 79-87.

Secoy, D. M. Smith, A. E. (1983) Use of plants in control of agriculture and domestic pest. Econ. Bot. 37, pp. 28-57.

Shapiro, M., Stock, R.D. and Ignoffo, C.M. (1969) Haemocyte changes in larvae of the bollworm, *Heliothis zea*, infected with a nucleopolydrosis virus. J. Inver. Pathol. 14 pp. 28-30.

Shirk, P.D., Bean, D.W. and Brookes, V.J. (1990) Ecdysteroids control vitellogenesis and egg maturation in pharate adult female of the Indian meal moth *Plodia interpunctella*. Arch. Insect Biochem. Physiol. 15 pp.183-199.

Shukla, A., Pathak, S.C. Agrawal, R.K. (1996) Efficiency and economics of some insecticides and plant products against the infestation of okra shoot and fruit borer, *Earias vitella* Fab. Crop-Research (Hisar) 12 (3) pp. 367-373.

Siddiqui, S. (1942) A note on the isolation of 3 new bitter principles from the nim oil. Current. Sci. 11 pp. 278-279.

Siddiqui, S., Faizi, S., Siddiqui, B.S. (1986) Studies on the chemical constituents of azadirachta indica A.Juss (Meliaceae). Z. Naturforsch 42b pp. 922-924.

Sieber, K.P. and Rembold, H. (1983), The effects of azadirachtin on the endocrine control of moulting in *Locusta migratoria*, J. Insect Physiol., 29, pp. 523-527.

Singh, B. and Singh, A.P. (1987), Neem seed kernel suspension as an oviposition deterrent to the desert locust, *Schistocerca gregaria* F., Plant Prot. Bull., 39,9-16.

Singh, G.J.P., Schonest, L.P. Jr and Gill S.S. (1986) The toxic action of *Bacillus thuringiensis* var.issaelensis in *Aedes aegypti* in vivo I. The relevance of midgut lesions to its poisoning syndrome. Pestic. Biochem. Physiol. 26 pp. 36-46.

Singh, R.P. (1984), Effect of water extract of deoiled neem kernel on second instar larvae of *Culex fatigans* Wiedemann. Neem Newsletter, 1(2), 16-17.

Singh, R.P. (1987), Comparison of antifeedant efficacy and extract yields from different parts and ecotypes of neem (*Azadirachta indica* A.Juss), In: Eds, Schmutterer, H., Ascher, K.R.S., Natural pesticides from the neem tree and other tropical plants; Proceedings of the Third International Neem Conference, GTZ, Eschborn,185-194.

Singh, R.P. (1993), Bioactivity against insect pests, Neem Research and Development, Eds. Randhawa, N.S. and Parmar, B.S., Society of pesticides science, India, 3, 109-122.

Singh, R.P. and Kataria, P.K. (1986), Deoiled neem kernel powder as protectant of wheat seed against, *Trogoderma granarium*, Indian J. Ent., 48(1), 119-120.

Singh, R.P., and Srivastava, B.G. (1983), Alcohol extract of neem (*Azadirachta indica* A. Juss) seed oil as oviposition deterrent for *Dacus cucurbitae* Coq., Indian J. Ent., 45(4), 497-498.

Sinniah, B. Sinniah, D. Ibrahim, J. (1994) Effect of neem oil and mosquito larvae. Mosquito-Borne-Disease-Bulletin 11(3) pp. 90-93.

Slama, K. (1978) The principles of antihormone action in insects. Acta. Ent. Bohemoslov, 75 pp. 65-82.

Smith, B. D., (1998). Origins of Agriculture: Between Foraging and Farming. Science 279(5357) pp.1651-52.

Stark, J.D., Vargas, R.I., Thalman, R.K. (1990) Azadirachtin: effects on metamorphosis, longevity and reproduction of three tephritid fly species (Diptera: Tephritidae). J. Econ. Entmol. 83 pp. 2168-2174.

Steiner, H., Hultmark, D., Engstrom, A., Bennich, H., Boman, IIG. (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 292 pp. 246-48.

Stryer, L. (Ed) (1988) *Biochemistry*, Third Edition pp. 201-203.

Su, TY. and Mulla, MS. (1998) Antifeedancy of neem products containing azadirachtin against *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: culicidae). *J. of Vector Ecol.* 23(2) pp. 114-122.

Su, TY. and Mulla, MS. (1998) Ovicidal activity of neem products (azadirachtin) against *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: culicidae). *J. of The American Mosquito Control Association* 14(2) pp. 204-209.

Subrahmanyam, B. and Rembold, H. (1989) Effect of azadirachtin A on neuroendocrine activity in *Locusta migratoria*, *Cell Tissue Res.*, 256, 513-517.

Subrahmanyam, S.; Muller, T. and Rembold, H. (1989) Inhibition of turnover of neurosecretion by azadirachtin in *Locusta migratoria*, *J. Insect Physiol.*, 35, 6, 493-500.

Sunn, S., Asling, B. and Faye, I. (1991) Organisation and expression of the immunoresponsive lysozyme gene in the giant silk moth, *Hyalophora cecropia*. *J. Biol. Chem.* 266 pp. 6644-6649.

Taqi, A., (1997). Press release, International Labour Organisation, Geneva. UNICEF (ILO/97/23)

Tawfik, A.I., Mat'hova' A., Schnal, F., Ismail, S.H., (1997) Haemolymph ecdysteroids and the prothoracic glands in the solitary and gregarious adults of *Schistocerca gregaria*. *Arch. Insect. Bioch and Physiol.* 43 pp. 485-493.

Telfer, W.H. (1960) The selective accumulation of blood proteins by the oocytes of saturniid moths, *Bilo. Bull. mar. biol. Lab. Woods Hole*, 118, 338-351.

Telfer, W.H., (1954): Immunological studies of insect metamorphosis II. The role of a sex-limited blood protein in eggs of the cecropin silkworm. *J. Gen. Physiol.* 37 pp. 539-558.

Timmins, W.A. and Reynolds, S.E. (1992) Azadirachtin inhibits secretion of trypsin in midgut of *Manduca sexta* caterpillars: reduced growth due to impaired protein digestion, *Entomologia Exp. Appl.*, 63, pp. 47-54.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellular sheets. Procedure and Applications. *Proc. Natu. Acad. Sci. U.S.A* 76 pp. 4350-4354.

Uebel, E.C., Warthen, J.D. and Jacobson, M. (1979) Preparative reversed phase liquid chromatographic isolation of azadirachtin from neem seed kernals , *J. Liq. Chrom.*, 2, 875-882.

UHL, M. and Rembold, H. (1987) Effects of azadirachtin A on oogenesis in *Locusta migratoria*. (Reproduction) In: *The Neem Tree Azadirachta indica* A.Juss. and other Meliaceous Plants. VCH Weinheim, New York, Cambridge, Tokyo 1995 Schmutterer, H. 1995 (Ed). pp. 200-201.

Uvarov. B. (1966) *Grass-hoppers And Locusts Vol I*, Cambridge University Press, Cambridge UK.

Uvarov. B. (1977) *Grass-hoppers And Locusts Vol II*, Cambridge University Press, Cambridge UK. pp. 85

Vallc, D. (1993) Vitellogenesis in insects and other groups. A review. *Mem. Inst. Oswaldo Cruz* 88 pp. 1026.

Van der Horst, D.J., Van Doorn, J.M., Pener, M.P., Van den Broek, A.T.M., Van Marrewijk, W.J.A. and Beenackers, A.M.T. (1989), Development of imaginal competence to adipokinetic hormone in *Locusta* : lipophrin conversions in precocene-induced adultiforms and in azadirachtin-induced over-aged nymphs, *Comp. Biochem. Physiol.*, 92B, 133-136.

Vanderberg, J.P. (1963) Synthesis and transfer of DNA, RNA and protein

vitellogenesis in *Rhodnius prolixus*, Biol. Bull., Wood Hole, 125, 556-575.

VanhenHeuvel, JFJM., Hogenhout, SA. Verbeek, M., Vanderwilk, F (1998) *Azadirachta indica* metabolites interfere with the host-endosymbiont relationship and inhibit the transmission of potato leafroll virus by *Myzus persicae*. Entomologia Experimentalis et Applicata 86(3) pp. 253-260.

Verna, S.P., Singh, B. and Singh, Y.P. (1985), Studies on the comparative efficacy of certain grain protectants against, *Sitotroga cerealella* Oliver., Bull. Grain Tech., 24, pp. 37-42.

Voller, A., Bidweel, D.E., Bartlett, A. (1979) The enzyme linked immunosorbant assay (ELISA) Alexandria VA: Dynatech Laboratory.

Voss, G. and Neumann, R., (1992) in Neurotox 91: Molecular Basis of Drug & Pesticide Action, (Duce, I.R. ed.) pp. vii-xx, Elsevier Applied Science London.

Wang, Z., Chen, X., Hauserland, N.H., (1993) Flight Muscle development in juvenile and adult forms of the desert locust *Schistocerca gregaria*. J. Insect. Physiol. 39(4) pp. 325-333.

Warthen, J.D., (1989), Nccm (*Azadirachta indica* A. Juss): Organisms affected and reference list update, Proc. Entoml. soc. Wash., 91, pp. 367-388.

Warthen, J.D.; Stokes, J.B.; Jacobson, M. and Kozempel, M.F. (1984) Estimation of azadirachtin content in neem extract and formulations, J. Liq. Chroma., 7, pp. 591-598.

Warthen, J.H.Jr. (1979), *Azadirachta indica*, a source of insect feeding inhibitors and growth regulators. USDA Sci. & Educ. Adm. Agric. Reviews and Manuals North-eastern Ser. 4, pp. 1-21.

Wigglesworth, V. B. (1984) Insect Physiology, 8th ed, Chapman and Hall. London pp. 39-52 (The Circulatory System and Associated Tissues).

Wigglesworth, V.B., (1972) The principles of Insect Physiology, 7th ed. Chapman and Hall, London.

Wilps, H., Kirkilionis, E. and Muschenich, K. (1992), The effect of neem oil and azadirachtin on mortality, flight activity, and energy metabolism of *Schistocerca gregaria* (Forsk.) - a comparison between laboratory and field locusts, *Comp. Biochem. Physiol.*, 102c, pp. 67-71.

Wu, D., Yan, Y., Cui, J., (1996) Chemical Structure and application of sex pheromone of cotton bollworm in China. *Proc. 5th Internat. Workshop "Practice Oriented Results on Use and Production of Nccm Ingredients and Pheromones" 1996* (Eds.: H. Kleeberg & C. P. W. Zebitz 1997), pp. 269-74.

Wyatt, G.R. (1988) Vitellogenin synthesis and the analysis of juvenile action in locust fat body, *Can. J. Zool.*, 66, pp. 2600-2610.

Wyatt, G.R.; Chen, T.T.; and Couble, P. (1976) Juvenile hormone induced vitellogenin synthesis in locust fat body *in vitro* . In " *Invertebrate Tissue culture Application in Medicine, Biology, and Agriculture*": Eds: E. Kurstak and K. Maramorosch, pp. 195-202, Academic press, New york.

Yamasaki, R.B. & Klocke, J.A. (1987) Structure bio-activity relationships of azadirachtin, a potent insect control agent, *J. Agric. Food Chem.*, 35, pp. 467-471.

Yamasaki, R.B.; Klocke, J.A.; Lee, S.M.; Stone, G.A. and Darvington ,M.V.; (1968) Isolation and purification of azadirachtin from neem (*Azadirachta indica*) seeds using flash chromatography , *J. Chroma.*, 356, pp. 220.

Yamasaki, R.B.; Klocke, J.A.; Lee, M.Z.; Stone, G.A, and Darlington, M.V. (1986) Isolation and purification of azadirachtin from neem (*Azadirachta indica*) seeds using flash chromatography and high performance liquid chromatography, *J. Chromatography*, 356, pp. 220-226.

Zachary, D. and Hoffmann, D. (1984). Lysozyme is stored in the granules of certain haemocyte types in *Locusta*. *J. Insect Physiol.* 30(5), pp. 405-411.

