A STUDY OF SOME PARAMETERS RELATING TO MYCOCIDAL ACTIVITY OF *BEAUVERIA BASSIANA* FOR THE CORN EARWORM *HELIOTHIS ZEA*

BY

PETER YEE-KONG CHEUNG II

Bachelor of Science
Oklahoma Baptist University
Shawnee, Oklahoma
1974

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1977

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the degree of
DOCTOR OF PHILOSOPHY
July, 1981

Thesis
1981D
C526s

OKLAHOMA STATE UNIVERSITY LIBRARY

A STUDY OF SOME PARAMETERS RELATING TO MYCOCIDAL ACTIVITY OF BEAUVERIA BASSIANA FOR THE CORN EARWORM HELIOTHIS ZEA

Thesis Approved :

E. A. [illegible]
Thesis Adviser

Mark R Lambert

John R. Sauer

Robert L Burton

Norman N Durham
Dean of the Graduate College

1099195

ACKNOWLEDGEMENTS

Gratitude is expressed to Dr. Edward A. Grula for his guidance, friendship and patience during my years of study at Oklahoma State University. In addition, appreciation is extended to Drs. Robert L. Burton, Norman N. Durham, Mark R. Sanborn and John R. Sauer for their advice and support as committee members.

Thanks are also extended to Dr. K. D. Berlin, Dept. of Chemistry, Oklahoma State University, for his valuable assistance in interpretation of the IR and nmr spectra. I also thank Dr. T. H. Liao, Dept. of Biochemistry, Oklahoma State University, for performing the amino acid analyses.

To my fellow graduate students, I wish to express my sincere appreciation for their companionship and contributions throughout this study.

Special gratitude is extended to my family for their dedicated support and encouragement.

TABLE OF CONTENTS

PART THREE

IN VIVO EVENTS ASSOCIATED WITH ENTOMOPATHOLOGY OF BEAUVERIA BASSIANA FOR THE CORN EARWORM (HELIOTHIS ZEA)

LIST OF TABLES

Table Page

PART FOUR

PART FIVE

LIST OF FIGURES

Figure | Page

PART FOUR

PART FIVE

PREFACE

Recent enthusiasm pertaining to the use of entomophathogenic fungi, as agents of biological pest control, has lead to a renewed interest in these fungi. Ferron (1978), listed seventeen genera of Deuteromycetes, six genera of Phycomycetes and two genera of Ascomycetes as being pathogenic to various insects. This represents a substantial increase from the three major genera reported years ago (Madelin, 1963) and the four genera in 1965 by Pramer (1965).

Among the many entomogenous fungi, the genus Beauveria, having the two species bassiana and tenella, is the best known and most studied. The reason Beauveria enjoys so much attention is probably due to the fact that it has long been known to be an insect pathogen and it was the first fungus to be used in the field on a large scale basis.

Diseased silkworm larvae infected by B. Bassiana are transformed into white, mummified specimens indicative of what has come to be called the white muscardine disease. Being the most widespread and the first acknowledged disease of the silkworm (Bassi, 1835), the nature of this disease must have baffled many curious minds throughout the centuries. Outbreaks of the disease were frequently reported throughout the world wherever silkworms were reared for raw silk. It was reported (Kobayasi, 1977) that 2000 years ago, the Chinese used white mummified silkworm larvae as drugs. This was presumably because of the antibiotics produced by

the fungus, although the Chinese did not understand the existence of such molecules as we know them today. In the eighteenth century, Boissier des Sauvagges postulated that the white muscardine disease was due to a particular state of the atmosphere which preceeds thunderstorms (Steinhaus, 1949). In the early nineteenth century Dandola proposed that the disease actually resulted from abnormal physiological conditions and the white effluorescence on cadavers was the so called "original minerals" (Steinhaus, 1949).

It was not until 1835 that Bassi showed that the disease is not caused by any agents pertaining to meteorological or rearing conditions, but rather by a parasitic and contagious fungus that multiplied in and on the body of the silkworm (Pramer, 1965). From this point on, many studies were done which eventually established the fungus as the etiological agent for the muscardine disease and the fungus came to be called Beauveria bassiana in honor of Bassi. Comprehensive reviews of the history of the muscardine disease and B. bassiana can be found in Steinhaus' Principles of Insect Pathology (Steinhaus, 1949) and a review by MacLeod (1954).

Most recently, interest in Beauveria has focused on the applicability of the organism as a biological pest control agent. The fungus was the first to be field tested in the United States (Roberts, 1973). In the U.S.S.R., a conidial preparation of the fungus (Boverin) is widely used to control potato beetles in the field (Ignoffo, et al., 1979). Such extensive field application naturally leads to a demand for more knowledge of the pathogenicity

of the fungus in order to gain a better understanding of how to best utilize the fungus as an agent of insect pest control

To be a successful pathogen, a parasite (whether a virus, bacterium, fungus, or protozoan) has to accomplish three essential processes: (1) entrance into the host; (2) establishment and multiplication within the host and (3) exit of the parasite from the host.

The three main mechanisms by which B. bassiana initiates infection of the host include entrance via the integument, the respiratory tracheal system and the alimentary tract. Direct penetration of the insect integument by B. bassiana is well documented (Lefebvre, 1934; Vey and Fargues, 1977; Pekrul and Grula, 1979). Initiation of infection by entrance through the digestive tract has also been reported (Broome et al., 1976) as well as through the tracheal system (Clark et al., 1968; Hedlund and Pass, 1968; Pekrul and Grula, 1979). However, it appears that the latter two routes are only secondary when compared with penetration through the integument (Madelin, 1963; Ferron, 1978; Pekrul and Grula, 1979).

To be able to penetrate the integument, B. bassiana and other entomogenous fungi must possess the ability to break down the integument. The insect integument is essentially made up of a chitin-protein complex associated with some lipids and phenolic compounds in layers (Rudall, 1963; Wigglesworth, 1974). Enzymatic attack of this chitin-protein complex is therefore essential for penetration (Madelin, 1963; Ferron, 1978) and B. bassiana does

possess the correct enzymes to achieve this goal. Included are proteases, chitinases and lipases (Gabriel, 1968; Samsinakova et al., 1971; Mapes, 1974; Grula et al., 1978).

After B. bassiana passes through the exoskelton, the fungus faces the defense reactions of the host. Humoral and cellular reactions are the two main host defense mechanisms the invading fungus must cope with (Jones, 1964; Alekseyev, 1971; Whitcomb et al., 1974). A survey of the literature shows that no antifungal factors have been reported in the haemolymph and cellular defense is apparently the only protection an insect has against pathogenic fungi. If an antifungal factor(s) does indeed exist, it is certainly not always effective in stopping the proliferation of intruding fungi.

Haemocytes which are responsible for removing foreign matter will attack the pathogen by either phagocytosis and/or cyst formation (Whitcomb et al., 1974) but with virulent entomogenous fungi such as B. bassiana and B. tenella, neither process is effective. Rapid mycelial development and production of toxins allow mycelia to grow out of these cysts and invade deeper tissues (Steinhaus, 1949; Madlin, 1963; Ferron, 1978). Free cells or hyphal bodies are released further populating the haemolymph and possibly enhancing the spread of the fungus to other organs and tissues. Although production of toxic elements is considered to be an important aspect of B. bassiana's pathogenicity, and indeed a number of beauveria toxins have been identified, evidence for their production in in vivo are vague. The fact that toxin production

by B. bassiana (both quantitatively and qualitatively) varies in different cultural media and environmental conditions further emphasizes the need for caution to be exercised when interpreting the importance of the involvement of any particular toxin in an in vivo situation (Dresner, 1950; Kucera and Samsinakova, 1968; West and Briggs, 1968; Huang and Shapiro, 1971).

After development of mycosis and death of the host insect, saprophytic growth of the pathogen will quickly lead to mummification of the cadaver. Competition with the bacterial flora is kept to a minimum apparently due to antibiotics produced by the fungus. Depending on the humidity, mycelia will penetrate the integument again from inside out forming the white mummy. Conidiophores will soon produce more infective conidia which are dispersed into the environment by rain, wind or even other insects. Transmittance and contraction of the disease is basically by direct contact with the cadaver, e.g. in silkworm cultures, or by contact with contaminated matter, e.g. plant debris and soil. In either case, the pathogenic cycle of B. bassiana is completed.

Using corn earworm, Heliothis zea (Boddie), larvae as the target insect, the pathogenicity of B. bassiana is being studied in this laboratory. The ability of the germinating conidia of the fungus to penetrate the intact cuticle of corn earworm larvae has been clearly shown (Pekrul and Grula, 1979). The purpose of this study is to assess the various characteristics of the fungus as they pertain to its pathogenicity.

This dissertation is made up of five parts. Parts One to

Three deal with the host/parasite interactions (B. bassiana and corn earworm larvae). In Part One, the entomopathogenicity of selected B. bassiana mutants, when topically applied to the larval surface, is reported using probit analysis to establish their relative virulence. After the pathogenicity of the mutants had been determined, attempts were made to relate the infectivity and virulence of B. bassiana to its exocellular enzymatic content. Included were the proteolytic, chitinolytic and lipolytic activities. These data are reported in Part Two. Part Three consists of investigations relating to the events occurring after the fungus has gained access to and established itself within the insect host. Preliminary studies relating to an anticoagulant produced by B. bassiana are presented in Part Four.

In Part Five, the isolation and identification of a polyamine present in the haemolymph of corn earworm larvae is reported.

SELECTED BIBLIOGRAPHY

Alekseyev, A. N. 1971. Immunity in insects. Entomol. Rev., 50:420-426.

Bassi, A. 1835. Parte 1, Teorica Tip. Orcesi, Lodi. (Quoted in Ignoffo et al., 1979.)

Broome, J. R., P. P. Sikorowkis and B. R. Norment. 1976. A. mechanism of pathogenicity of Beauveria bassiana on larvae of the imported fire ant, Solenopsis richteri. J. Invertebr. Pathol., 28:87-91.

Clark, F. R., W. R. Kellen, T. Fukuda and J. E. Lindegren. 1968. Field and laboratory studies of the pathogenicity of the fungus Beauveria bassiana to three genera of mosquitoes. J. Invertebr. Pathol., 11:1-7.

Dresner, E. 1950. The toxic effect of Beauveria bassiana (Bals.) Vuill. on insects. J. N. Y. Entomol. Soc., 58:269-278.

Ferron, P. 1978. Biological control of insect pests by entomogenous fungi. Ann. Rev. Ento., 23:409-442.

Gabriel, B. P. 1968. Enzymatic activities of some entomophthorous fungi. J. Invertebr. Pathol., 11:70-81.

Grula, E. A., R. L. Burton, R. Smith, T. L. Mapes, P. Cheung, S. Pekrul, F. R. Champlin, M. Grula and B. Abegaz. 1978. Biochemical basis for the entomopathogenicity of Beauveria bassiana. pp. 192-216. In, C. M. Ignoffo (ed), Proceedings of the First Joint US/USSR Conference on the Production, Seclection and Standardization of Entomopathogenic Fungi of the US/USSR Joint Working Group on the Production of Substances by Microbiological Means. 293 pp.

Hedlund, R. C. and B. C. Pass. 1968. Infection of the alfalfa weevil, Hypera postica, by the fungus, Beauveria bassiana. J. Invertebr. Pathol., 11:25-34.

Huang, H. T. and M. Shapiro. 1971. Insecticidal activity of microbial metabolites. Progress in Ind. Microbiol., 9:79-112.

Ignoffo, C. M., C. Garcia, O. A. Alyoshina and V. V. Lappa. 1979. Laboratory and field studies with Boverin: a mycoinsecticidal preparation of Beauveria bassiana produced in the USSR. J. Econ. Entomol., 72:562-565.

Jones, J. C. 1964. The circulatory system of insects, pp. 1-107. In, M. Rockstein (ed.), The Physiology of Insecta, Vol. III. Academic Press. N. Y.

Kobayasi, Y. 1977. Miscellaneous notes on the genus Cordyceps and its allies. J. Jpn. Bot., 52:269-272.

Kucera, M. and A. Samsinakova. 1968. Toxins of the entomophagous fungus Beauveria bassiana. J. Invertebr. Pathol., 12:316-3220.

Lefebvre, C. L. 1934. Penetration and development of the fungus, Beauveria bassiana, in the tissue of the corn borer. Ann. Bot. (London), 48:441-452.

MacLeod, D. M. 1954. Investigations on the genera Beauveria Vuill. and Tritirachium Limber. Can. J. Botany, 32:818-890.

Madelin, M. F. 1963. Diseases caused by Hyphomycetous fungi. pp. 233-271. In, E. A. Steinhaus (ed.), Insect Pathology Vol. 2. Academic Press, N. Y.

Mapes, T. L. 1974. Exocellular enzymes of Beauveria bassiana and their possible relationship to entomopathgenicity. Masters Thesis. Okla. State University.

Pekrul, S. and E. A. Grula. 1979. Mode of infection of the corn earworm (Heliothis zea) by Beauveria bassiana as revealed by scanning electron microscopy. J. Invertebr. Pathol., 34:238-247.

Pramer, D. 1965. Symposium on microbial insecticides. III. Fungal parasites of insects and nematodes. Bacteriol. Rev., 29:382-387.

Roberts, D. W. 1973. Means for insect regulation: Fungi. N. Y. Acad. Scs., 217:76-84.

Rudall, K. M. 1963. The chitin-protein complexes of insect cuticles. Adv. Insect Physiol., 1:257-313.

Samsinakova, A., S. Misikova and J. Leopold. 1971. Action of enzymatic systems of Beauveria bassiana on cuticle of the greater wax moth larvae (Galleria melonella). J. Invertebr. Pathol., 18:322-330.

Steinhaus, E. 1949. Principle of Insect Pathology. pp. 318-416. McGraw-Hill Book Company, N. Y.

Vey, A. and J. Fargues. 1977. Histological and ultrastructural studies of Beauveria bassiana infection in Leptinotarsa decemlineta larvae during ecdysis. J. Invertebr. Pathol., 30:207-215.

West, E. J. and J. D. Briggs. 1968. In vitro toxin production by the fungus Beauveria bassiana and bioassay in greater wax moth larvae. J. Econ. Entomol., 61:684-687.

Whitcomb, R. F., M. Shapiro and R. R. Granados. 1974. Insect defense mechanisms against micro-organisms and parasitoids. pp. 447-536. In, M. Rockstein (ed.), The Physiology of Insecta, Vol. V. Academic Press, N. Y.

Wigglesworth, V. B. 1974. The Principles of Insect Physiology. 7th ed. Chapman and Hall, London.

PART ONE

PROBIT ANALYSIS OF THE RELATIVE VIRULENCE OF *BEAUVERIA BASSIANA* MUTANTS

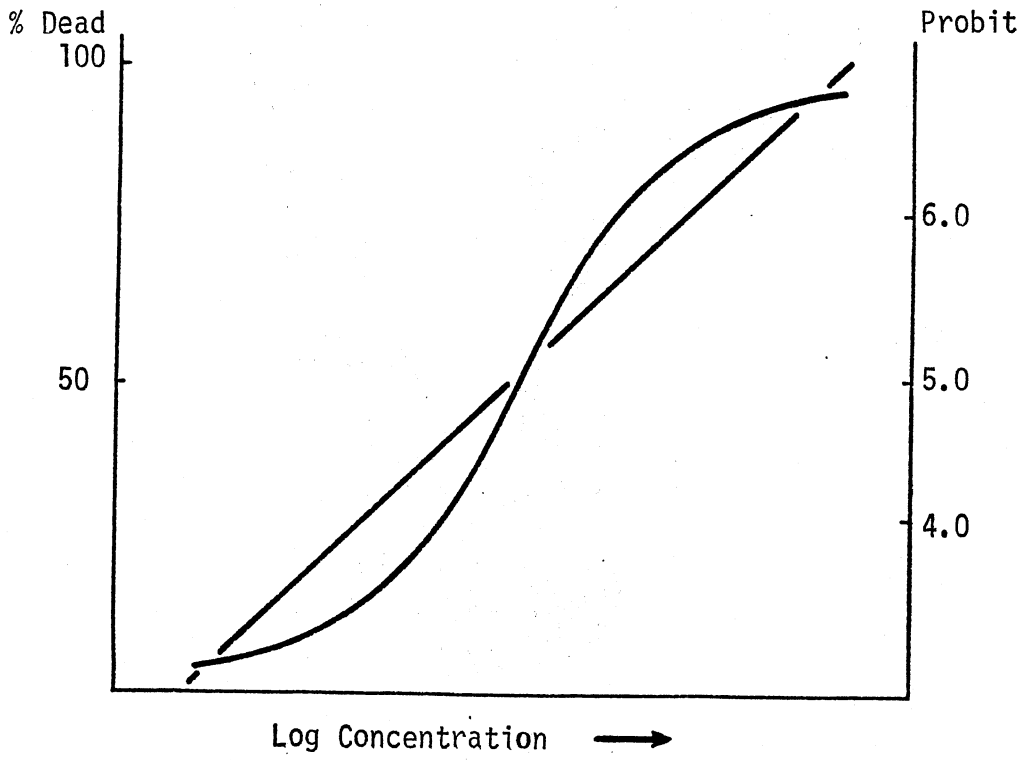
CHAPTER I

INTRODUCTION

Probit analysis is a biological method for assaying the responses of a population of organisms to a given stimulus. When the percentage of individuals responding to the stimulus is plotted against the logarithm of the concentrations applied, a sigmoid curve is obtained as seen in figure 1; a straight line is obtained when the percent values are converted to probit (probability units).

To evaluate different stimuli, a dose vs. response assay is performed on each stimulus and a different probit line is obtained through a series of calculations for each stimulus. Evaluations of the stimuli are then made by comparing several features of the probit lines. Concentrations that give 50% or 90% responses are common parameters. The slope of the line can also be used to indicate how responsive a population is to a particular stimulus. For an indepth discussion concerning the theory and applications of the probit analysis, one should read the two books by Finney (1947, 1971).

Figure 1. Transformation of Percentage to Probits.


% Dead
100
50
Probit
6.0
5.0
4.0
Log Concentration

CHAPTER II

MATERIALS AND METHODS

Test Organisms

All B. bassiana mutants were originally obtained by UV irradiation in our laboratory (Grula et al., 1978) except R_1 which was obtained during a visit to Russia in 1978 (NSF-Sponsored Group I Science Exchange Team of which Dr. E. Grula was a member). Wild type conidia (strain 16) were suspended in water and exposed to UV light at a distance of one and a half feet for 5 min. Mutants were then selected for varying degrees of proteolytic activity by plating on Sabouraud dextrose agar (SDA) containing litmus-milk (casein proteins). All cultures of the fungus were grown on Sabouraud dextrose agar (SDA) with 0.3% yeast extract at 25°C until sporulation. Stocks were maintained by suspending washed conidia (see section on conidial suspensions) in sterile distilled water and stored at 4°C.

Target Insect

The corn earworm, Heliothis zea (Boddie), was used as the target insect in these studies. Larvae were reared on the corn-soy flour-milk powder (CSM) diet developed by Burton (1970). Individual, newly-hatched first instar larvae were placed in diet cups and maintained at 25°C under conditions of high humidity. Larvae were

then selected for the various experiments when they reached the desired instar and weight.

Conidial Suspension

Freshly sporulated cultures were harvested by washing the conidia off the agar surface with 0.03% Triton X-100 (V/V) then washing 2X with sterile distilled water. This procedure effectively removes the detergent and conidia are easily suspended throughout the procedure to allow titer manipulations and plate counting. In most instances an optical density of 1.0 at 540 nM (Spectronic 20 spectrophotometer) indicates a concentration of approximately $2X10^8$ live conidia/ml.

Condial suspensions were prepared fresh each time and appropriate dilutions were made immediately prior to each experiment. In all cases, plate counts were done to determine the concentration of viable conidia in the suspension.

Modified CSM Diet

The antimicrobial substances (p-hydroxymethylbenzoate and sorbic acid) normally present in the regular CSM diet were found to be inhibitory towards B. bassiana and therefore interfered with pathogenicity testing. To eliminate this problem, the two chemicals were replaced with D-chloramphenical (0.5 gm per 3.5 liter diet) which inhibits bacterial, but not fungal growth. To reduce fungal contamination, all water used in preparing the diet was boiled for 10 minutes immediately prior to use.

Probit Analysis

To determine and compare the virulence of the different mutants against corn earworm larvae using topical application, first instar larvae were allowed to crawl on pieces of Whatman 3MM filter paper (8.25 cm in diameter) saturated with 2.0 ml of a conidial suspension in petri plates. After five minutes, individual larvae were placed in diet cups containing modified CSM diet and maintained under normal rearing conditions for 4 days, at which time mortalities were scored. For each mutant, a total of four concentrations were tested and 150-200 larvae were used for each dilution.

At the same time as infected larvae were being put into diet cups, groups of five larvae were taken off the filter paper and put into 5.0 ml aliquots of 0.03% Triton X-100. The larval suspensions were then votexed for one minute to wash conidia from the larval surface. Plate counts were done using 1.0 ml volumes of each of these suspensions to estimate the number of viable conidia each larva had actually acquired on its surface. Five replicas were tested on each dilution to obtain an average number of viable conidia per larva at that particular concentration.

Probit analysis was performed after all the data were collected. The median lethal concentration (LC_{50}) was determined and the number of conidia each larva had acquired at the LC_{50} was also extrapolated. A total of 13 mutants of B. bassiana were tested.

The following terms are used in the calculation of probit:

x: The logarithms of the concentrations of stimulus.

n: Number of test organisms exposed to concentration x.

p: Percent of individuals responding to concentration x.

Y: Probability unit (Probit) of p. This value can be determined from Table I or similar tables in textbooks.

w: Weighting coefficient; because of the sigmoid nature of the response curve, points approaching p=100 and p=0 will carry less weight than those closer to p=50. w can be determined from Table II or similar tables in textbooks.

At this point, x is plotted against Y and a straight line is drawn to fit the points. The value of x at which Y=50 is determined and designated m. When m is converted back to antilogarithms it will give the LC_{50} of the median lethal concentration. To improve the estimation of m and its variance, a regression calculation is performed by reading off the probit for each x from the probit line. These values are then designated as expected probit (y). Then the values of the following terms are determined: Σnw, Σnwx, Σnwx^2, $\Sigma nwxy$ and Σnwy. From these, the following are calculated:

$\bar{x}$: mean of x, $\Sigma nwx / \Sigma nw$

$\bar{y}$: mean of y, $\Sigma nwy / \Sigma nw$

Sxx: $\Sigma nwx^2 - (\Sigma nwx)^2 / \Sigma nw$

Sxy: $\Sigma nwxy - (\Sigma nwx)(\Sigma nwy) / \Sigma nw$

b: slope of the regression line, it is equal to

$\Sigma nw(x-\bar{x})(y-\bar{y}) / \Sigma nw(x-\bar{x})^2$ or Sxy / Sxx

TABLE I

TRANSFORMATION OF PERCENTAGES TO PROBITS

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
—	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

TABLE II

THE WEIGHTING COEFFICIENT, w.

Y	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1	0.001	0.001	0.001	0.002	0.002	0.003	0.005	0.006	0.008	0.011
2	0.015	0.019	0.025	0.031	0.040	0.050	0.062	0.076	0.092	0.110
3	0.131	0.154	0.180	0.208	0.238	0.269	0.302	0.336	0.370	0.405
4	0.439	0.471	0.503	0.532	0.558	0.581	0.601	0.616	0.627	0.634
5	0.637	0.634	0.627	0.616	0.601	0.581	0.558	0.532	0.503	0.471
6	0.439	0.405	0.370	0.336	0.302	0.269	0.238	0.208	0.180	0.154
7	0.131	0.110	0.092	0.076	0.062	0.050	0.040	0.031	0.025	0.019
8	0.015	0.011	0.008	0.006	0.005	0.003	0.002	0.002	0.001	0.001

For the regression line formula, b, $\bar{x}$ and $\bar{y}$ are substituted into the following equation;

$$Y = \bar{y} + b(x-\bar{x})$$

To determine m, set Y=5

To estimate the variance of m, use

$$V_m = \frac{1}{b^2}\left[\frac{1}{\Sigma nw} + \frac{(m-\bar{x})^2}{Sxx}\right]$$

$$s_m = \sqrt{V_m} \quad \text{(Standard deviation of m)}$$

Since V_m and s_m are both on a logarithmic scale, a rough value can be obtained by using the following equation;

$$10^m \times \log_e \times s_m$$

Therefore; $LC_{50} = 10^m \pm 2.3 \times s_m \times 10^m$

CHAPTER III

RESULTS AND DISCUSSION

The results of probit analyses establishing the relative virulence of the different B. bassiana mutants are listed in Table III. The LC_{50} represents the concentration of conidia/ml at which 50% of the exposed larvae are killed within four days. The LD_{50} represents the average number of conidia each larva had acquired at the LC_{50}. These are the two major parameters used to compare the virulence of the 13 mutants that have been studied in depth.

The virulence of the B. bassiana mutants can roughly be divided into three categories. Mutants E_1, E_{1-2} and HP_1 are mutants of high virulence. Less than 20 conidia/larva are required to kill 50% of the larval population and their LC_{50} value is smaller than $2X10^5$ conidia/ml. Mutants E_{1-1}, 7, 16, 23 and R_1 are categorized as having medium virulence. Their LD_{50} is between 20-100 conidia/larva and LC_{50} value lies between $2X10^5$ to $1X10^6$ conidia/ml. Mutants having an LD_{50} of greater than 100 conidia/larva and LC_{50} value greater than $1X10^6$ conidia/ml are considered to have low virulence. Included are mutants 10, 8, 1, 14 and 9.

The relative virulence of B. bassiana mutants when topically applied to the larvae was established using probit analysis. It has been important in our study of B. bassiana to establish such a relative virulence of the fungal mutants. Fungal characteristics

that are related to the infectivity and virulence of the parasite include exocellular enzymes and production of toxins. By correlating these parameters with the relative virulence of the different mutants, a mechanism for the virulence of B. bassiana can perhaps be established.

TABLE III

THE VIRULENCE RANKING OF *BEAUVERIA BASSIANA* MUTANTS

Mutants	LC_{50} (conidia/ml)	LD_{50} (conidia/larva)
E_1	$4.11 \pm 1.10 \times 10^4$	<15
E_{1-2}	$1.07 \pm 0.10 \times 10^5$	~10
HP_1	$1.48 \pm 0.14 \times 10^5$	~10
E_{1-1}	$3.89 \pm 0.27 \times 10^5$	~20
7	$6.31 \pm 0.78 \times 10^5$	35-40
16	$6.46 \pm 1.00 \times 10^5$	35-40
23	$4.79 \pm 0.55 \times 10^5$	70-90
R_1	$3.43 \pm 0.36 \times 10^5$	100
10	$2.92 \pm 0.32 \times 10^6$	150-180
8	$1.39 \pm 0.20 \times 10^6$	180-200
1	$1.66 \pm 0.33 \times 10^6$	~250
14	$9.82 \pm 0.83 \times 10^5$	~320
9	1.00×10^7	~500

SELECTED BIBLIOGRAPHY

Burton, R. L. 1970. A low-cost artifical diet for the corn earworm. J. Econ. Entomol., 63:1969-1970.

Finney, D. J. 1964. Statistical Method in Biological Assay. Hafner Publishing Comp., New York.

Finney, D. J. 1971. Probit Analysis. 3rd ed. pp. 1-66. Cambridge Univ. Press, Great Britian.

Grula, E. A., R. L. Burton, R. Smith, T. L. Mapes, P. Cheung, S. Pekrul, F. R. Champlin, M. Grula and B. Abegaz. 1978. Biochemical basis for the entomopathogenicity of Beauveria bassiana. pp. 192-216. In, C. M. Ignoffo (ed.), Proceedings of the First Joint US/USSR Conference on the Production, Selection and Standardization of Entomopathogenic Fungi of the US/USSR Joint Working Group in the Production of Substances by Microbiological Means. pp. 293.

PART TWO

EXOCELLULAR ENZYMATIC ACTIVITIES OF *BEAUVERIA BASSIANA*

CHAPTER I

INTRODUCTION

The biochemical compositions of the various components of insect cuticles are revealed in recent reports by Hackman (1974, 1976); and Rudall (1963, 1976). In general, the structure of insect cuticles can roughly be described as depicted in figure 1 and it is agreed that the major constituents are proteins and chitin. These two constituents are complexed with each other through covalent and other weaker bonds and these protein-chitin structures are further cross-linked by quinones upon sclerotization. For entomopathogens that initiate an infection by penetrating the integument, the protein-chitin complex will present a formidable barrier. It has been suggested that enzymatic attack is essential (Madlin, 1963; Ferron, 1978) to penetrate this complex structure and the enzymes involved are probably proteases, chitinases and lipases. In our laboratory, Beauveria bassiana has been shown to possess all three of these exocellular enzymes (Mapes, 1974; Grula, et al., 1978) and enzymatic penetration of the intact cuticle has been demonstrated (Pekrul and Grula, 1979).

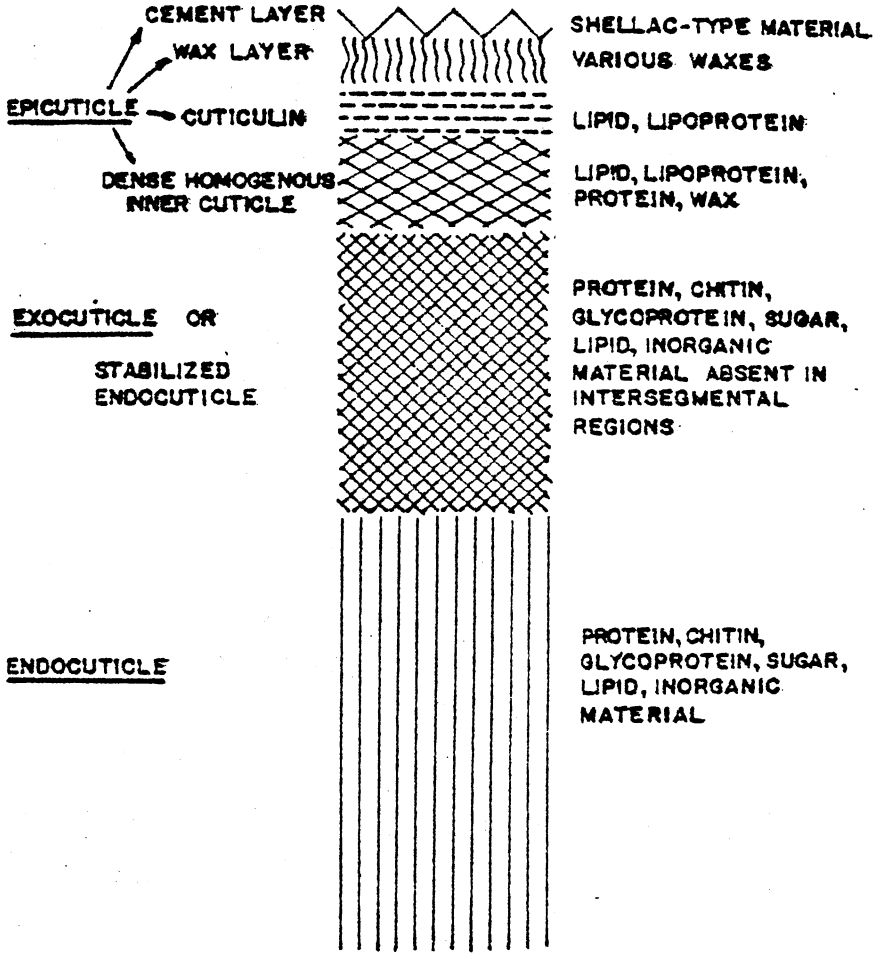
In this study, attempts have been made to relate the infectivity and virulence of B. bassiana to its exocellular enzymatic activities. The problem was approached by comparing the killing of corn earworm larvae by B. bassiana mutants possessing different

exocellular enzymatic activities. If infectivity of the fungus is due only to surface-related phenomena such as germination, growth and enzyme secretion, then mutants having lower pathogenicity by topical application might exhibit elevated levels of virulence when the cuticle barrier is by-passed by injection of conidia. By the same line of reasoning, mutants possessing high pathogenicity when topically applied should not show a drastic change in virulence when directly introduced into the haemocoel since the fungus would not have to by-pass any barrier it cannot overcome by itself.

A second approach is to obtain mutants that are lacking in exocellular proteolytic, chitinolytic or lipolytic activities. If any one of these enzymes is essential for penetration of the cuticle, a negative mutant should be non-pathogenic (or exhibit a significant reduction in virulence) when infection must occur through the host cuticle because it is now enzymatically handicapped.

Figure 1. Composition of the Insect Cuticle. (Chapman, 1969)

INSECT INTEGUMENT



CHAPTER II

MATERIALS AND METHODS

Test Organisms

See Part One, Chapter II.

Colloidal Chitin Preparation

To 20 grams of commercial chitin (Sigma, powdered crab shells) 200 ml of cold concentrated hydrochloric acid was added. The slurry formed was then stirred for two hours on a magnetic stirrer. Colloidal chitin was precipitated by adding the preparation to 2 L of distilled water. The water was removed after the colloidal chitin settled overnight and this washing procedure was repeated several times. After the last washing, the colloidal chitin suspension was dialyzed against large volumes of distilled water to remove the hydrochloric acid. When pH of the water reached 5.0-6.0, the chitin suspension was centrifuged at 3000 g for 15 min and the pellet, consisting of colloidal chitin, was lyophilized and stored at room temperature.

Media and Buffers Used

Solid Media for Assay of Proteolytic Enzymatic Activity

Two types of media were used to assay for exocellular proteo-

lytic enzyme activities. Litmus-milk agar plates consisted of 65 gm Sabouraud dextrose agar (SDA) and 52 gm litmus-milk per L. Gelatin agar consisted of 65 gm SDA and 2 gm gelatin per L. The media were sterilized at 15 pounds pressure for 15 minutes.

Hydrolysis of gelatin was determined by flooding the plates with saturated ammonium sulfate. Proteolytic activities in both media are reported as the ratio of the diameter of clearing to the diameter of the colony.

Solid Media for Assay of Lipolytic Enzyme Activity

Two types of media were used to assay exocellular lipolytic enzyme activity. Tributyrin agar consisted of 65 gm SDA and 10 ml of tributyrin per L. Tween-80 agar consisted of 65 gm SDA, 2 gm $CaCl_2$ and 10 ml Tween-80 per L.

Lipolytic activity on tributyrin agar is reported as the ratio of the diameter of clearing to colony size. For Tween-80 agar, calcium is precipitated directly around colonies due to the acids produced by lipid hydrolysis. Thus hydrolytic activity is reported as the ratio of the diameter of the calcium precipitation ring to colony size.

Phosphate Buffered Saline (PBS)

PBS contains 0.07M NaCl, 0.039M $Na_2HPO_4.7H_20$ and 0.02M KH_2PO_4. This is adjusted to pH 6.5 (with 2N HCl) which is compatible to the pH of the haemolymph (Burton et al., 1972). This buffer was mainly used for injection into corn earworm larvae.

Mineral Salts Solution

The basic salts solution consists of the following: 0.03% $MgSO_4 \cdot 7H_2O$, 0.3% K_2HPO_4 and 0.03% NaCl (added in the order given). Specific carbon, energy and nitrogen sources for germination and growth were sterilized separately, then added to the salts solution after cooling to room temperature.

Chitin Growth Medium

Five types of media containing different preparations of commercial chitin were checked for their ability to support growth of B. bassiana.

Regular chitin medium: 4% commercial chitin in mineral salts solution.

Colloidal chitin medium: 4% colloidal chitin (prepared by concentrated HCl washing) in mineral salts solution.

Regular chitin medium (after hydrolysis in 4N HCl): Commercial chitin was hydrolyzed with 4N HCl at 100°C for 4 hours. The residue was resuspended in mineral salts solution.

Chitin filtrate medium: Regular chitin medium was autoclaved twice (15 min., 15 lbs., 225°C). Supernatant from the second autoclaving was filtered through Whatman No. 1 filter paper and centrifuged at 59,000 g for 20 min., to remove any non-soluble particles. The supernatant was then tested for its ability to support growth of B. bassiana.

Sephadex G-15 gel filtration medium using the chitin filtrate: Chitin filtrate medium prepared as described above and concentrated

by lyophilization. The lyophilizate was redissolved in water (20X concentrated) and passed through a Sephadex G-15 column (150 ml bed volume, 20 ml/hr flow rate, distilled water was used as the eluant). The void volume was collected, lyophilized and redissolved in the mineral salts solution.

Amino Acid-Mineral Salts Medium

Aspartic acid, phenylalanine and alanine at the concentration of 0.1 mg/ml each were made up in the mineral salts solution. This medium was used as a chemically defined medium for germination and growth of the fungus.

Acid Hydrolysis

All five chitin preparations were completely hydrolyzed with 6N HCl at 100^{o}C for 24 hours under vacuum. Presence of amino acids and glucosamine were determined using 2-dimensional cellulose thin-layer chromatography (TLC) and the amino acid analyzer. For details of the TLC and amino acid analyzer procedure, see Part Three, Chapter II.

Assay for Chitinolytic Enzyme Activity

Exocellular chitinase activity of B. bassiana mutants is being studied by a co-worker, Rebecca J. Smith. Cell free supernatants were obtained from the chitin (10%) mineral salts medium at different days post-inoculation and concentrated by dialyzsis against 20% carbowax 6000. The procedure of Jeuniaux (1966) is utilized to assay chitinase activities.

Conidial Injection

Injections of live conidia into the haemocoel of corn earworm larvae (CEW), described in Part One, Chapter II under Target Insect, were accomplished using a Hamilton 10 µl microsyringe. The appropriate number of conidia was usually adjusted to be contained in 1 µl of PBS. This amount was injected into the haemocoel of larvae at the base of the second proleg. Before withdrawing the needle, pressure due to holding of the larvae was released to prevent bleeding. Larvae were discarded whenever excessive bleeding occurred.

Groups of larvae were injected with either 50, 100 or 200-300 conidia. These larvae were reared under normal conditions and mortality was scored at 7 days post-injection. A total of five different mutants were tested. These included E_1, HP_1, R_1, 8 and 14.

Isolation of Negative Mutants

Conidial suspensions were prepared as described in Part One, Chapter II under Conidial Suspension. Five ml of the suspension was placed in a sterile petri plate. Mutation was induced by exposing the conidia to UV light at a distance of 18 inches for five minutes. Mutants surviving the UV irradiation were screened for protease, lipase and chitinase activity.

Protease and Lipase Negative Mutants

After UV irradiation, appropriate dilutions of the conidial suspension were made so that the suspension contained approximately

10^3 live conidia/ml. Aliquots (0.1 ml) of the suspension were plated out on litmus-milk or tributyrin agar plates that also had 0.03% Triton X-100 added. Presence of the detergent prevents spreading of the fungal colonies thus allowing more colonies to be screened per plate. Colonies exhibiting either a reduction or a complete absence of exocellular enzymes were transferred onto fresh media immediately to re-check for enzymatic activity.

Chitinase Negative Mutants

A different approach was tried to obtain chitinase-negative mutants. After conidia were exposed to UV light, they were transferred to a medium containing "chitin" (poly-β-1,4-N-acetylglucosamine) as the sole carbon, nitrogen and energy source. At the same time, a fungicide or antimetabolite or combinations of both were added to kill off all the germinating/growing conidia. Conidia that did not germinate (due to their inability to utilize chitin) were then back-selected by removing the fungicide and supplying these conidia with the amino acid-mineral salts medium. The following antimetabolites and fungicides were used: 6-Mercaptopurine, 6-Methylpurine, 5-Fluorouracil, Hexadecyl Pyridinium chloride, Nystatin, Actinomycin D and Amphotericin B.

CHAPTER III

RESULTS AND DISCUSSION

Exocellular Enzymes

The exocellular proteolytic, lipolytic and chitinolytic activities of selected mutants of B. bassiana are presented in Table I. The mutant strains are arranged in descending order of pathogenicity as determined by probit analysis (see Part One). As can be seen, although different mutants possess different abilities to hydrolyze casein, gelatin, tributyrin, Tween-80 and chitin, no positive correlation can be drawn between the pathogenicity of the fungus and any of its enzymatic activities we have studied.

When these data are arranged according to the order of virulence as established by injecting conidia into the larvae directly (Table II and III), a positive correlation between virulence (by injection) and proteolytic activities becomes evident. Mutants with high proteolytic activities (R_1, E_1 and 14) show high virulence while mutants with low proteolytic activities (HP_1 and 8) show low virulence. Similar correlations cannot be drawn with lipolytic or chitinolytic activities. This suggested that protease activity maybe important in determining the virulence of the fungus once it gained access to the haemocoel.

Furthermore, the increase in virulence of strains R_1 and 14 as compared to E_1 and HP_1 (injection vs. topical application) indicates that the ability to penetrate the cuticle is an over-riding parameter in the pathogenicity of the fungus. Mutant strains R_1 and 14 although having high proteolytic activities (highly invasive), cannot penetrate the cuticle effectively (Pekrul and Grula, 1979) and thus exhibit low virulence when topically applied to the larvae.

Isolation of Negative Mutants

Attempts to isolate negative mutants lacking (or decreased) in proteolytic, lipolytic and chitinolytic activities are presented.

Protease Negative Mutants

Mutant strain HP_1 (a UV-induced mutant of E_1) has decreased hydrolytic activities towards casein and gelatin (Table I). Mutant strain 8 has completely lost its ability to hydrolyze casein, but retains some hydrolytic activities towards gelatin (Table I).

Both mutants still possess the ability to infect the CEW, and both exhibit higher virulence than some mutant strains that possess much higher proteolytic activities. This tends to rule out general proteolysis as the important parameter in the pathogenicity of *B. bassiana*. The fact that gelatin is an extended β-sheet protein whereas casein is a globular protein suggested that infectivity of the fungus may be related to specific hydrolytic activity against the chitin-protein complex of the cuticle.

TABLE I

EXOCELLULAR ENZYME ACTIVITIES OF B. BASSIANA MUTANT STRAINS

Mutants	Casein Hydrolysis 4 day	Casein Hydrolysis 7 day	Gelatin Hydrolysis 4 day	Gelatin Hydrolysis 7 day	Tributyrin Hydrolysis 4 day	Tributyrin Hydrolysis 7 day	Tween-80 Hydrolysis 4 day	Tween-80 Hydrolysis 7 day	Chitinase** 5 day
E_1	1.27	2.00	1.20	1.87	1.20	1.14			55
E_{1-2}	1.28	1.61	1.52	1.15	1.35	1.15	1.55	1.52	
HP_1	0.00	1.17 hz*	1.10 hz	1.20 hz	1.30	1.00	1.30	1.36	49
E_{1-1}	1.21	2.77	1.43	3.30			0.00	1.45	
7	1.27	2.13	1.77	1.87	1.64	1.50	1.25	1.39	
23	1.10 hz	1.61	1.49	1.39	1.28	1.00	1.60	1.49	
R_1	1.30	2.16	2.06	3.00	1.32	1.10	1.38	1.52	51
8	0.00	0.00	1.21	1.08	1.53	2.52	1.25	1.72	
1	1.28	2.32	2.89	3.66	1.00	1.05	2.70	1.64	
14	1.46	2.72	2.08	3.14	1.28	1.14	1.43	1.69	23
9	0.00	1.77	2.02	3.39	1.83	1.15	0.00	1.41	

* hz means zone is hazy instead of clear.

** Activity is expressed as µg N-acetylglucosamine liberated (in 6 hr) by 1 ml (200X conc.) of 5 day B. bassiana spent growth medium (Jeuniaux, 1966).

TABLE II

7 DAY MORTALITY OF 4TH INSTAR CORN EARWORM LARVAE AFTER INJECTION OF B. BASSIANA CONIDIA

	No. Conidia (±10%) / Larva		
Mutant	50	100	200-300
R_1	48% (70 larvae)*	76% (172 larvae)	
E_1	24% (165 larvae)	35% (64 larvae)	
14		29% (215 larvae)	32% (73 larvae)
HP_1		14% (49 larvae)	51% (73 larvae)
8	4% (50 larvae)	10% (112 larvae)	26% (73 larvae)

* Number of larvae injected.

TABLE III

COMPARISON OF VIRULENCE AND PROTEOLYTIC ACTIVITIES AMONG FIVE *B. BASSIANA* MUTANTS

Mutant	Virulence (by injection)[a]		Virulence (LC_{50})[b]		Powdered-milk[c]		Gelatin[c]	
R_1	High	76%	Medium	$3.4X10^5$	High	2.44	High	3.00
E_1	Medium	35%	High	$4.1X10^4$	Medium	2.00	Medium	1.87
14	Medium	23%	Low	$1.0X10^6$	High	2.72	High	3.14
HP_1	Low	14%	High	$1.5X10^5$	Low	1.17	Low	1.20
8	Low	10%	Low	$1.4X10^6$	Low	0.00	Low	1.08

[a] Numbers given are the percent mortality at 7 days after injection of live conidia (100±10%) into 4th instar larvae.

[b] Obtained by Probit Analysis (150-200 larvae/conc.) of the number of dead larvae at 4 days after larvae were allowed to crawl on conidia suspensions on filter paper. Numbers given are conidia/ml.

[c] Numbers given are the ratio of clearing zone (enzymatic activities) to colony size at 7 days after inoculation. Activities were determined using litmus-milk/SDA and gelatin/SDA agar plates.

Lipase Negative Mutant

We were not able to isolate any mutant that completely lacks lipolytic activity.

Chitinase Negative Mutant

Isolation of chitinase negative mutants was not possible for two reasons. Firstly, of all the antimetabolites and fungicides tested, none had a cidial effect towards B. bassiana. The three nucleic acid analogs were not taken up by the fungus at all. Concentrations up to 500 ppm were tested with no effect. Hexadecylpyridinium chloride was toxic to both germinating and non-germinating conidia at 10 ppm. However, at lower concentrations (less than 2.5 ppm) the conidia could overcome the toxic effect of the compound. With nystatin, actinomycin D and amphotericin B the effects were static and back-selection was not applicable. Secondly, of the five chitin preparations tested, none was pure poly β-1,4-N-acetylglucosamine. Ability of B. bassiana conidia (strain R_1) to germinate and grow in all five preparations is summarized in Table IV. Amino acid analyses after complete acid hydrolysis of the commercial, colloidal and 4N HCl-treated chitin are presented in Table V. As shown in Table V, none of these are pure "chitin". Using TLC, hydrolyzates of the filtered chitin material and Sephadex eluates of these chitin filtrates were shown to possess large amounts of amino acids. Germination and growth of conidia in these two chitin preparations (Table IV) suggested that the fungus is actually growing on the

proteins washed off the commercial chitin during autoclaving. Inability of conidia to utilize colloidal chitin and 4N HCl-treated commercial chitin, which have large amounts of protein stripped off, gives support to the belief that the fungus does not grow (at least initially) on the poly-N-acetylglucosamine contained in regular chitin but rather the proteins associated with the chitin.

Therefore, unless we can come up with a pure chitin preparation, an exocellular chitinase negative mutant would have to be screened using the brute force method (purification and assay of colonies selected at random for chitinase activity).

TABLE IV

GERMINATION AND GROWTH OF *B. BASSIANA* (STRAIN R_1) IN DIFFERENT PREPARATIONS OF CHITIN

	Germination	Growth[a]
Regular Chitin Medium	+	+
Colloidal Chitin Medium	-[b]	-[b]
Regular Chitin after 4N HCl Treatment	-	-
Chitin Filtrate Medium	+	+
Sephadex G-15 Gel Filtration of the Chitin Filtrate	+	+
Colloidal Chitin plus Chitin Filtrate Medium	+	+

[a] Growth was defined as having long hyphae (50-100 μm) at 24 hours post-inoculation.

[b] Only about 5% of the conidia germinated and these failed to show any elongation. Hyphal tips started to wither within 24 hours after conidia were first observed to germinate and no new germination was observed thereafter.

TABLE V

AMINO ACID COMPOSITIONS OF THE THREE DIFFERENT CHITIN PREPARATIONS USED TO TEST THE GERMINATION AND GROWTH OF *B. BASSIANA* CONIDIA

Amino Acid	Regular[a] Chitin	Colloidal[a] Chitin	4N HCl treated[b] Regular Chitin
Asp	11	13	
Thr	5	5	
Ser	8	8	
Glu	13	13	
Pro	7	7	
Gly	12	13	
Ala	13	12	
Val	6	5	
Met	1	0.5	
Ile	2	2	
Leu			
Tyr			
Phe	5	3	
Trp			
His	5	6	
Lys	2	2	
Arg	6	6	
Glu-NH_3	330	980	160

[a] Numbers given are % of total amino acids as determined by amino acid analysis.

[b] Amount of amino acids in this chitin preparation is extremely small. Their presence was detected by spotting large amount of the sample on TLC plates. In both TLC and amino acid analyses glucosamine is the predominant compound.

CHAPTER IV

SUMMARY

In summary, once B. bassiana passes the cuticle and gains access to the haemocoel, virulence of the fungus appears to be related to its ability to secrete exocellular protease. Fungal activities such as germination, growth and enzyme secretion on the larval surface were shown to be important in the penetration of the larval integument by the fungus (Pekrul and Grula, 1979). However, except for the proteases, results of this study did not point to any positive relationship between the other exocellular enzymatic activities (Lipolytic and chitinolytic) of the fungus and its entomopathogenicity. Data to establish this point were obtained using protease-negative and protease-deficient mutants which exhibited poor or minimal virulence when injected into the haemolymph directly. This was in contrast to mutant strains secreting large amounts of proteases.

The apparent correlation between exocellular proteolytic activities and virulence (by injection) of the fungus is probably due to the enhancement of infectivity in the presence of proteases secreted by the pathogen. Large amounts of exocellular proteases will definitely enable the fungus to infiltrate and invade tissues more effectively once it reaches its target organ, the alimentary tract (see Part Four for preferential invasion of gut tissues).

SELECTED BIBLIOGRAPHY

Burton, R. L., D. G. Hopper, J. R. Sauer and J. H. Frick. 1972. Properties of haemolymph of the corn earworm. Comp. Biochem. Physiol., 42B:713-716.

Chapman, R. F. 1969. The Insect: Structure and Function. p. 427 American Elsevier Publishing Company Inc., N. Y.

Ferron, P. 1978. Biological control of insect pests by entomogenous fungi. Ann. Rev. Entomol., 23:409-442.

Grula, E. A., R. L. Burton, R. Smith, R. L. Mapes, P. Cheung, S. Pekrul, F. R. Champlin, M. Grula and B. Abegaz. 1978. Biochemical basis for the entomopathogenicity of Beauveria bassiana. pp. 192-216. In, C. M. Ignoffo (ed.), Proceedings of the First Joint US/USSR Conference of the Production, Selection and Standardization of Entomopathogenic Fungi of the US/USSR Joint Working Group on the Production of Substances by Microbiological Means. 293 pp.

Hackman, R. H. 1974. Chemistry of the insect cuticle. pp. 216-270. In, M. Rockstein (ed.), The Physiology of Insecta, Vol. IV. Academic Press, N. Y.

Hackman, R. H. 1976. The interactions of cuticular proteins and some comments on their adaptions to function. pp. 107-120. In, H. R. Hepburn (ed.), The Insect Integument. Elsevier Scientific Publ. Co., Amsterdam.

Jeuniaux, C. 1966. Chitinases. pp. 644-650. In, E. Neufeld and V. Gensburg (ed.), Methods in Enzymology, Vol. 8. Academic Press, N. Y.

Madelin, M. F. 1963. Disease caused by hyphomycetous fungi. pp. 233-271. In, E. A. Steinhaus (ed.), Insect Pathology, Vol. 2. Academic Press, N. Y.

Mapes, T. L. 1974. Exocellular enzymes of Beauveria bassiana and their possible relationship to entomopathogenicity. Masters thesis, Oklahoma State University.

Pekrul, S. and E. A. Grula. 1979. Mode of infection of the corn earworm (Heliothis zea) by Beauveria bassiana as revealed by scanning electron microscopy. J. Invertebr. Pathol., 34:238-247.

Rudall, K. M. 1963. The chitin-protein complexes of insect cuticles. Adv. Insect Physiol., 1:257-313.

Rudall, K. M. 1976. Molecular structure in arthropod cuticles. pp. 21-41. In, H. R. Hepburn (ed.), The Insect Integument. Elsevier Scientific Publ. Co., Amsterdam.

PART THREE

IN VIVO EVENTS ASSOCIATED WITH ENTOMOPATHOLOGY OF *BEAUVERIA BASSIANA* FOR THE CORN EARWORM (*HELIOTHIS ZEA*)

CHAPTER I

INTRODUCTION

The basis for entomopathogenicity of Beauveria bassiana has been under investigation for many years. Ability of germinating fungal conidia to enzymatically penetrate the intact cuticle of corn earworm (CEW) larvae has now been clearly shown (Grula et al., 1978; Pekrul and Grula, 1979). Not much information is available, however, with regard to how the fungus eventually causes death of an insect larva once it gains access to the haemocoel.

Production of toxic elements is considered to be an important aspect of pathogenicity of B. bassiana. Although a number of B. bassiana toxins have been identified (Dresner, 1950; Kucera and Samsinakova, 1968; West and Briggs, 1968; Huang and Shapiro, 1971), convincing evidence is lacking for their production during an actual infection. Work from this laboratory has shown that the killing of CEW larvae does not involve the in vivo release of any nonpolar ionophore-like toxin by the fungus; indeed, even the release of water-soluble toxin(s) into the haemolymph during infection can be questioned (Champlin and Grula, 1979).

Another possible mechanism of pathogenicity could involve invasion of haemocoel tissues and/or organs leading to malfunction of vital physiological activities. Alimentary tract, body muscle, silk gland, nervous system, fat body and tracheal system have all

been reported to be invaded by B. bassiana (Broome et al, 1976; Wasti and Hartman, 1975; Lefebvre, 1934) and malfunction of any of these organs would probably result in death. Additionally, depletion of nutrients (starvation) by the fungus during mycosis or alteration of haemocoel pH could also be possible causes of death.

The primary purpose of this investigation was to study events occurring after the fungus had gained access to and established itself within the haemocoel of the insect. Physiological, cytological and biochemical changes must result from interactions between metabolic activities of the fungus and defense mechanisms of the host. Since haemolymph is the physiological linkage between organs and tissues in the insect, this fluid component should reflect changes that occur after infection. These changes could be due to degradation and depletion of host haemolymph components by the fungus and/or attempts by the host to combat the pathogen. Since B. bassiana can readily utilize many proteins, amino acids and carbohydrates for germination and growth (Smith and Grula, 1981) and these substances are known to be present in larval haemolymph, our study of infected haemolymph centered on these particular components as well as the possible invasion of specific tissues.

CHAPTER II

MATERIALS AND METHODS

Beauveria bassiana Cultures and Conidial Suspensions

B. bassiana was cultured on Sabouraud's Dextrose Agar (SDA) supplemented with 0.3% yeast extract. Five different mutant strains were used in this study. They were chosen because of differences in virulence when topically applied (rated by probit analysis with 1st instar larvae; see Table III, column 2 and Part One) as well as differences in proteolytic activities (rated by ability to hydrolyze powdered-milk and gelatin; see Table II, column 3 and 4; also see Results of Part Two).

Freshly sporulated cultures were harvested by removing conidia from the agar surface using 0.03% (v/v), of the non-ionic detergent, Triton X-100, followed by 2 washes in sterile distilled water. This procedure effectively wets and disperses the conidia allowing quantitative manipulations such as titer determinations and plate counting. Conidial suspensions were prepared fresh each time and appropriate dilutions were made immediately prior to each experiment. To ensure uniformity in conidial dosage, plate counts were done to determine the number of viable conidia in a given suspension. Triton X-100 (0.03%) was always present in agar media. Presence of the detergent

reduces spreading of the fungal colonies and permits reliable counts of up to about 300 colonies per plate. An optical density of 1.0 at 540 nm (Spectronic 20 spectrophotometer) indicates a concentration of approximately $2X10^8$ conidia/ml.

Injections were accomplished using a Hamilton 10 µl microsyringe. The appropriate number of conidia was usually adjusted to be contained in 1 µl of phosphate buffered saline (PBS; 0.07M NaCl, 0.03M $Na_2HPO_4.7H_20$ and 0.02M KH_2PO_4) at pH 6.5 which is compatible with pH of the haemolymph (Burton et al., 1972). This amount was injected into the haemocoel of larvae at the base of the second proleg. Before withdrawing the needle, pressure due to holding of the larvae was released to prevent bleeding (Cheung et al., 1978). Larvae were discarded whenever excessive bleeding occurred.

Selection of Larvae

Corn earworm larvae were reared on the CSM artifical diet of Burton (1970). Middle 4th instar larvae, 8-9 days old and weighing 90-100 mg. were stored at 10^oC in the dark. This treatment retards physiological development and feeding thus allowing for the collection of sufficient numbers of larvae (50-60) having the same weight and development to perform each individual experiment. Importance of such synchronization will be discussed.

Larval Development

To study changes in haemolymph components during development, selected larvae (end of the 3rd molting) were stored in the cold until 25 larvae had been collected. These early 4th instar larvae were then taken out of the cold and allowed to warm to room temperature. Their weight gain and haemolymph protein development at 25°C were followed for 50 hours until just prior to the 4th molting. Results are shown in figures 3 and 4.

Starvation of Larvae

Fifty synchronized larvae of the same weight (90-95 mg) were utilized. Forty were supplied with only water for 24 hours. The other ten were placed on CSM diet and served as controls. Weight gain of both control and starved larvae was followed. At the same time, haemolymph samples were collected from both groups at 6, 12, 18 and 24 hours and their haemolymph proteins were compared using polyacrylamide gel electrophoresis (PAGE). After the larvae had been starved for 24 hours, haemolymph from 25 starved larvae was collected and utilized for amino acid and carbohydrate analyses.

Haemolymph Activity as Related to Infection

One µl of a conidial suspension (OD_{540}=1.0) was injected into middle 4th instar larvae. This injection allowed each larva to receive $\sim 2X10^5$ conidia which was necessary to ensure that death of larvae occurred before they molted (see Figure 3). After the

live conidia had been injected, the same suspension was boiled for 10 min cooled to room temperature and 1 µl of such heat-killed conidia injected into additional larvae (controls).

At 6, 12, 18 and 24 hr postinjection (PI), haemolymph from infected and control larvae was collected in capillary tubes pre-coated with a small amount of phenylthiourea to prevent melanization. Haemocytes were removed by centrifugation at 1000 g for 10 min and proteins in infected and non-infected haemolymph samples compared using PAGE. Additionally, in the 24 hr PI samples, proteins were precipitated 2X by addition of ethanol (50-50 v/v each time) and the final supernatant (66% ethanol) was analyzed for amino acids and carbohydrates.

Cytological Events Related to Infection

Conidial suspensions having an optical density of 1.0 at 540 nm were diluted to 10^{-2} and 10^{-3} with PBS. Groups of larvae were then injected with 0.5, 1.0 and 2.0 µl of the 10^{-3} dilutions; thus introducing either 50, 100 or 200-300 live conidia into each larval group respectively. These larvae were reared under normal conditions and mortality was scored at 5, 6 and 7 days PI. Virulence, (in this case penetration of the integument was by-passed by injection) of the various fungal mutants, was compared and data are presented in Table II.

Another group of larvae was injected with 1 µl of the 10^{-2} dilution introducing $\sim 2X10^{3}$ live (strain R_1) conidia into each

larva. At 15 min followed by every 24 hr PI, haemolymph was taken from the larvae for study of phagocytosis using a phase-contrast microscope. Stained slides were also prepared by drawing a drop of haemolymph over a clean slide with the edge of another slide followed by vapor-fixation in 40% acetic acid in formalin for 6 to 6.5 min prior to flooding with Giemsa stain. Larvae that had shown symptoms of sickness (cessation of feeding, sluggishness and weight loss) were dissected and the silk glands, gonads, alimentary tract, Malpighian tubules, body wall and fat bodies were excised. These organs were studied under the phase-contrast microscope to determine which had been invaded by the fungus. They were also fixed in alcoholic Bouin's fixative and serial paraffin sections were obtained and stained with Mayer's hemalum and eosin (Grimstone and Skae, 1972).

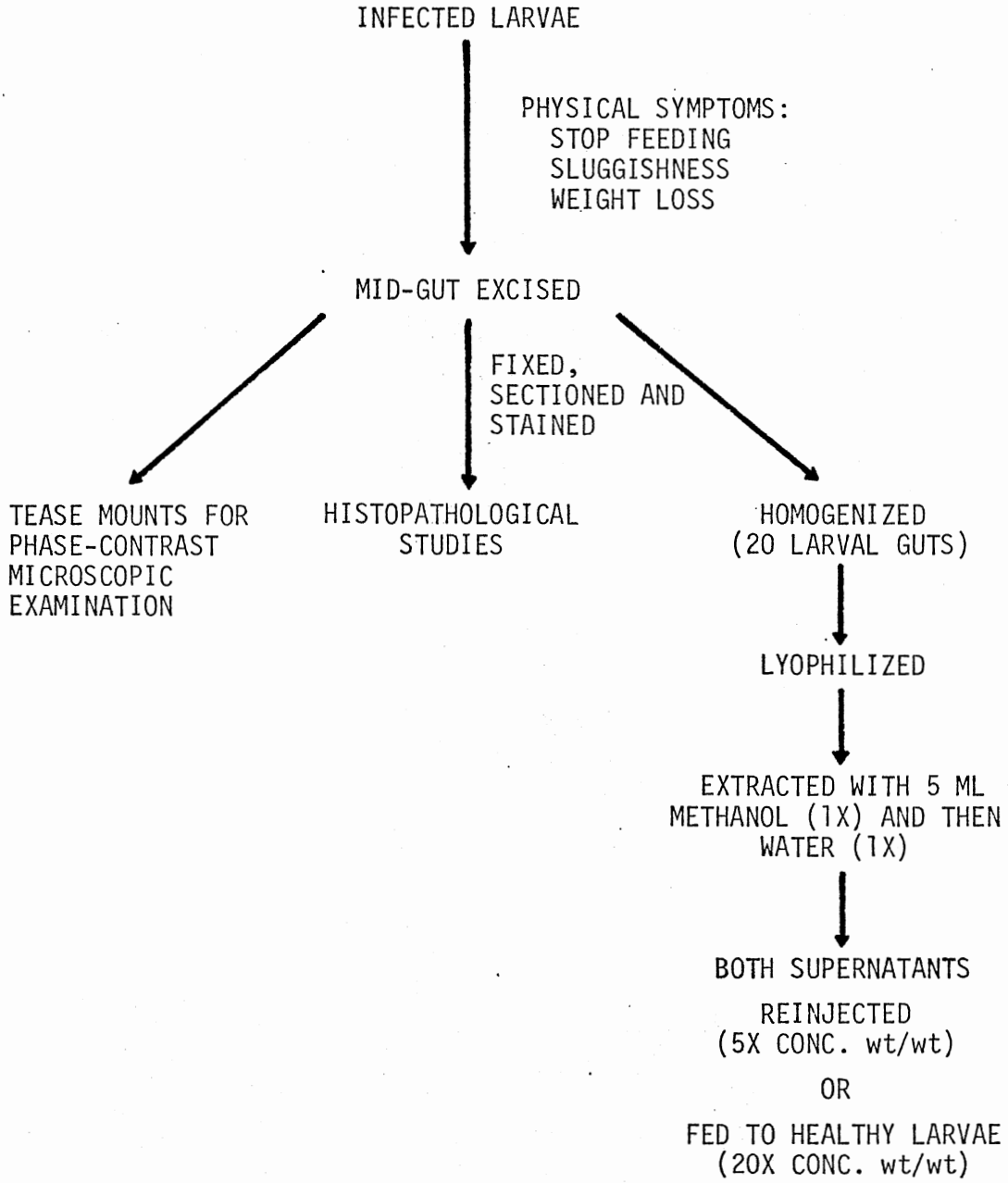
In Vivo Toxin Production

To study possible production of toxin by B. bassiana in vivo, gut tissues were excised and treated as shown in Figure 1.

Polyacrylamide Gel Electrophoresis (PAGE)

Slab polyacrylamide gels were prepared having the following composition: 6.5% acrylamide, 2.22 mg/ml bisacrylamide, 0.31% glycine, 0.25 mg/ml Tris. 0.7 mg/ml ammonium persulfate and 0.35 µl/ml TEMED. The gels were allowed to polymerize and individual haemolymph samples (2.5 µl) were layered into separate wells on the slab. Electrophoresis was performed using a glycine/Tris

Figure 1. Flow Chart for Study of Toxin Production During Infection by *B. bassiana*.


INFECTED LARVAE
PHYSICAL SYMPTOMS:
STOP FEEDING
SLUGGISHNESS
WEIGHT LOSS
MID-GUT EXCISED
FIXED,
SECTIONED AND
STAINED
TEASE MOUNTS FOR
PHASE-CONTRAST
MICROSCOPIC
EXAMINATION
HISTOPATHOLOGICAL
STUDIES
HOMOGENIZED
(20 LARVAL GUTS)
LYOPHILIZED
EXTRACTED WITH 5 ML
METHANOL (1X) AND THEN
WATER (1X)
BOTH SUPERNATANTS
REINJECTED
(5X CONC. wt/wt)
OR
FED TO HEALTHY LARVAE
(20X CONC. wt/wt)

1

buffer (0.25% glycine, 0.06% Tris), pH 8.0, at 10 mA, 100 V for one hour. Gels were stained with 0.2% Coomassie Blue for protein visualization.

Amino Acid Analysis

In addition to thin-layer chromatography, free amino acids in all haemolymph samples were identified and quantitated using an amino acid analyzer. The analyses were performed at 60°C on a Durum DC6A column using a citrate buffer (Liao et al., 1973).

Thin-Layer Chromatography (TLC)

Two types of TLC were performed. First, TLC plates coated with Silica Gel H (50 gm/125 ml 0.15M KH_2PO_4) were used for separation of carbohydrates. The plates were developed in an acetone:n-butanol:water (5:4:1) solvent at 37°C to avoid the separation of solvent components. Sugar visualization reagents (Dawson et al., 1979) included periodate/permanganate spray (for all carbohydrates), aniline/diphenylamine (for reducing sugars) and acetylacetone/p-dimenthylaminobenzaldehyde spray (for amino sugars). Second, TLC plates with powdered cellulose MN300 (15 gm/100 ml water) were used for separation of amino acids. The cellulose plates were pretreated by developing overnight in isopropanol:water:formic acid (80:20:4). This treatment will accumulate impurities present in cellulose at the leading edge of the plates (solvent front) where they can be scraped off with a razor blade. Two-dimensional chromatography was then performed using the solvent system developed by Heathcote and

Jones (1965). Amino acids and other amines were detected by spraying the plates using 0.4% ninhydrin in acetone.

CHAPTER III

RESULTS

Haemolymph Development

Haemolymph from 4th instar larvae contains at least 24 proteins (Figure 2). Major proteins include bands 4, 5, 6 and 8. All four proteins stained positive with lipid stains (oil red or Sudan Black B) while only 4, 6 and 8 stained positive with Periodate-Schiff glycoprotein stain (Dawson et al., 1979). In addition, all or one of the bands running to the bottom of the gel (22-24) also gave a positive glycoprotein reaction. Because these bands did not separate well we cannot be sure which band is the glycoprotein. Protein bands 4 and 6 are the two major pigments within the haemolymph; band 4 is green and 6 is blue before staining.

After the third molt, an increase in haemolymph protein concentration and larval weight was observed (Figures 3 and 4). Prior to the 4th molt (50 hr), the proteins rapidly decrease in concentration; most notably bands 4, 5, 6 and 8. This suggested to us that these lipo- and glyco- proteins serve as carbon, nitrogen and energy reservoirs for larval developmental processes. Because of this rapid change in haemolymph proteins and gain in weight, larvae used in infection experiments had to be carefully selected. Middle 4th instar larvae, weighing 90-95 mg and having a well developed

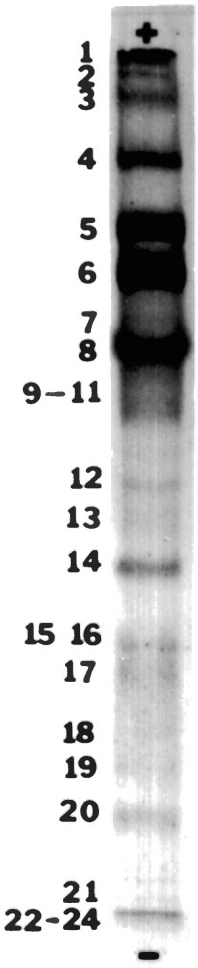
haemolymph protein pattern (16-20 hrs; Figure 4) were chosen and their body weight was synchronized to a satisfactory degree by cold storage until sufficient larvae had been collected. If larval weight (which was used in these experiments as the major index of physiological development) is not controlled, differences in individual haemolymph proteins during growth will interfere with the final results, making interpretation of data virtually impossible.

Haemolymph Components After Infection

Figures 5, 6 and 7 show a comparison of haemolymph samples from control and infected larvae. The most prominent feature is the decrease in protein concentration after larvae have been infected. At 6 hr PI the decrease in haemolymph proteins is obvious, and by 24 hr PI the decrease is so drastic that some of the bands disappear. Although infections by three strains of B. bassiana having different proteolytic activities (Table III, column 4 and 5) are presented, the decrease in haemolymph proteins was observed with all strains.

Free amino acids present in the haemolymph of healthy and infected larvae are shown in Table I. A high level of basic amino acids is present in healthy haemolymph. Histidine, lysine and arginine add up to approximately 30% of the total. Other major amino acids are threonine, serine and proline. This particular distribution of amino acids was also observed in infected insects, except that an increase in the total amount of basic amino acids occurs after infection by four B. bassiana strains; however, there

Figure 2. Proteins Present in the Haemolymph of 4th Instar Corn Earworm Larvae. Lipoprotein (Lp), Glycoprotein (Gp).



Pigment(green) Lp Gp

Lp

Pigment(blue) Lp Gp

Lp Gp

Pigment (red) Gp

2

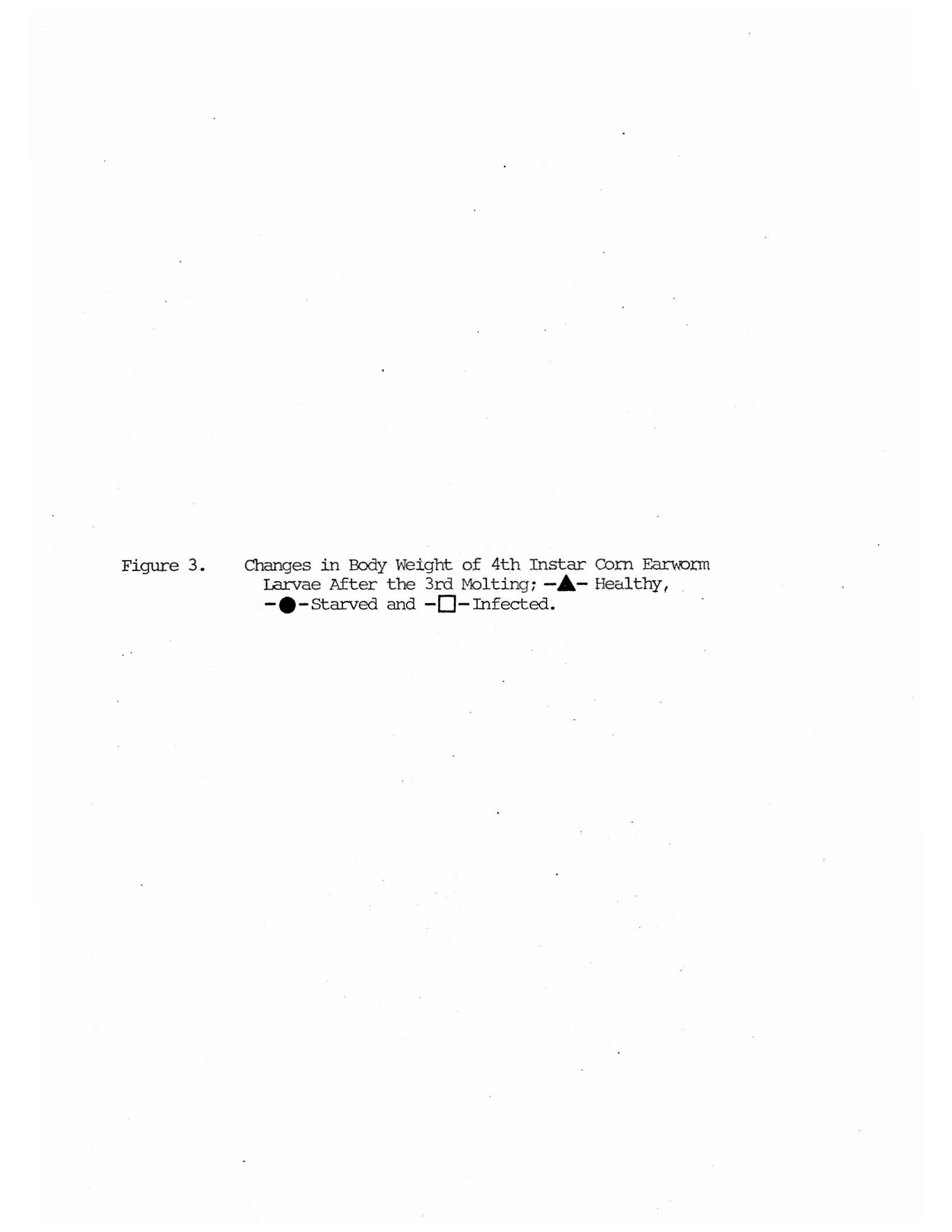
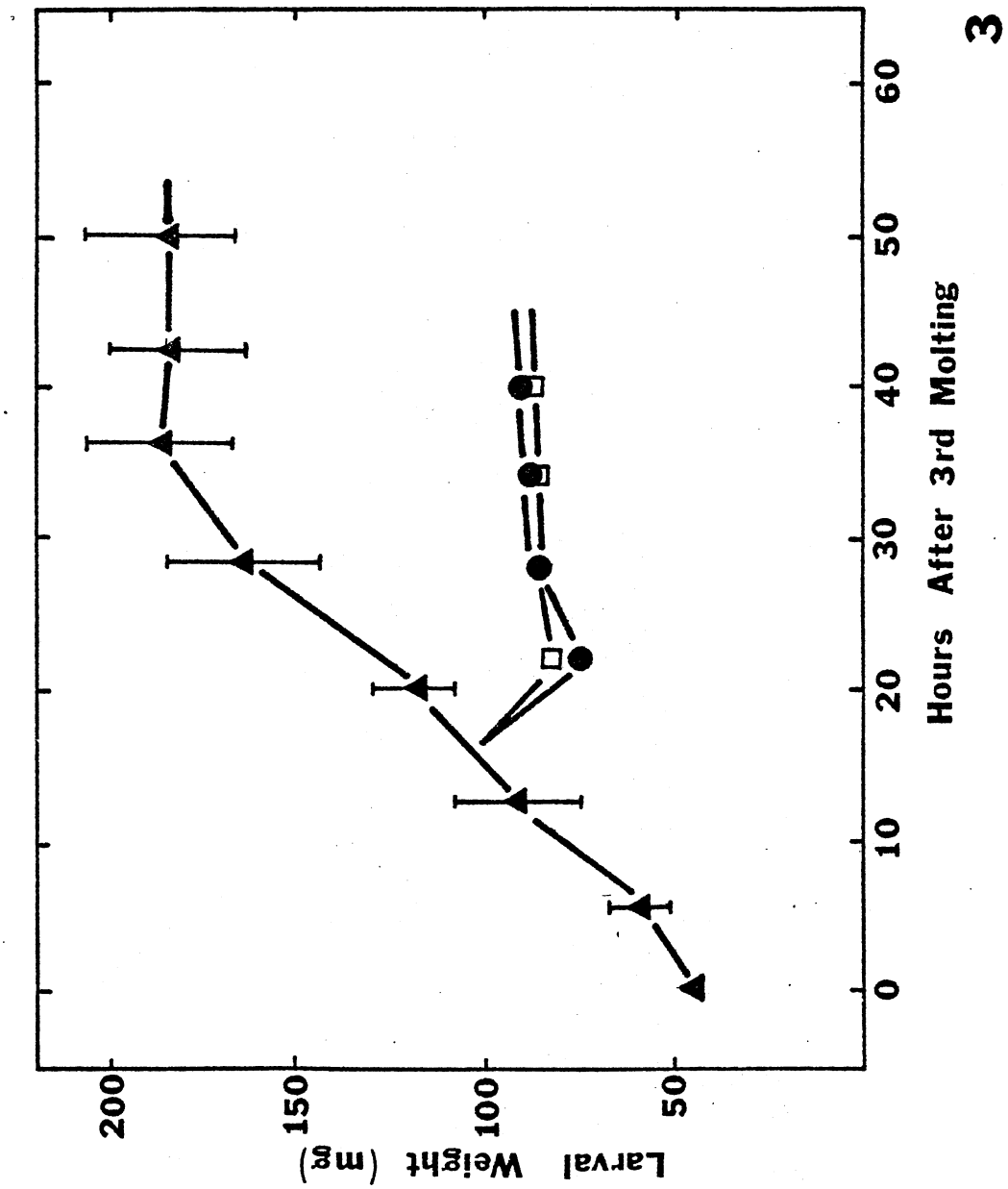


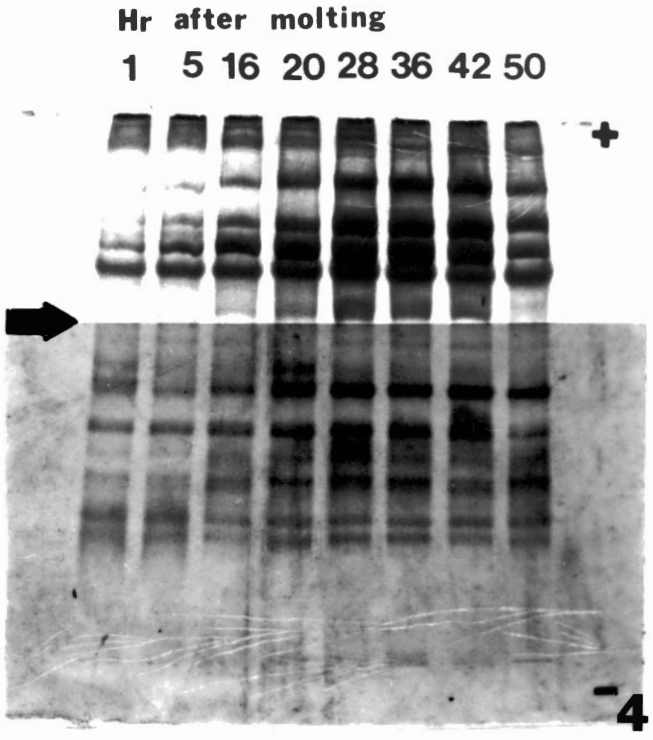
Figure 3. Changes in Body Weight of 4th Instar Corn Earworm Larvae After the 3rd Molting; -▲- Healthy, -●-Starved and -□-Infected.

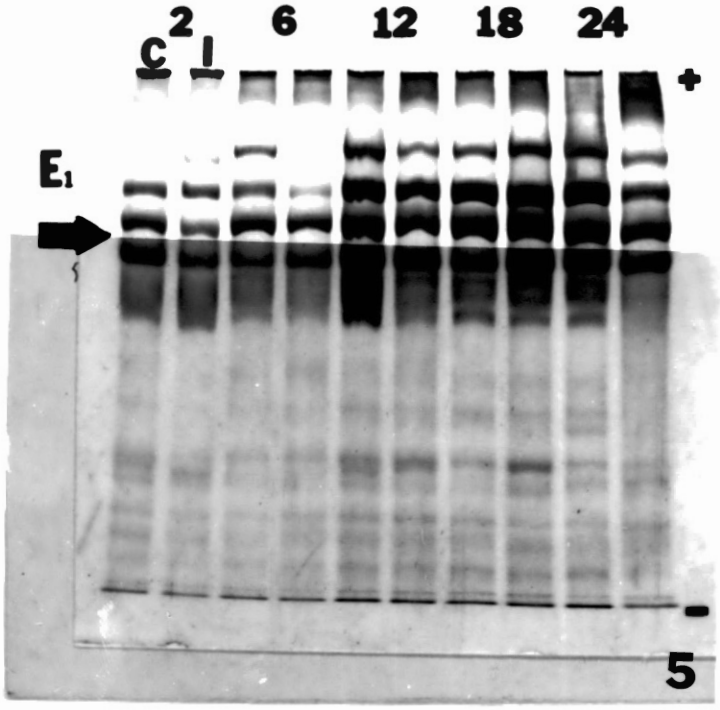

Larval Weight (mg)
200
150
100
50
0
10
20
30
40
50
60
Hours After 3rd Molting

3

Figure 4. Changes in Haemolymph Proteins During Development of the Corn Earworm Larva. Data Shown Were Obtained at Different Hrs After the 3rd Molting. To Better Resolve the Light Bands on the Botton One-half of the Gels, After a 3X Increase in Exposure Time Was Made During Photographic Development of the Lower Half (Areas Below Arrow) of the Picture.

Figure 5. Electrophoretic Analysis of Larval Haemolymph Proteins After Injection of Conidia from Mutant E_1. Haemolymph from Control (C) and Infected (I) Larvae are Presented in Pairs at 2, 6, 12, 18 and 24 Hr Postinjection. Photographic Treatment was Given in Fig. 4.


Hr after molting
1 5 16 20 28 36 42 50
+
-
4


C 2 1
6
12
18
24
+
E1
-
5

is no selective depletion of any particular amino acid. In addition to all of the regular amino acids identified using the amino acid analyzer, several unknown amines are also present in the haemolymph (Figure 10); however, their concentrations do not seem to vary greatly during infection or starvation. One of the major amines has been identified as putrescine and is the subject of a separate chapter in this thesis (Part Five).

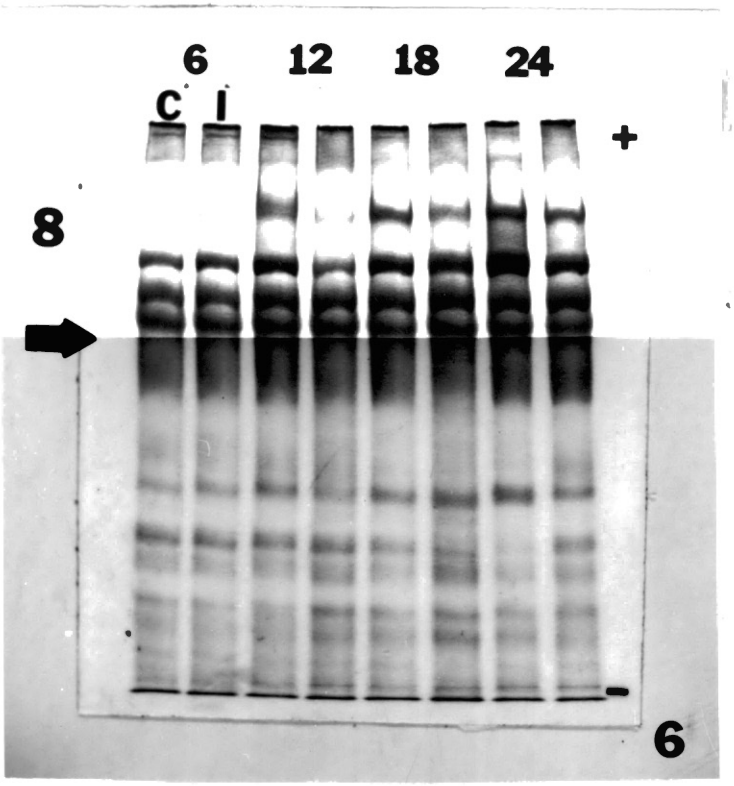
TLC chromatography (Figure 9) reveals that a total of 3 major and 1 minor sugar components are present. Spot number 2 appears to be trehalose; the other three have not been identified. All four sugars react negatively in reducing sugar tests. Except for loss of the minor sugar (spot 4) and slight gain in glucose (usually not observed in the healthy larvae), infected larvae do not show any significant loss or alteration of haemolymph carbohydrates.

Haemolymph Components After Starvation

Throughout these experiments, infected larvae were observed to stop feeding on the artifical diet. Larvae were therefore starved for 24 hrs and haemolymph from such larvae compared with that of non-starved insects. A decrease in haemolymph proteins soon occurs in starved larvae (Figure 8). The fairly rapid decrease in protein bands 4, 5, 6 and 8 lends support to the idea that these lipo- and glyco- proteins are reservoirs of carbon, nitrogen and energy. An important point to be made is that in no case did starvation result in the death of any larvae, even when continued

Figure 6. Electrophoretic Analysis of Larval Haemolymph Proteins After Injection of Conidia from Mutant 8. Haemolymph from Control (C) and Infected (I) Larvae are Presented in Pairs at 6, 12, 18 and 24 Hr Postinjection. Photographic Treatment was a Given in Fig. 4.

Figure 7. Electrophoretic Analysis of Larval Haemolymph Proteins After Injection of Conidia from Mutant 14, Haemolymph from Control (C) and Infected (I) Larvae are Presented in Pairs at 6, 12, 18 and 24 Hr Postinjection. Photographic Treatment was as Given in Fig. 4.


6
12
18
24
C
I
+
8
-
6

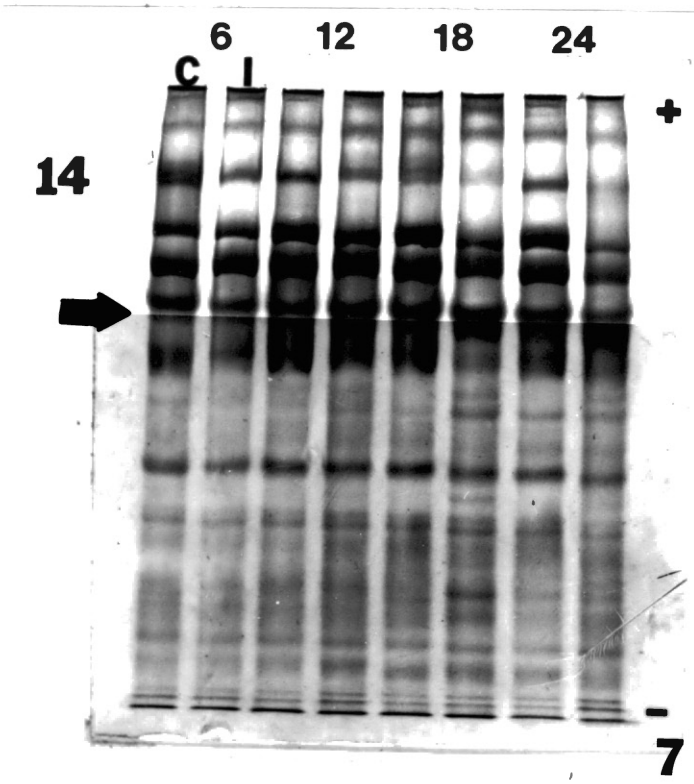
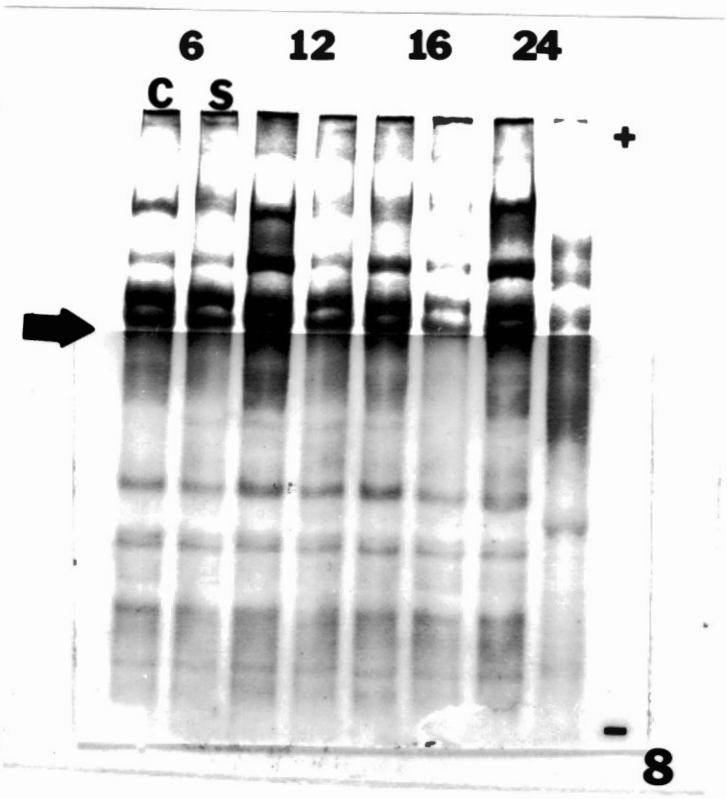

6
12
18
24
C
I
+
14
-
7

Figure 8. Electrophoretic Analysis of Haemolymph Proteins During Starvation of 4th Instar Corn Earworm Larvae. Haemolymph from Control (C) and Starved (S) Larvae are Presented in Pairs at 6, 12, 18 and 24 Hr of Starvation. Photographic Treatment was as Given in Fig. 4.

Figure 9. Carbohydrates Present in the Haemolymph (After Removal of Haemocytes and Proteins) of Healthy, Starved and Infected Larvae. Mutants Used Included E_1, HP_1, R_1, 8 and 14. Solvent: Acetone:n-Butanol:Water (5:4:1). Rhamnose (Rha), Xylose (Xyl), Glucose (Glu) and Trehalose (Tre) are Shown as Sugar Standards.


6
12
16
24
C
S
+
-
8

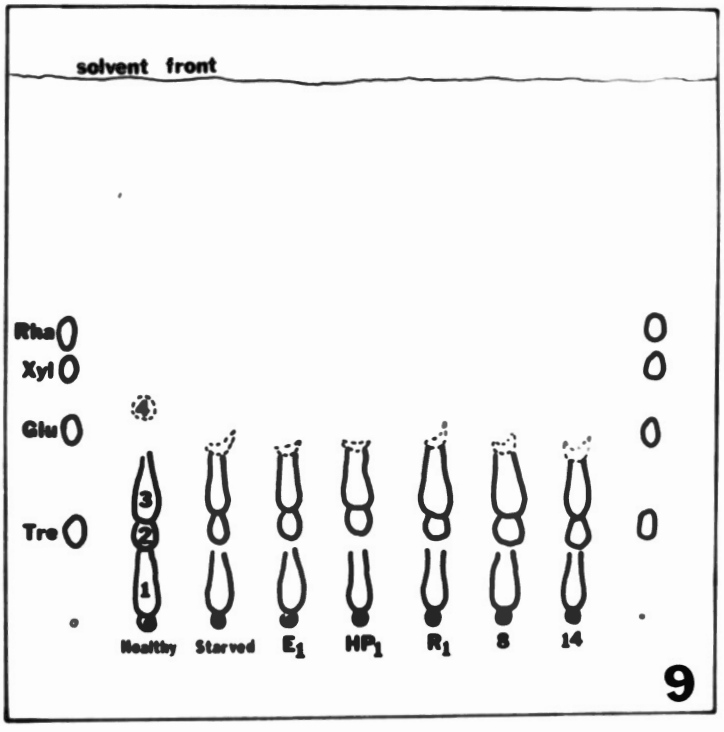

solvent front
Rha
Xyl
Glu
Tre
4
3
2
1
Healthy
Starved
E1
HP1
R1
8
14
9

Figure 10. Amino Acids and Amines of Haemolymph (After Removal of Haemocytes and Proteins) from Corn Earworm Larvae. Solvent 1: Isopropanal: Water: Formic Acid (80:20:4); Solvent 2: Tert-Butanol: Methyl Ethyl Ketone: Water: 88% NH_4OH (50:30:10:10). Compounds Marked With an X are Unidentified Ninhydrin-Positive Components. Putrescine (Put).

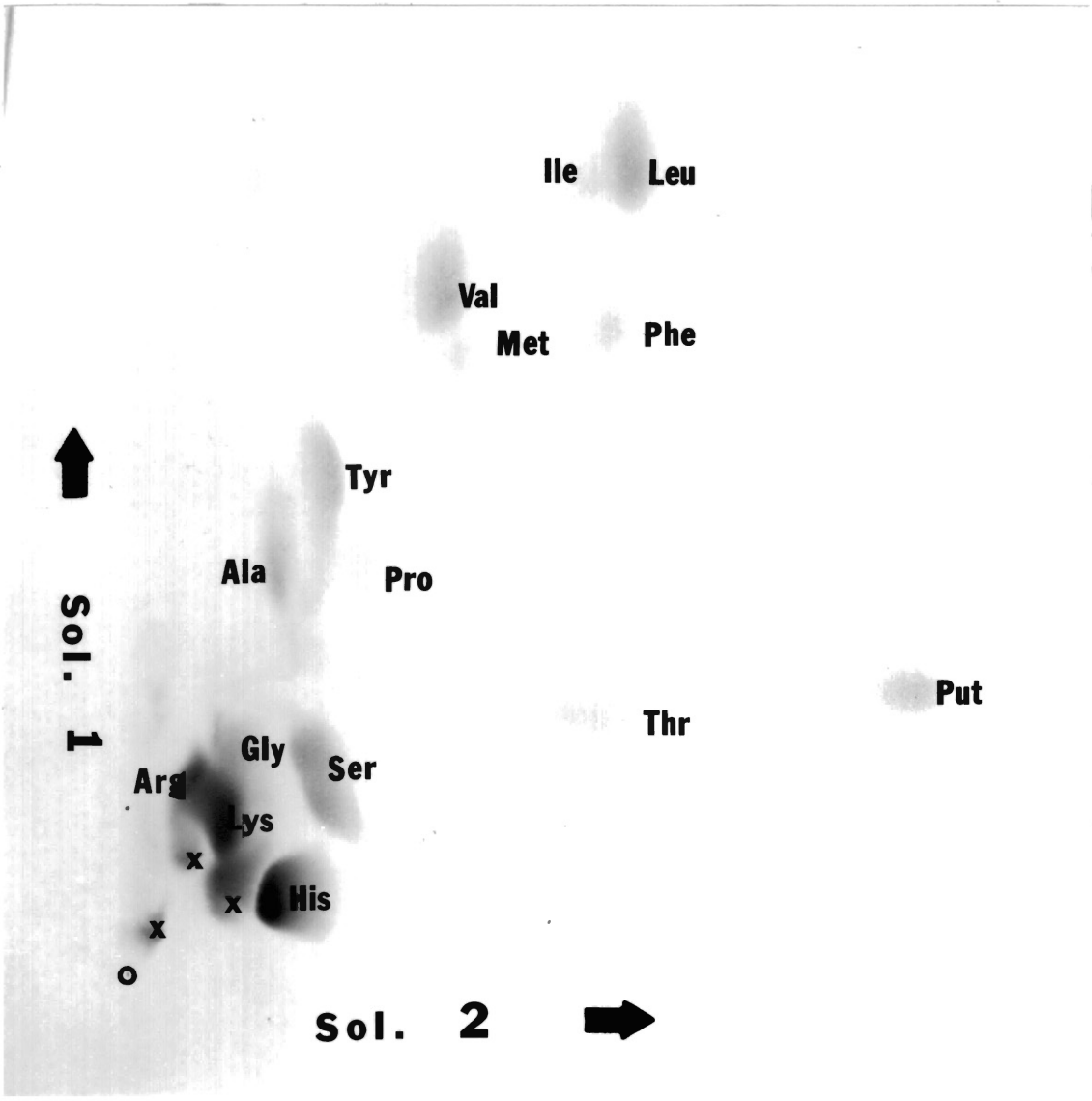

Ile
Leu
Val
Met
Phe
Tyr
Ala
Pro
Put
Thr
Gly
Ser
Arg
Lys
His
Sol. 1
Sol. 2

TABLE I

AMINO ACIDS IN THE HAEMOLYMPH OF 4TH INSTAR CORN EARWORM LARVAE BEFORE AND AFTER INFECTION BY DIFFERENT MUTANTS OF *B. BASSIANA* [a]

Amino Acids	Healthy Larvae (2)	Starved Larvae (1) [b]	Mutant Strain [c] E_1 (3)	R_1 (2)	8 (1)	14 (3)
Asp	0.7	0.3	< 0.1	0.2	1.5	0.5
Thr+Ser	28.0	21.3	34.0	31.0	27.8	23.4
Glu	0.1	1.4	0.5	0.3	0.1	0.6
Pro	13.0	11.1	10.7	12.1	11.3	13.1
Gly	4.3	3.8	4.2	4.1	3.9	4.0
Ala	2.7	3.4	2.5	2.4	2.7	2.8
Cys	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Val	5.0	2.3	2.2	3.7	5.1	4.4
Met	0.7	0.6	0.7	0.6	0.4	0.8
Ile	1.6	1.0	1.0	1.4	1.7	1.7
Leu	4.6	2.8	3.3	3.7	4.0	4.2
Tyr	5.5	6.9	5.0	4.8	5.3	4.5
Phe	1.7	1.2	1.1	1.6	2.4	1.5
Trp	0.4	1.7	< 0.1	< 0.1	< 0.1	< 0.1
His	13.6	21.1	14.0	14.7	13.0	15.0
Lys	12.0	16.6	13.2	12.2	12.6	15.0
Arg	5.6	4.2	7.3	7.3	7.4	8.0
(Total μM/ml)	882	557	584	775	878	672

[a] Larvae were injected with ~$2X10^5$ conidia/larva, and haemolymph samples were collected at 24 hr postinjection. Data are given as percent of total amino acids.

[b] Larvae were taken off diet and given only water for 24 hours.

[c] Numbers in parenthesis indicate number of experiments and analyses performed and percent figures reported represent the averages obtained.

TABLE II

7 DAY MORTALITY OF 4TH INSTAR CORN EARWORM LARVAE AFTER INJECTION OF *B. BASSIANA* CONIDIA

	No Conidia (±10%) / Larva		
Mutant	50	100	200-300
R_1	48% (70 larvae)*	76% (172 larvae)	
E_1	24% (165 larvae)	35% (64 larvae)	
14		29% (215 larvae)	32% (73 larvae)
HP_1		14% (49 larvae)	51% (73 larvae)
8	4% (50 larvae)	10% (112 larvae)	26% (73 larvae)

* Number of larvae injected.

TABLE III

COMPARISON OF VIRULENCE AND PROTEOLYTIC ACTIVITIES AMONG FIVE B. BASSIANA MUTANTS

Mutant	Virulence (by injection)[a]		Virulence (LC_{50})[b]		Powdered-milk[c]		Gelatin[c]	
R_1	High	76%	Medium	$3.4X10^5$	High	2.44	High	3.00
E_1	Medium	35%	High	$4.1X10^4$	Medium	2.00	Medium	1.87
14	Medium	23%	Low	$1.0X10^6$	High	2.72	High	3.14
HP_1	Low	14%	High	$1.5X10^5$	Low	1.17	Low	1.20
8	Low	10%	Low	$1.4X10^6$	Low	0.00	Low	1.08

[a] Numbers given are the percent mortality at 7 days after injection of live conidia (100±10%) into 4th instar larvae.

[b] Obtained by Probit Analysis (150-200 larvae/conc.) of the number of dead larvae at 4 days after larvae were allowed to crawl on conidia suspensions on filter paper. Numbers given are conidia/ml.

[c] Numbers given are the ratio of clearing zone (enzymatic activities) to colony size at 7 days after inoculation. Activities were determined using litmus-milk/SDA and gelatin/SDA agar plates.

for 48 or more hours. Furthermore, examination of haemolymph amino acids and carbohydrates from starved and infected larvae reveals little or no distinction between the two (see Table I and Figure 9).

Injection of B. bassiana Conidia

Results of injection with small numbers of conidia (50-300) are given in Table II. The five B. bassiana strains showed definite differences in virulence. Mortality at 7 days PI ranged from 80% with R_1 to 10% with mutant strain 8 (at 100 conidia/larva).

When larvae were injected with $2X10^3$ live conidia (strain R_1), the spores were cleared (based on microsopic examination) from the haemolymph circulation with 30 min PI. By 24 hr PI, however, germinating conidia were observed to be emerging from adipohaemocytes (the major phagocytic cells of the CEW; Figure 11). By 48 hr PI, larvae showed signs of sluggishness and extensive weight loss. At this time, free blastospores and short hyphal bodies were present in large amounts in the haemolymph. When the gut wall was excised and examined microscopically (tease mount), it was observed to be infiltrated with long hyphae (40-70 um), indicating that the fungus must have been growing there for at least 24 hr or more (Figure 12). Although many larval tissues and organs have been reported to be the target of attack by B. bassiana (see Introduction), we have not been able to find significant numbers of hyphae in the silk glands, tracheal system, fat bodies, gonads, Malpighian tubules or body wall at this stage of infection (48 hr PI). Fungal infiltration was further confirmed by teasing all tissues and organs apart and

streaking their contents on agar media. Significant numbers of viable conidia were obtained only from the gut wall tissues.

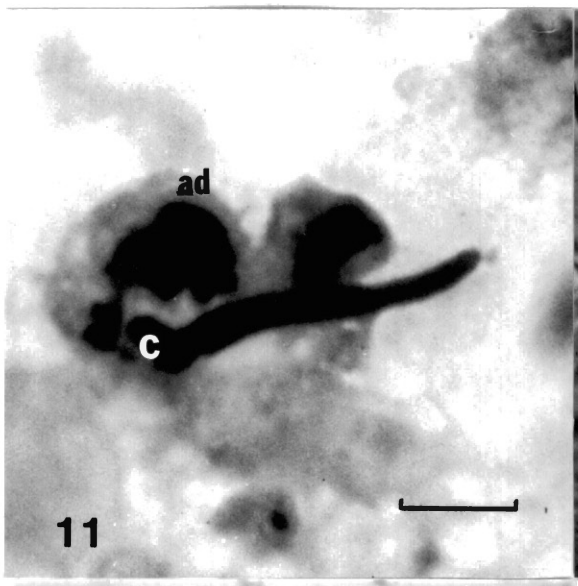
Histopathological examination of organs and tissues revealed that, except for the digestive tissues, none of the others exhibited any significant cytological changes. Cells in the mid-gut tissues (columnar cells) were swollen after infection and, in many cases, they were heavily vacuolated and detached from the peritrophic membranes (Figures 13 and 14). In contrast, none of these effects were observed with healthy gut tissues (Figures 15 and 16).

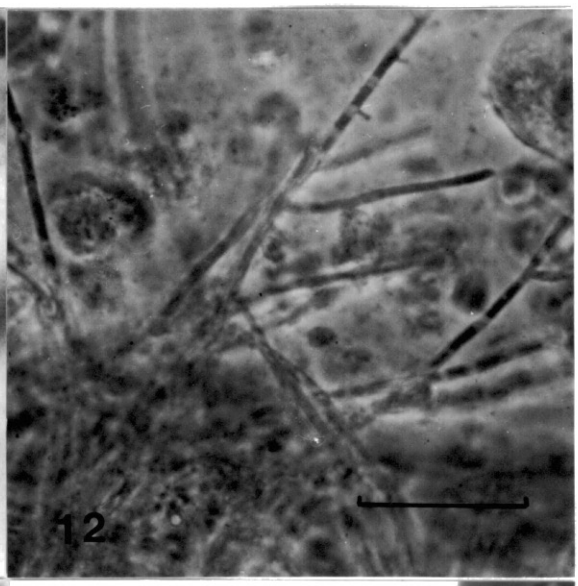
Figure 11. Germination of Phagocytized Conidia (c) from Within an Adipohaemocyte (ad). Bar Represents 5 µm. Mutant Strain R_1. 24 Hr PI.

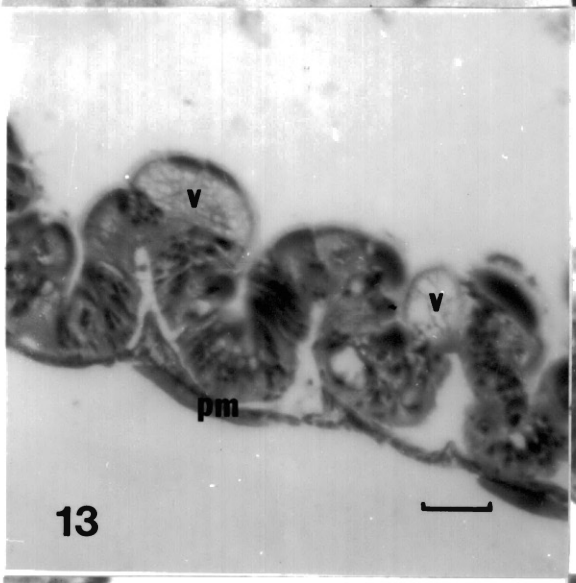
Figure 12. Infiltration of Gut Tissues by B. bassiana Hyphae After Injection of Conidia (Mutant R_1). Tease Mount at 48 Hr PI. Bar Represents 50 µm.

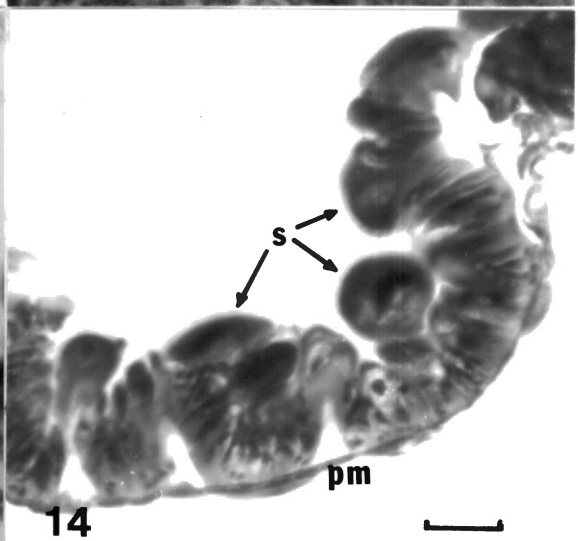
Figure 13 and 14. Stained Paraffin Sections of Infected Gut Tissues from Corn Earworm Larvae. Cytotoxic Effects Observed Include Swelling (s), Vacuolation (v) and Separation from Peritrophic Membranes (pm). Bars Represent 50 µm. R_1, 48 Hr PI.

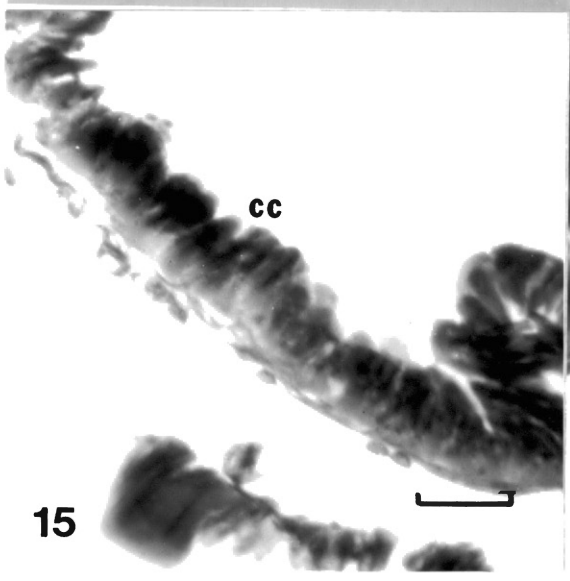
Figure 15 and 16. Stained Paraffin Sections of Healthy Larval Gut Tissues. Note how Columnar Cells (cc) and Goblet Cells (gc) are Tightly Packed. Bars Represent 50 µm.

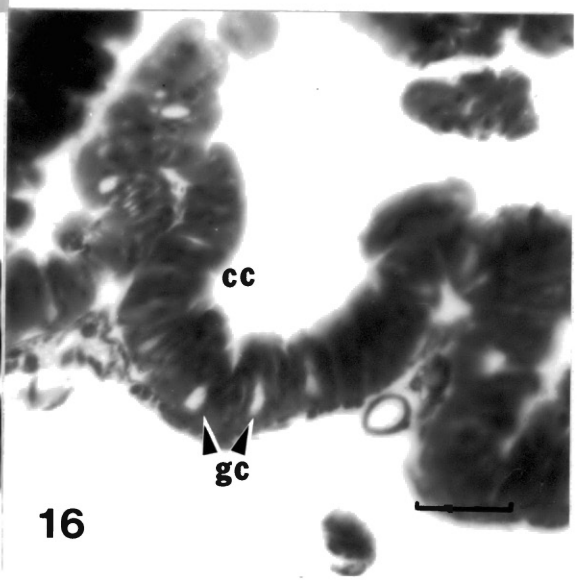

ad
c
11


12


v
v
pm
13


s
pm
14


cc
15


cc
gc
16

CHAPTER IV

DISCUSSION AND SUMMARY

Study of haemolymph components in CEW larvae during infection is a relatively complex problem, since changes, particularly in proteins, which occur during the rapid physiological development, interfere with the interpretation of data. For this reason, synchronization of the development of individual larvae at the beginning of experiments is vital for any comparisons to be made after an infection has been induced.

Changes in haemolymph components after infection with B. bassiana conidia include a relatively rapid decline in some proteins, decrease in total amino acids (amounts differ depending on the mutant strain used) with an increase in the total amount of basic amino acids, disappearance of a minor sugar and, possibly a small increase in glucose. These changes in haemolymph components, together with the cessation of feeding when larvae were infected, suggested they were experiencing difficulty in processing food materials and the observed changes were the result of starvation rather than the immediate consequence of infection by the fungus. This conclusion received further support from our finding that the wall of the alimentary tract is invaded at an early stage of infection (possibly 24 hr PI or earlier). The observed cytological damage to the digestive tissues further suggested that some cytotoxic

element(s) may be produced by the fungus during growth in the gut wall. The effects observed (both cytological and physiological) are very similar to those reported by Endo and Nishiitsutsuji-Uwo (1980) for Bacillus thuringiensis δ-endotoxin; however, this does not necessarily mean that a δ-endotoxin-like compound is produced by B. bassiana.

Physical presence of the fungus in the alimentary tract tissues and/or toxin liberation in the immediate area most likely leads to a malfunction of the digestive tract and subsequent starvation. The ultimate cause of death, however, is not starvation alone since uninfected larvae survive without food for 48 hr and beyond.

To study the hypothetical "gut-toxin", infected gut tissues were homogenized and centrifuged. The supernatant and pellet were both frozen and lyophilized prior to extraction with methanol and then water (Figure 1). Neither injection 5X conc.) nor feeding (20X conc.) of these extracts (wt/wt) resulted in any malfunction of the larvae. We cannot conclude that a toxin is not produced only that our experiments to date have turned up no evidence to support it.

Examination of the exocellular proteolytic activities of the B. bassiana mutants shows a fairly direct correlation between virulence (by injection) and proteolytic activities (Table III, columns 3 and 4). Strains with high proteolytic activities show high virulence (R_1, E_1 and 14) while strains with low proteolytic activities show low virulence (8 and HP_1). When virulence by topical

application (Table III, column 2) is compared with virulence by injection, one can see that the order is changed (see also Results of Part Two), particularly as regards mutant number 14. These data strongly imply that in addition to their ability to invade the gut tissues and resist larval defense mechanism, highly virulent strains of B. bassiana possess additional abilities which allow them to gain access to the haemocoel. If this were not true, then those mutants judged to be very poor pathogens by topical application (strain 14 is an example) should not exhibit any significant increase in virulence when the integument barrier is by-passed by injection.

A special note needs to be made regarding strains E_1 and HP_1. HP_1, a UV-induced mutant of E_1, exhibits a marked decrease in virulence (by injection) which appears to be correlated to a decrease in proteolytic activity of this mutant since other exocellular enzymes are not appreciably affected (Smith and Grula, unpublished). This kind of simple and direct correlation between virulence and proteolytic activities cannot be made when conidia are applied topically (Pekrul and Grula, 1979). Unquestionably, proteolytic activity is important in penetration of the integument by fungal hyphae; however, such penetration appears to involve a complex cascade of sequential events and proteolytic activity is merely one of the events in the series (Smith et al., 1981).

In summary, these studies have focused on two important parameters involved in the entomopathogenicity of B. bassiana. One

involves ability to penetrate the cuticle whereas the other involves killing ability of the fungus once access to the haemocoel has been gained. A positive correlation appears to exist between exocellular proteolytic activity and virulence when fungal conidia are injected into the haemocoel. Once the fungus gains access to the haemolymph of the CEW, defense mechanisms (including phagocytosis) appear to be incapable of overcoming the intruding microbes and preferential invasion of the digestive tract is a major event. Whether or not a paralytic type toxin is produced in the gut tissues is yet an open question. The histopathological changes seen are strongly suggestive that some type of toxin(s) is produced by those fungi that are actively growing in the gut wall; however, it is not known if such toxin exists or that it is even necessary to cause the observed inhibition of feeding. Biochemical changes can be seen in haemolymph components after infection has occurred; but, these are viewed as being due to general starvation rather than to specific activities of the fungi, at least up to the time that a general mycosis is established. With the host larva under physiological stress (starvation, depletion of some haemolymph components, and, possibly, toxin production by the invading fungus) and failure of defense mechanisms, the fungi grow rapidly and a terminal mycosis results.

SELECTED BIBLIOGRAPHY

Broome, J. R., P. Sikorowski, and B. Norment. 1976. A mechanism of pathogenicity of Beauveria bassiana on larvae of the imported fire ant, Solenopsis richteri. J. Invertebr. Pathol., 28:87-91.

Burton, R. L. 1970. A low-cost articial diet for the corn earnworm. J. Econ. Entomol., 63:1969-1970.

Burton, R. L., D. G. Hopper, J. R. Sauer and J. H. Frick. 1972. Properties of haemolymph of the corn earworm. Comp. Biochem. Physio., 42B:713-716.

Champlin, F. R. and E. A. Grula. 1979. Noninvolvement of beauvericin in the entomopathogenicity of Beauveria bassiana. Appl. Envir. Microbiol., 37:1122-1125.

Cheung, P. Y. K., E. A. Grula and R. L. Burton. 1978. Haemolymph responses in Heliothis zea to inoculation with Bacillus thuringiensis or Micrococcus lysodeikticus. J. Invertebr. Pathol., 31:148-156.

Dawson, R. M., D. C. Elliott, W. H. Elliott and K. M. Jones. 1979. Data for Biochemical Research. 2nd ed. Clarendon Press, Oxford.

Dresner, E. 1950. The toxic effect of Beauveria bassiana (Bals.) Vuill. on insects. J. N. Y. Entomol. Soc., 58:269-278.

Endo, Y., and J. Nishiitsutsuji-Uwo. 1980. Mode of action of Bacillus thuringiensis δ-endo-toxin: Histopathological changes in the silk worm midgut. J. Invertebr. Pathol., 36:90-103.

Grimstone, A. V. and R. J. Skae. 1972. A Guide to Microscopical Methods. pp. 23-41. Cambridge Univ. Press, London/New York.

Grula, E. A., R. L. Burton, R. Smith, T. L. Mapes, P. Cheung, S. Pekrul, F. R. Champlin, M. Grula and B. Abegaz. 1978. Biochemical basis for the entomopathogenicity of Beauveria bassiana. pp. 192-216. In, C. M. Ignoffo, (ed.), "Proceedings of the First Joint US/USSR Conference on the Production, Selection and Standardization of Entomopathogenic Fungi of the US/USSR Joint Working Group on the Production of Substances by Microbiological Means". 293 pp.

Heathcote, J. G. and K. Jones. 1965. The rapid resolution of complex mixtures of the naturally occurring amino acids by two-dimensional thin-layer chromatography on cellulose powder. Biochem. J., 97:15P.

Huang, H. T. and M. Shapiro. 1971. Insecticidal activity of microbial metabolites. Prog. Indust. Microbiol., 9:79-112.

Kucera, M. and A. Samsinakova. 1968. Toxins of the entomophagous fungus Beauveria bassiana. J. Invertebr. Pathol., 12:213-320.

Lefebvre, C. L. 1934. Penetration and development of the fungus, Beauveria bassiana, in the tissues of the corn borer. Ann. Bot. (London), 48:441-452.

Liao, T. H., G. W. Robinson and J. Salinkow. 1973. Use of narrow-bore columns in amino acid analysis. Anal. Chem., 45:2286-2288.

Pekrul, S. and E. A. Grula. 1979. Mode of infection of the corn earworm (Heliothis zea) by Beauveria bassiana as revealed by scanning electron microscopy. J. Invertebr. Pathol., 34:238-247.

Smith, R. and E. A. Grula. 1981. Nutritional requirements for conidial germination and hyphal growth of Beauveria bassiana. J. Invertebr. Pathol., 37:000.

Smith, R., S. Pekrul and E. A. Grula. 1981. Requirements for sequential enzymatic activities for penetration of the integument of the corn earworm (Heliothis zea). J. Invertebr. Pathol., In press.

Wasti, S. S. and G. C. Hartmann. 1975. Experimental parasitization of larvae of the gypsy moth, Porthetria dispar (L), with the entomogenous fungus Beauveria bassiana (Balsamo) Vuill. Parasitology, 70:341-346.

West, E. J. and J. D. Briggs. 1968. In vitro toxin production by the fungus Beauveria bassiana and bioassay in greater wax moth larvae. J. Econ. Entomol., 61:684-687.

PART FOUR

PRELIMINARY STUDIES INVOLVING AN ANTICOAGULANT PRODUCED BY *BEAUVERIA BASSIANA*

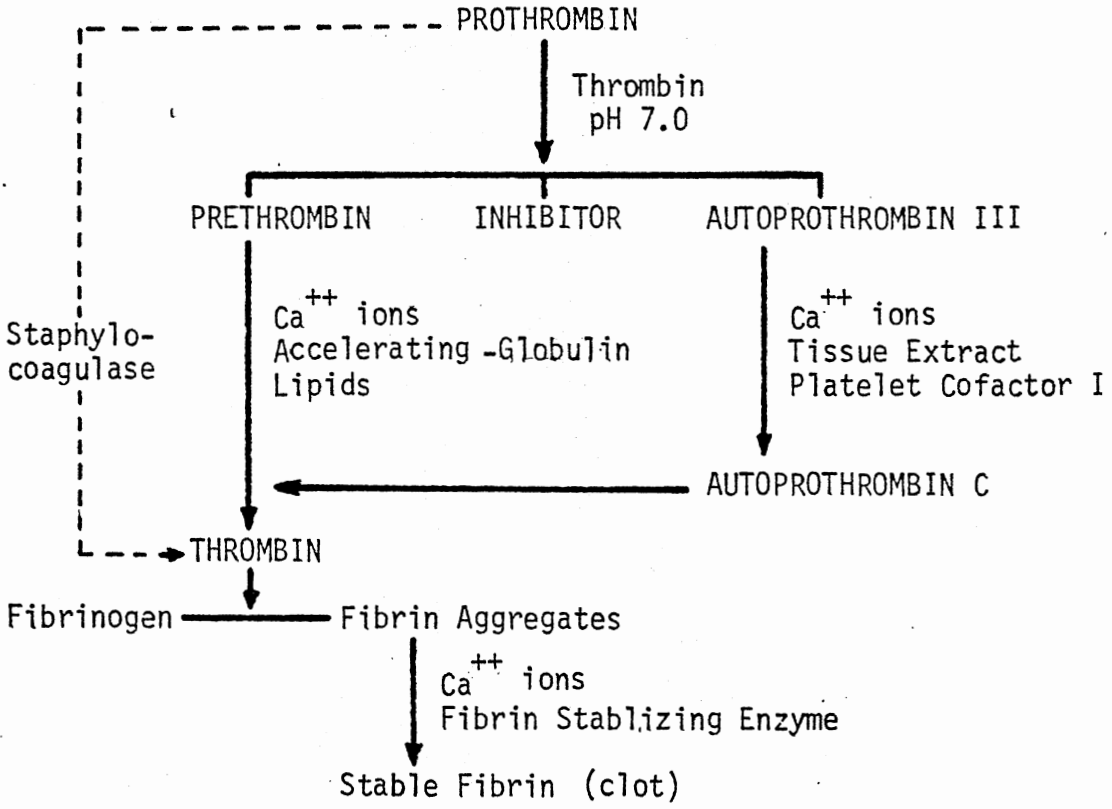
CHAPTER I

INTRODUCTION

Production of biologically active compounds during the life cycle of microorganisms is a well studied subject. Each year thousands of chemicals are isolated and studied. These compounds usually affect biochemical reactions and therefore the physiological functions of other life forms and many have been found useful in the service of man. The best known of these would be the antibiotics which possess antimicrobial activities, e. g. penicillin. Others, such as the alkaloids and halucinogens of mushrooms have intrigued humans for centuries. Many have been used as diet supplements and flavorings, e. g. vitamins and amino acids. Many more find their ways into research, adding to our knowledge in the various areas of life sciences.

Work related to blood coagulation covers a broad range of fields, e. g. pharmacology, clinical applications, blood metabolism, etc. Advances in these areas have furnished us with a picture of the fundamental nature of the blood coagulation mechanisms. An in-depth description and review of the subject can be found in the book Blood Clotting Enzymology (Seegers, 1967a). A simplified graphic description of the coagulation process is presented in figure 1. Prothrombin is first activated to form prethrombin and the autoprothrombins by autocatalysis. Activation involves a large

Figure 1. Activation of Prothrombin and Polymerization of Fibrinogen. (Modified from Seegers, 1967b; p. 52 & 63).


PROTHROMBIN
Thrombin
pH 7.0
PRETHROMBIN
INHIBITOR
AUTOPROTHROMBIN III
Staphylo-
coagulase
Ca++ ions
Accelerating -Globulin
Lipids
Ca++ ions
Tissue Extract
Platelet Cofactor I
AUTOPROTHROMBIN C
THROMBIN
Fibrinogen
Fibrin Aggregates
Ca++ ions
Fibrin Stablizing Enzyme
Stable Fibrin (clot)

number of substances and cofactors. Thrombin, the enzyme that mediates the polymerization of fibrinogen, is formed from action of autoprothrombin C on prethrombin. In the presence of thrombin, fibrinogen is polymerized to fibrin (the clot) which is stablized by a stablizing enzyme in the blood and inactivation of thrombin.

In the course of our study of Beauveria bassiana, several biologically active substances that are excreted into the environment of the fungus have come to our attention. Two compounds possessing antimicrobial properties are being isolated and studied. Several extracellular enzymes (proteases, lipases and chitinase) that are important to the entomopathogenicity of the fungus are also being investigated. The possible existence of a compound that prevents skin sheading (leads to death of the larva) and a toxin that may paralyze the gut of corn earworm larvae await further study.

The existence of an anticoagulant was first suspected when injection of spent growth medium into 5th instar corn earworm larvae resulted in extensive bleeding of the insect. Although the coagulation of insect haemolymph and mammalian blood are two completely different processes, testing with human plasma quickly revealed that the concentrated growth medium of B. bassiana interferes with the normal coagulation process.

In this study, isolation of the anticoagulant from B. bassiana spent growth media and some preliminary characterization of the compound are presented.

CHAPTER II

MATERIALS AND METHODS

Test Organisms

B. bassiana mutant strain 2 was used in this study. Details of how mutants were obtained and maintained are given in Part One, Chapter II.

Growth Medium

Sabouraud Fructose Broth (SFB; 10 gm neopeptone and 40 gm fructose per L.) at pH 7.5 was inoculated with spores from B. bassiana mutant strain 2 and incubated at 25^{o}C on a rotary shaker for 5 days. After the incubation period spent growth medium was frozen and lyophilized.

Coagulation Plasma

Sodium citrate (0.8 gm) and citric acid (0.02 gm) were placed in a clean centrifuge bottle into which fresh blood (100 ml) was collected. Red blood cells were removed by centrifugation at 1000 g for 10 min. The citrated plasma was divided into 10 ml aliquots and kept frozen until needed. Three types of plasma were collected and tested (human, dog and turkey).

Staphylocoagulase Test

Cultures of a coagulase-positive Staphylococcus aureus strain were maintained on Tryptic Soy Agar. To test staphylocoagulase activity, 50 µl of a 24 hr 37°C Tryptic Soy Broth culture of S. aureus was added to 300 ul of plasma. The mixture was then incubated at 37°C and examined periodically. Any degree of clotting in three to four hours was regarded as a positive test (coagulation present).

Thrombin Coagulation Test

To assay anticoagulant activity, human fibrinogen (Sigma, 4 mg/ml) plus citrated dog plasma (10%, to provide cofactors needed) was used. Human thrombin (Sigma, 1/20 NIH unit; each NIH unit of thrombin is defined as the amount of thrombin that can clot 1 ml of standard fibrinogen in 15 sec at 37°C) was added to 300 µl of the fibrinogen/plasma solution and incubated at 37°C. Clotting of fibrinogen will occur within 10 min. Samples of anticoagulant to be tested were added to this assay mixture. A positive anticoagulant test is defined as complete inhibition of coagulation within 10 min at 37°C.

The use of thrombin instead of staphylocoagulase in coagulation tests allowed us to perform the tests more quickly (reduced from 12-18 hr to 10 min). It also eliminated the problem of clot digestion by contaminating bacteria (e.g. Pseudomonas) when the tests have to be incubated at 37°C over a long period of time (12-18 hr).

Extraction of Anticoagulant

The lyophilized spent growth medium was washed 2X with chloroform and then acetone to remove lipids and fatty acids. The precipitates were dissolved in methanol and the methanol precipitates removed by centrifugation. The supernatant was air-dried and re-dissolved in water which was then frozen and stored.

Column Chromatography

Gel Filtration

Sephadex G-15 gel filtration was performed with the crude extract from the spent growth medium. The column had a 160 ml bed volume and a 60 ml void volume. Flow rate was at 1 ml/min and distilled water was used as eluant. Twenty fractions, 5 ml each, were collected after the void volume had passed through. These fractions were lyophilized and their anticoagulation activities tested.

Anion Exchange

Anion exchange chromatography (10 ml bed vol., 6 ml void vol.) was performed using AG 3-A4X resin (Bio-Rad). The resin was first converted to the OH^- form with 30 ml of 0.5N NaOH, and then washed with distilled water at pH 10.5 until pH of column reaches 10.5. Distilled water at pH 10.5 (adjusted with 0.5N NaOH) was used as eluant. A twenty ml fraction was collected after the void volume. This process effectively removed all phosphorylated compounds and

any small peptides.

Molecular Weight Estimation

The following compounds were filtered through the Sephadex G-15 column: blue dextran, >1500 daltons; flavin adenine dinucleotide (FAD) 786 daltons; flavin mononucleotide (FMN), 456 daltons; phenol red, 354 daltons; cyclic adenosine monophospate (cAMP), 329 daltons; flavin 246 daltons and tryptophan (trp), 204 daltons. The elution volume of each compound was plotted against the logarithm of their molecular weight and the elution profile ($A_{280\ nm}$) of the crude extract of the spent growth medium.

Heat Stability

Aliquots of the crude extract of spent growth medium were heated in a steambath for 30 min 1 hr and also autoclaved (15 psi for 15 min). The heat-treated anticoagulant samples were then tested for inhibition of coagulation.

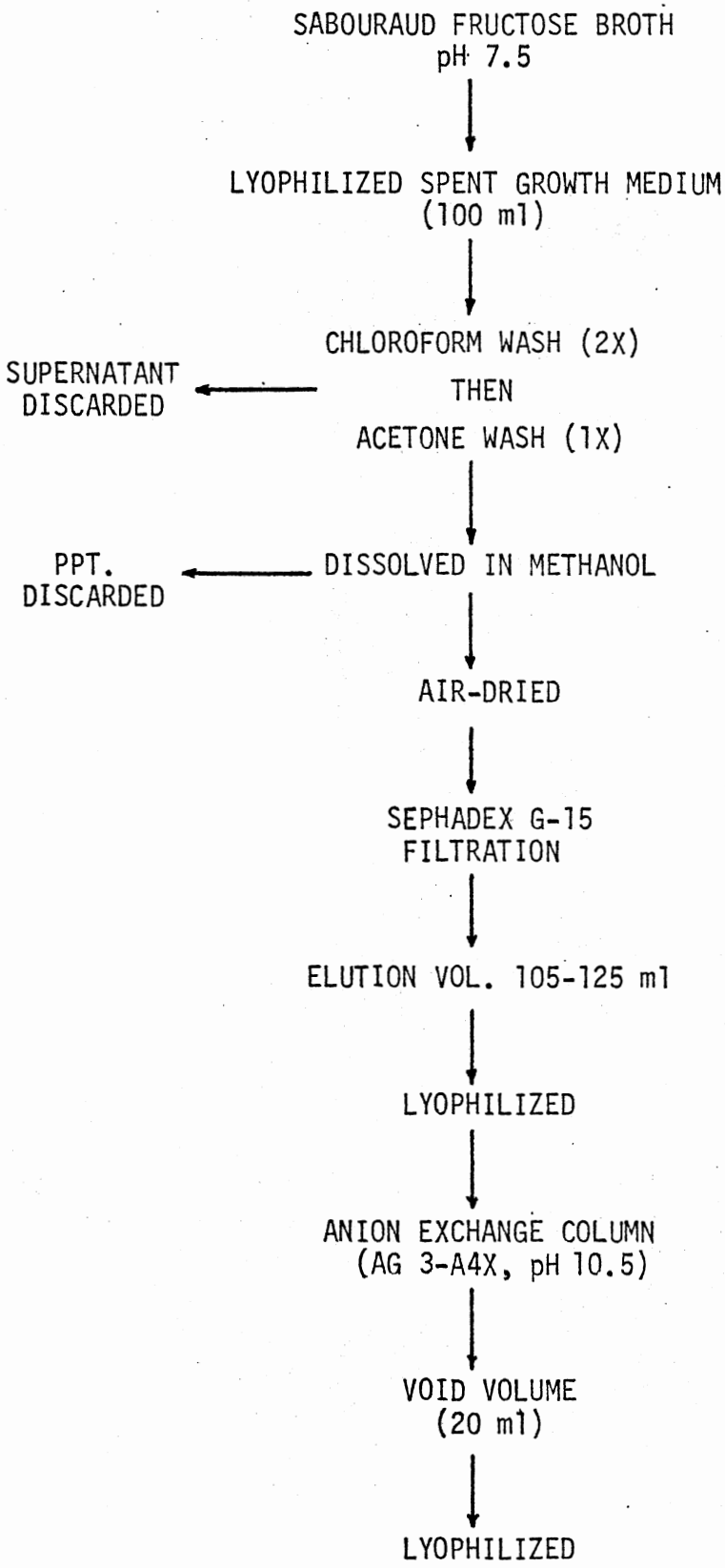
Acid Hydrolysis

After the MeOH extract had been purified (see Figure 2), the anticoagulant preparation was hydrolyzed with 1N HCl at 100°C under vacuum for 1, 2, 4 and 8 hr. The hydrolyzates were air-dried three times (to remove HCl) and chromatographed to detect components of the anticoagulant.

Thin-Layer Chromatography (TLC)

TLC plates coated with powdered cellulose MC300 (15 gm/100 ml water) were used to characterize the anticoagulant. Chromatograms were developed in isopropanal:methanol:water (60:30:10). After air-drying, the plates were sprayed with the following reagents (Dawson et al., 1979): ninhydrin (for amino acids and free amines), periodate/permanganate (for carbohydrates), aniline/diphenylamine (for reducing sugars), acetylacetone/p-dimethylaminobenzaldehyde (for amino sugars) and $FeCl_3$-sulfosalicylic acid (for phosphate and other chelating compounds).

Figure 2. Flow Chat for Purification of Anticoagulant from *B. bassiana* Spent Growth Medium.


SABOURAUD FRUCTOSE BROTH
pH 7.5
LYOPHILIZED SPENT GROWTH MEDIUM
(100 ml)
CHLOROFORM WASH (2X)
THEN
ACETONE WASH (1X)
SUPERNATANT
DISCARDED
DISSOLVED IN METHANOL
PPT.
DISCARDED
AIR-DRIED
SEPHADEX G-15
FILTRATION
ELUTION VOL. 105-125 ml
LYOPHILIZED
ANION EXCHANGE COLUMN
(AG 3-A4X, pH 10.5)
VOID VOLUME
(20 ml)
LYOPHILIZED

CHAPTER III

RESULTS AND DISCUSSION

Results of coagulation tests with various preparations of the anticoagulant are summarized in Table I. The compound inhibits coagulation by both staphylocoagulase and thrombin. However, since staphylocoagulase exerts its coagulation activity through thrombin (by complexing with and thus activating thrombin so that thrombin is not activated through the normal Ca^{++} dependent process, see Figure 1) action of the anticoagulant must be solely on thrombin. The compound is effective against thrombin from human, dog and turkey sources. It is also very heat stable since neither heating at 100°C nor autoclaving resulted in loss of activity.

A schematic of the procedure for isolation and purification of the anticoagulant is presented in figure 2. Filtration through Sephadex G-15 column shows that the anticoagulant has a molecular weight of about 350±50 daltons (Figure 3). Samples collected at the end of the purification process (after anion exchange chromatography) are anticoagulant positive. TLC shows that the sample is free of phosphate or other chelating compounds. Except for a very small amount of ninhydrin-positive material at the origin of the chromatogram, the preparation is composed of an aminosugar mainly. Chemical and physical characteristics of this compound on TLC are listed in Table II.

TABLE I

ACTIVITIES OF DIFFERENT ANTICOAGULANT PREPARATIONS FROM SPENT GROWTH MEDIUM OF *B. BASSIANA*

Citrated Plasma (300 µl)	Fibrinogen/Plasma* Solution (300 µl)	Anticoagulant Samples (100 µl)	Thrombin (1/20 NIH unit)	Staphylo-coagulase (50 µl)	Coagulation 10 min, 37°C
Human				+	+ (4 hr)
Human		Crude Extract		+	- (4 hr)
Human			+		+
Human		Crude Extract	+		-
Dog				+	+ (4 hr)
Dog		Crude Extract		+	- (4 hr)
Dog			+		+
Dog		Crude Extract	+		-
Turkey				+	+ (4 hr)
Turkey		Crude Extract		+	- (4 hr)
Turkey			+		+
Turkey		Crude Extract	+		-
	+			+	+ (4 hr)
	+	Crude Extract		+	- (4 hr)
	+		+		+
	+	Crude Extract	+		-
	+	Autoclaved	+		-

TABLE I (Continued)

Citrated Plasma (300 µl)	Fibrinogen/Plasma* Solution (300 µl)	Anticoagulant Samples (100 µl)	Thrombin (1/20 NIH unit)	Staphylo-coagulase (50 µl)	Coagulation 10 min, 37°C
	+	Chloroform ppt. from Crude Ext.	+		-
	+	Acetone ppt. from Crude Ext.	+		-
	+	Methanol ppt from Crude Ext.	+		+
	+	Methanol super from Crude Ext.	+		-
	+	Elution Vol. 105-125 ml fraction from G-15 column	+		-
	+	First 20 ml after the void vol. from Anion Exchange Column	+		-

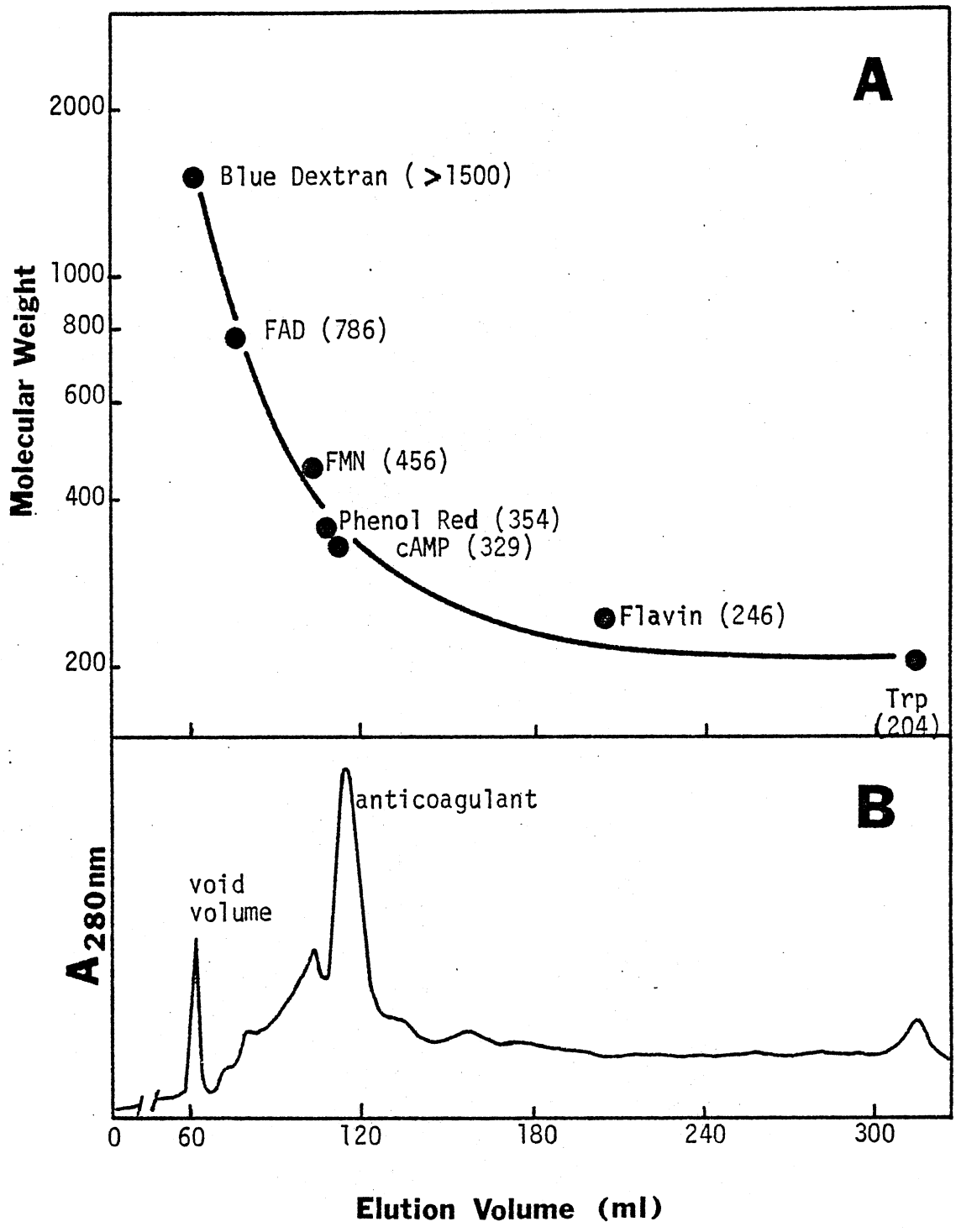
* Fibrinogen 4 mg/ml plus 10% dog plasma, pH of this solution is 6.0.

TABLE II

CHEMICAL AND PHYSICAL CHARACTERISTICS OF ANTICOAGULANT

Reagent	Reaction	
Ninhydrin	+	very slow, probably indicates a tert-amine
Periodate/ permanganate	+	carbohydrate component present
Aniline/ diphenylamine	+	brown, indicates presence of a aldo- or keto- hexose/pentose
Amino-sugar	+	purple, indicates that the amino group is not free
Sulfosalicylic acid	-	no phosphate; no chelating groups
UV Fluoresence	-	probably no conjugated double bonds
Heat Stability	+	survives autoclaving
Acid Hydrolysis	+	very acid labile, 1N HCl/100^{o}C/1Hr
Molecular Weight		350±50 daltons

Figure 3. Determination of the Molecular Weight of Anticoagulant Using a Sephadex G-15 Column. A. Reference Compounds Used. B. Effluent Pattern (A_{280} nm) of Crude Extract from Spent Growth Medium of B. bassiana. Anticoagulation Activity was Found in The Elution Volumes from 105-125 ml.


A
2000
1000
800
600
400
200
Molecular Weight
Blue Dextran (>1500)
FAD (786)
FMN (456)
Phenol Red (354)
cAMP (329)
Flavin (246)
Trp (204)
B
anticoagulant
void volume
A280nm
0
60
120
180
240
300
Elution Volume (ml)

SELECTED BIBLIOGRAPHY

Dawson, R. M., D. C. Elliott, W. H. Elliott and K. M. Jones. 1979. Data for Biochemical Research. 2nd ed. Clarendon Press, Oxford.

Seegers, W. H. 1967a. Blood Clotting Enzymology. Academic Press, New York.

Seegers, W. H. 1967b. Prothrombin in Enzymology, Thrombosis and Hemophilia. Charles C. Thomas-Publisher, Springfield.

PART FIVE

PRESENCE OF FREE PUTRESCINE IN THE HAEMOLYMPH OF CORN EARWORM (*HELIOTHIS ZEA*) LARVAE

CHAPTER I

INTRODUCTION

The biological importance of many aliphatic polyamines is indicated by their wide occurrence in animal and plant tissues and microorganisms. The physiologial and pharmacological significance of these compounds has been extensively studied and possible regulatory roles in biological systems reassessed (Tabor et al., 1961; Tabor and Tabor, 1964).

1,4-Butanediamine (putrescine) has been reported as a growth requirement for some bacteria and fungi (Martin et al., 1952; Herbst et al, 1955; Sneath, 1955). Ackermann (1955) reported the presence of putrescine in pupae of the silkworm (Bombyx mori L.) and sexual maturation of Artemia salina was shown to require the diamine (Provasoli and D'Agostina, 1962). Davis (1966) reported that putrescine functions as a growth promoting substance for larvae of the saw-toothed grain beetle (Oryzaephilus surinamensis) when added to the diet. Herbst and Dion (1970) showed putrescine levels in the tissues of Drosophila melanogaster change with different developmental stages of the insect.

In the present study, we report the isolation and identification of cell free putrescine from the haemolymph of healthy corn earworm (Heliothis zea) larvae. To our knowledge, this is the first time this compound has been found to be present in this insect.

CHAPTER II

MATERIALS AND METHODS

Insects

Corn earworm larvae were reared on the CSM artifical diet developed by Burton (1970). Individual, newly-hatched first instar larvae were placed in diet cups and maintained at 25°C with a 19 hr light and 5 hr dark photoperiod.

Haemolymph

Haemolymph was obtained from larvae by puncturing them at the base of their second proleg and collecting the extruded haemolymph in a seriological test tube (in an ice bath) precoated with a small amount of phenylthiourea to prevent melanization. Haemocytes were removed by centrifugation at 1000 g for 10 min. Proteins were removed by precipitating two times with absolute ethanol and the final supernatant (66% EtOH) was utilized for amino acid analysis and isolation of putrescine. All injections into the haemolymph were performed using phosphate buffered saline pH 6.5 PBS; 0.07M NaCl, 0.039M $Na_2HPO_4.7H_20$, 0.02M KH_2PO_4; (Burton et al., 1972) .

Thin-Layer Chromatography (TLC)

TLC plates coated with powdered cellulose MN300 (15 gm/100 ml

water) were used for separation of amines and amino acids. The cellulose plates were pretreated by developing overnight in isopropanol:water:formic acid (80:20:4). This treatment causes impurities present in the cellulose to migrate and accumulate at the leading edge of the plates where they can be scraped off with a razor. Two-dimensional chromatography of haemolymph was then performed using the solvent systems developed by Heathcote and Jones (1965). Amino acids and other amines were detected by spraying the plates with 0.4% ninhydrin in acetone.

Autoradiography

Uniformly labelled ^{14}C-arginine (173 µC/mM) was either injected (0.1 µC/larva) or fed (0.5 µC/larva) to two groups of 4th instar larvae. Fourteen hr after injection or feeding, haemolymph was collected and treated to precipitate proteins as described above. Two-dimensional TLC was performed and fluorography was accomplished by saturating the TLC plates with 22% 2,5-diphenyloxazole in acetone. After the TLC plates were air-dried, X-ray film (X-Omat R, Kodak) was placed in contact with the TLC plate for 2 weeks at -20^{o}C (modified procedure of Bonner and Laskey, 1974).

Isolation

Putrescine was isolated from the haemolymph preparations (haemocytes and protein free) using one-dimentional chromatography on Whatman No. 1 paper. Chromatograms were developed in a solvent system consisting of tert-butanol:methyl ethyl ketone:water:88%

NH_4OH (50:30:10:10) for five hrs. After air-drying, the spot to where putrescine had advanced was cut out and eluted overnight at 2-4°C with flowing distilled water. The resulting putrescine solution was then lyophilized and subjected to infrared (IR) and proton nuclear magnetic resonance (nmr) spectroscopy and gas chromatography (GC). IR spectra were obtained using a Perkin-Elmer Model X-97 Infrared Spectrophotometer. The KBr wafers were prepared using lyophilized samples of the haemolymph putrescine. Proton nmr spectra were obtained using a Varian XL-100 Spectrometer at 100 MHz. Deuterium oxide was used as the solvent and sodium trimethylsilypropionate-2,2,3-3-d_4 (TSP) was included as an internal standard. GC was performed using a Perkin-Elmer, Sigma 2 unit. The column was glass (182.9 X 0.2 cm ID) packed with Carbopack B/4% CW 20M/0.8% KOH (Supelco). Injection port temperature was 250°C and the oven temperature was programed from 150°C to 220°C at 8°C/min. Carrier gas utilized was nitrogen at 20 ml/min and detection was by flame ionization.

Chemical Derivatives

Because primary amines are known to absorb carbon dioxide (CO_2) through continued exposure to air (formation of carbamate), and it was suggested to us that they may also readily condense with ketones such as methyl ethyl ketone which was present in our isolation solvent (formation of the ketone adduct, ketimine) authentic putrescine was subjected to our isolation procedure, reacted with CO_2 and also condensed with methyl ethyl ketone to produce the desired derivatives

as follows:

Carbamate

One gram of putrescine (1,4-butanediamine) was dissolved in 10 ml of distilled water and the solution was bubbled with CO_2 gas at room-temperature for 2 hr. To ensure complete carbamate formation, the solution was kept under CO_2 overnight at room-temperature then frozen and lyophilized.

N,N-Dibutylidene-1,4-butanediamine (Ketimine)

To a mixture of 1,4-butanediamine (7.0 gm, 0.079M) and methyl ethyl ketone (5.69 gm, 0.079M) 1 ml of conc. HCl was added. The solution was allowed to stand at room-temperature for 24 hr. Anhydrous sodium hydroxide pellets (ca. 5.0 gm) were added and the solution was stirred for 2 hr at room-temperature. The aqueous layer was removed by aspiration, the mixture was filtered through a sintered glass funnel and the filtrate was distilled to yield the ketimine (2 gm, 15.57% yield, bp 75-77^{o}C/1.5 mg Hg). Anal. Calded for $C_{12}H_{24}N_2$: 196.1939 daltons; found 196.1917 daltons.

CHAPTER III

RESULTS

Amino acid analyses, using an amino acid analyzer and the procedure of Liao et al.,(1973), showed that haemolymph from 4th instar corn earworm larvae possesses all of the common amino acids plus small amounts of β-alanine, ornithine and gamma-aminobutyric acid (GABA). Major amino acids include threonine, serine and proline; a high level of the basic amino acids (histidine, lysine and arginine) is also observed. These 6 amino acids add up to approximately 71% of the total (Table I). Aspartic and glutamic acids are present in very small amounts (1% of the total).

In addition to all the regular amino acids observed and identified using regular programming of the amino acid analyzer, it was shown by additional testing using two-dimensional TLC, that there are a number of unknown amines present in haemolymph. Continued testing revealed that one of the major amines migrated to approximately the same position as commercial putrescine (Figure 1). Based on color intensity to ninhydrin of known depositions of putrescine on TLC plates, 4th instar larvae contain about 375±25 µg of putrescine per ml of haemolymph.

Gas chromatographic scans of authentic putrescine, our material isolated from haemolymph, CO_2-treated putrescine, putrescine that has been taken through the isolation procedure and the

methyl ethyl ketone adduct product of putrescine that we have synthesized (ketimine) are presented in Figure 2. Gas chromatographic analysis showed that authentic putrescine had a retention time and temperature of 7.4 min/206^{o}C (Figure 2A). Putrescine isolated from haemolymph, putrescine that was subjected to our isolation procedure and CO_2-treated putrescine all had the same retention time and temperature as authentic putrescine (7.4-7.5 min/206-207^{o}C, Figure 2 B, C and D). However, the ketimine showed much lower retention values (2.4 min/106^{o}C) when subjected to the same gas chromatographic analyses (Figure 2E).

The IR spectra of the various compounds are presented in Figure 3. Authentic putrescine (Figure 3A) showed a double absorption at 3400^{-1}cm (2.94 μ) and 3300^{-1}cm (3.01 μ) and absorption at 1600^{-1}cm (6.25 μ) for NH-stretching and NH-bending, respectively (Sadtler, 1973-1975). IR absorption of putrescine isolated from the haemolymph, CO_2-treated putrescine and putrescine that had been subjected to our isolation procedure (Figure 3 C, D and E) all had a single NH-stretching absorption at 3400^{-1}cm (2.94 μ) and NH-bending at 1600^{-1}cm (6.25 μ). The aliphatic CH at 3000^{-1}cm was very broad and the C=O absorption at 1700^{-1}cm (5.90 μ) probably resulted from presence of carbamate ions. The ketimine (Figure 3B) showed very weak NH-stretching and no NH-bending. The C=N absorption was at 1670^{-1}cm (6.05 μ); this did not coincide with either the C=O or the NH-bending of putrescine isolated from the haemolymph or CO_2-treated putrescine.

Proton nmr spectra are shown in figure 4. Two absorptions were observed with authentic putrescine, one at 1.44 ppm and the other at 2.62 ppm. A 1:1 ratio between the two was obtained upon integration (Figure 4A). Spectra of putrescine isolated from haemolymph, putrescine subjected to our isolation procedure and CO_2-treated putrescine also showed two absorptions with a 1:1 ratio, but they were shifted downfield to 1.70 ppm and 3.04 ppm respectively (Figure 4 B, C and D). The ketimine gave a very different proton nmr spectrum (Figure 4E). Besides the two groups of methylene protons of butanediamine (1.70 ppm and 3.30 ppm; shifted further downfield by the C=N), the methyl and ethyl group of the butylidene gave additional absorptions at 1.10 ppm (triplet, methyl of C_2H_5), 1.80 ppm (singlet, methyl) and 2.20 ppm (quartet, methylene of C_2H_5).

To obtain some evidence relating to synthesis of putrescine in CEW larvae, ^{14}C-arginine was either fed or injected into different groups of 4th instar larvae. After 14 hr, haemolymph samples were removed, deproteinized, run on 2-dimensional TLC plates and, after air-drying, placed against X-ray film for 2 weeks. These studies showed that when ^{14}C-arginine was fed, large amounts of label appeared both in putrescine and poline. Additionally, labeling was evident in several of the unidentified basic compounds (Figure 1).

When ^{14}C-arginine was injected directly into the haemolymph, amounts of radioactive proline and putrescine seen after 14 hr were small; however, some labeling was present (ratio of fed to injected was about 5:1).

TABLE I

AMINO ACIDS PRESENT IN THE HAEMOLYMPH OF 4TH INSTAR CORN EARWORM LARVAE*

Amino Acid	Percent of Total
Asp	0.7
Thr+Ser	28.0
Glu	0.1
Pro	13.0
Ala	2.7
Gly	4.3
Cys	<0.1
Val	5.0
Met	0.7
Ile	1.6
Leu	4.6
Tyr	5.5
Phe	1.7
Trp	0.4
His	13.6
Lys	12.0
Arg	5.6
β-Ala	<0.1
Ornithine	<0.1
GABA**	<0.1

Total = 882.0 μM/ml

* Amino acids in all haemolymph samples were identified and quantitated using an amino acid analyzer. The analyses were performed at 60°C on a Durum DC6A column with citrate buffer (for details of the procedure, see Liao et al., 1973).

** Gamma-aminobutyric acid

Figure 1. Cellulose TLC of Haemolymph (After Removal of Haemocytes and Proteins) from Corn Earworm Larvae. Indicates Compound of Interest, Identified as Putrescine. Solvent 1: Isopropanol: Water:Formic Acid (80:20:4). Solvent 2: Tert-butanol:Methyl Ethyl Ketone:Water:88% NH_4OH (50:30:10:10). Compounds Marked With an X Are Unidentified. Those in the Lower Left are Assumed to be Basic Because of Their Position.

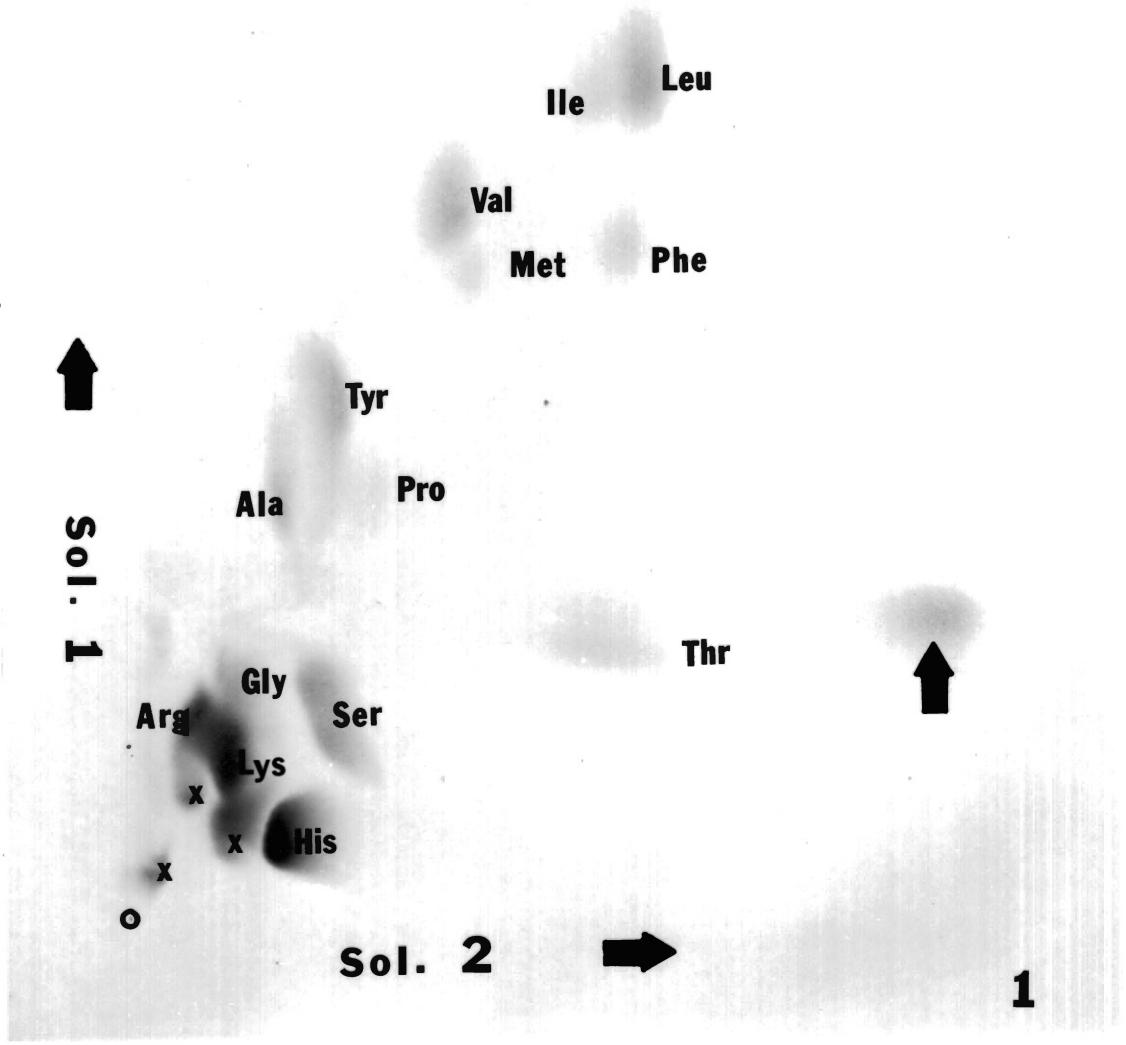

Ile
Leu
Val
Met
Phe
Tyr
Ala
Pro
Thr
Gly
Arg
Ser
Lys
His
Sol. 1
Sol. 2
1

Figure 2. Gas Chromatographic Scans of (A) Authentic Putrescine, (B) Haemolymph Isolate, (C) Authentic Putrescine that has been Subjected to Our Isolation Procedure, (D) Authentic Putrescine that has been Treated with Carbon Dioxide, and (E) Ketimine Adduct Product of Putrescine and Methyl Ethyl Ketone.

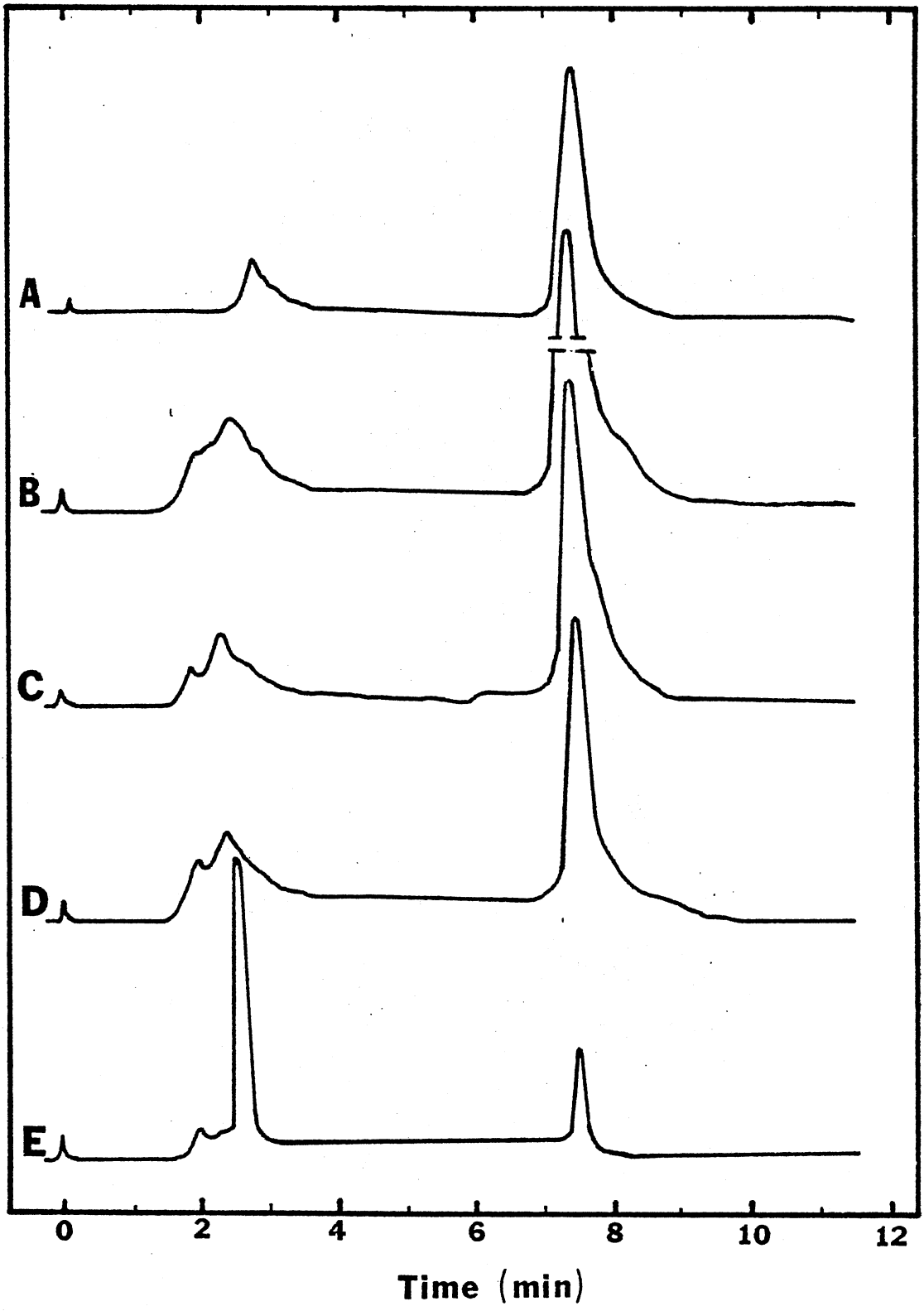

A
B
C
D
E
0
2
4
6
8
10
12
Time (min)

Figure 3. Infrared Spectra of the Different Putrescine Preparations. (A) Authentic Putrescine (Sadtler, 1973). (B) Ketimine, Condensation Product of Putrescine and Methyl Ethyl Ketone. (C) Haemolymph Isolate. (D) CO_2-Treated Putrescine. (E) Putrescine That Has Been Subjected to Our Isolation Procedure.

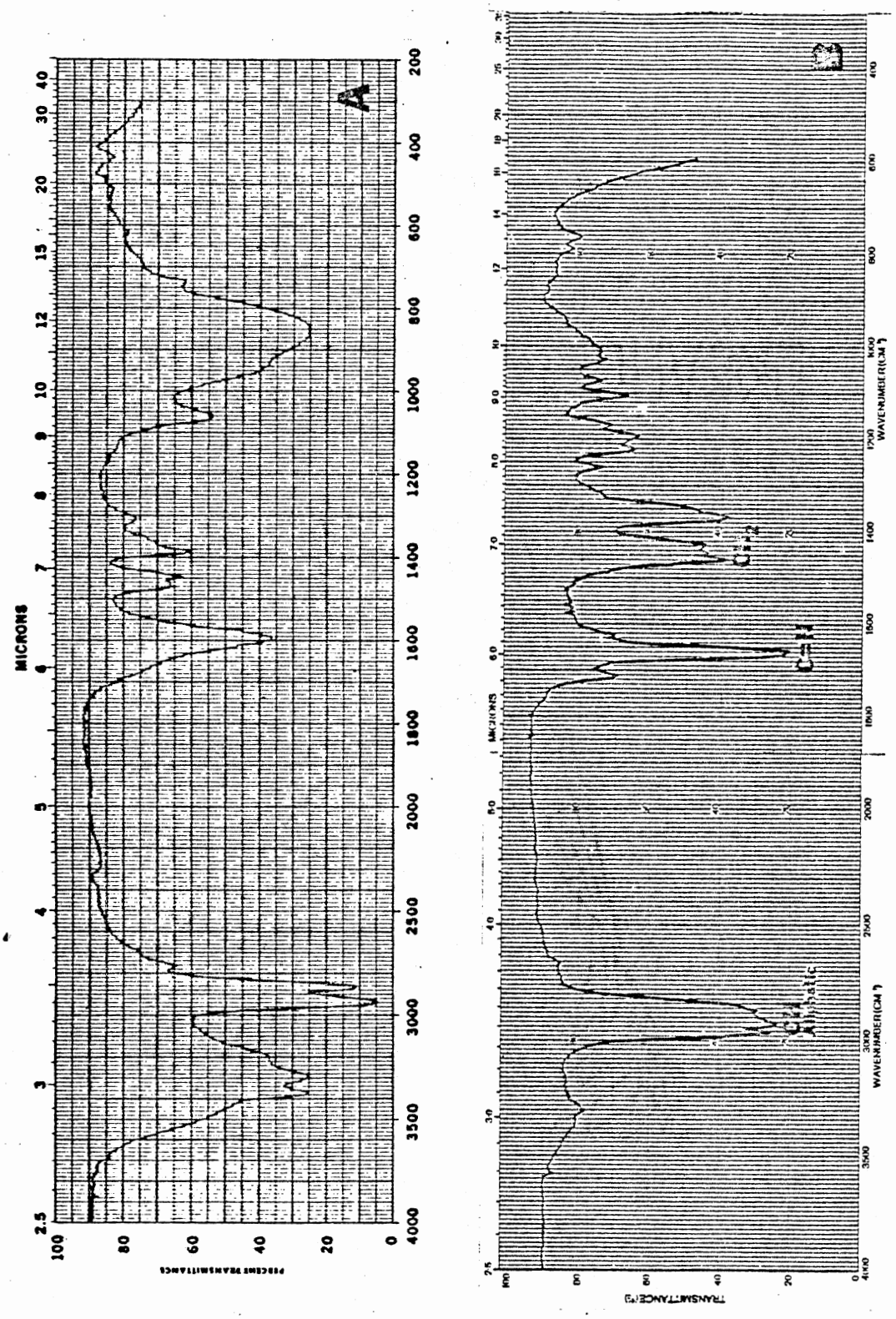

MICRONS
PERCENT TRANSMITTANCE
WAVENUMBER(CM⁻¹)
TRANSMITTANCE(%)
C-H
C=O
Aliphatic

Figure 3. Continued

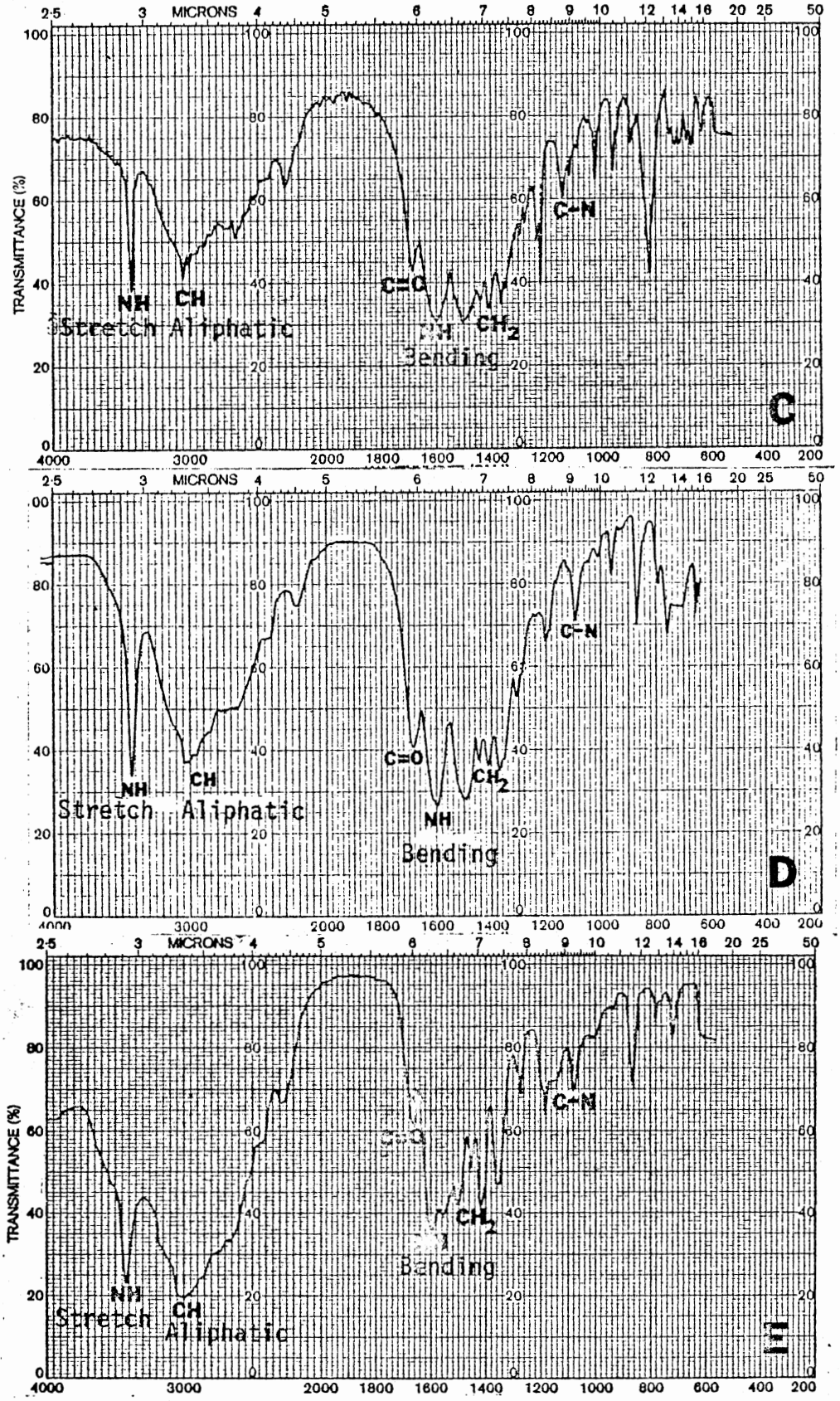
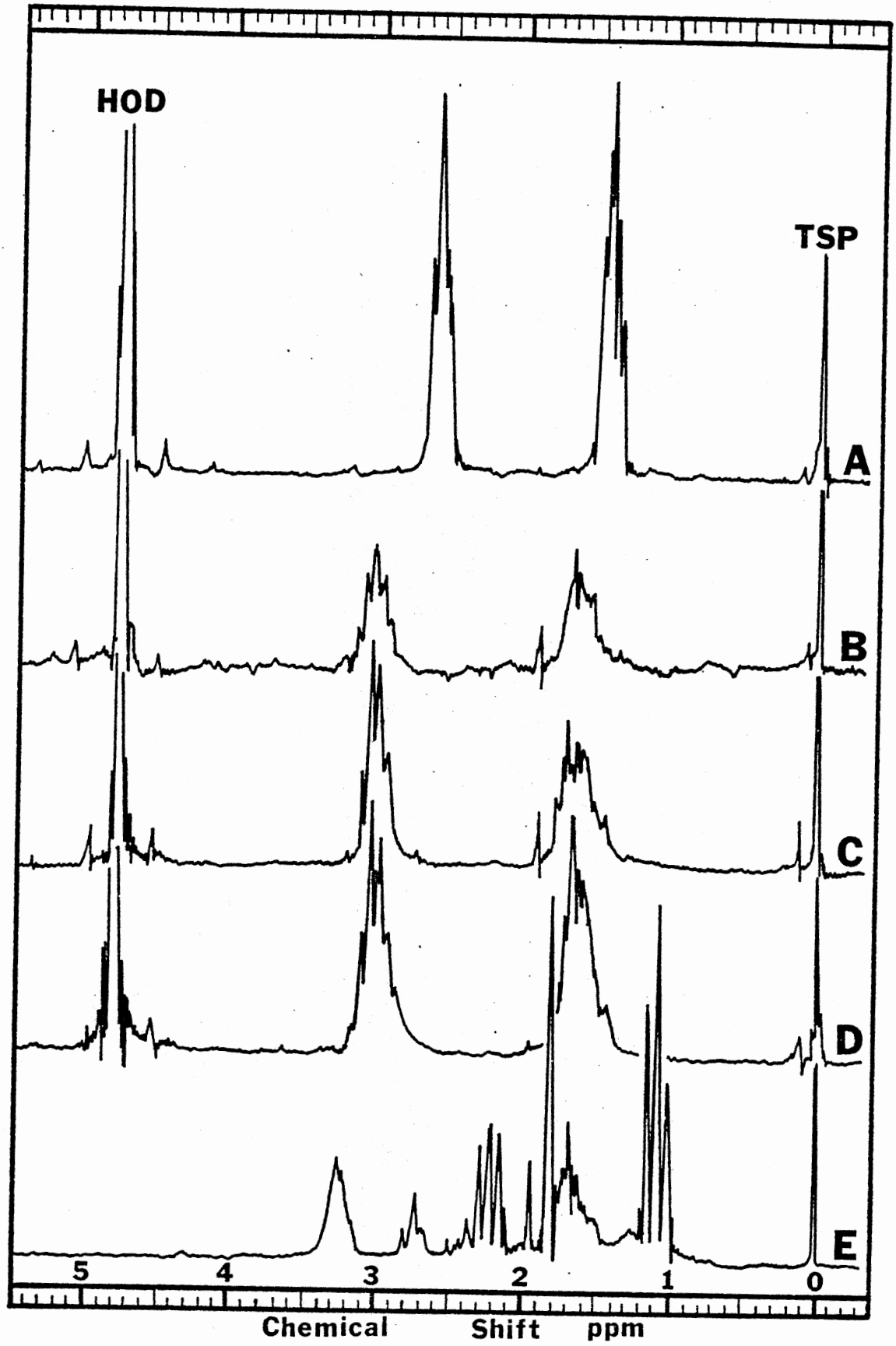

MICRONS
TRANSMITTANCE (%)
NH
CH
Stretch Aliphatic
C=O
NH
Bending
CH2
C-N
C
D
E

Figure 4. Proton nmr Spectra of (A) Authentic Putrescine, (B) Haemolymph Isolate, (C) Authentic Putrescine That Has Been Subjected to the Isolation Procedure, (D) CO_2-Treated Putrescine and (E) Ketimine.


HOD
TSP
A
B
C
D
E
5
4
3
2
1
0
Chemical Shift ppm

CHAPTER IV

DISCUSSION

Based on the data presented, free putrescine is present and appears to be one of the major amines in the haemolymph of corn earworm larvae.

Infrared and proton nmr analyses show that our isolation procedure alters the chemical nature of haemolymph putrescine. IR absorptions indicated the presence of C=O in haemolymph putrescine (Figure 3) while the downfield shifts of absorptions observed in the proton nmr studies suggested that the compound had an increased basicity. These data can be explained by the absorption of CO_2 by putrescine (therefore forming a carbamate) during isolation. Such a derivative was produced when we treated putrescine with CO_2 (Figure 3D and 4D). Our conclusion is supported by reports that, when exposed to air, amines will absorb large amounts of CO_2 to give the corresponding carbamate (Weiland and Trass, 1971 a, b; Fourier and Gouju, 1967). When subjected to analysis utilizing GC the carbamate form of putrescine isolated from haemolymph probably decomposed quickly (due to high column temperature) to yield putrescine which then gave the same retention values as authentic putrescine (Figure 2).

Analysis of ketimine synthesized in this study confirms that condensation of putrescine and methyl ethyl ketone does not occur

during chromatographic isolation of haemolymph putrescine. Although the ketimine (if formed) might have been further reduced to a secondary amine, data from GC and proton nmr spectroscopy do not support this possibility. The isolation procedure presented is thus suitable for isolation of polyamines from biological sources especially when working with small amounts of materials. One must, however, be aware of carbamate formation.

Based on the results obtained utilizing ^{14}C-arginine, it appears reasonable to conclude that the large bulk of haemolymph putrescine is produced by gut micro-flora. The small amounts of radioactive putrescine seen after injection of ^{14}C-arginine might occur as a result of enzymatic conversion directly in the haemolymph; however, this has to be determined. Two other possibilities exist. a) Small amounts of arginine normally enter the gut from the haemolymph. After conversion of this arginine to putrescine and proline, the compounds are secreted back into the haemolymph. b) Another mechanism would involve direct uptake of ^{14}C-arginine from the haemolymph by columnar cells which would utilize it for synthesis of enzymes that would then be secreted into the gut and used for digestive purpose. Normal turnover of these enzymes in the gut would result in making ^{14}C-arginine available to the gut micro-flora for conversion to putrescine and proline.

The presence of free putrescine in corn earworm haemolymph raises several interesting possibilities regarding functionality. It was shown that, in the larvae of brine shrimp (Provasoli and D'Agostina, 1962) and the saw-toothed grain bettle (Davis, 1966),

putrescine is of physiological and developmental importance. The growth and division of many microorganisms are known to be affected by polyamines (Tabor et al., 1961; Tabor and Tabor, 1964); thus the presence of free putrescine within the haemolymph could interfere with the proliferation of microorganisms that have gained access to the haemocoel. In this way, putrescine may be acting as a broad spectrum anti-microbial factor that aids in the humoral defense of the insect. A similar role is known to occur with spermine and spermidine in human semen (Davis et al., 1973).

Insect respiration at the tissue level generally involves direct gaseous exchange between tracheal system and various tissues through diffusion. Occurrence of a $0_2/CO_2$ carrier (e.g. hemoglobin) is restricted to only a few insects including the freshwater Notonecid, larvae of Gastrophilus intestinalis and three species of Hemiptera. The existence of a non-proteinous carrier has never been reported. Due to the high CO_2 affinity of putrescine (and other polyamines), it is possible that these compounds are acting as CO_2 scavengers in the haemolymph, tidying up CO_2 to maintain a balanced oxidation-reduction potential within the haemocoel. The existence of such a CO_2 scavenger may be of importance to the burrowing insects e.g. corn earworm larvae, that survive in an environment where oxygen supply is limited.

At this time, we have no definitive information relating to a functional role for putrescine in the haemolymph of corn earworm larvae. Judging by the color intensity with ninhydrin (Figure 1), it is one of the major amine components present in the haemolymph.

Its role in the infective process of fungi appears to be nil since infection with Beauveria bassiana does not cause either an increase or decrease in the putrescine content of haemolymph. Also, a direct role in development appears not to exist since haemolymph concentrations appear unaltered as corn earworm larvae progress through the third and fourth moltings.

SELECTED BIBLIOGRAPHY

Ackermann, D. 1955. Uberdie biogenen Amine der Puppe des Seidenspinners, Bombyx mori L. Zeit. Physiol. Chem., 302:87-91.

Bonner, W. M. and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem., 46:83-88.

Burton, R. L. 1970. A low-cost artifical diet for the corn earworm. J. Econ. Entomol. 63:1969-1970.

Burton, R. L., D. G. Hopper, J. R. Sauer and J. H. Frick. 1972. Properties of haemolymph of the corn earworm. Comp. Biochem. Physiol., 42B:713-716.

Davis, G. R. 1966. Putrescine and spermidine as growth-promoting substances for the saw-toothed grain beetle, Oryzaephilus surinamensis (L) (Coleoptera, Silvanidae). Comp. Biochem. Physiol., 19:619-627.

Davis, B., R. Dulbecco, H. Eisen, H. Ginsberg, W. Wood and M. McCarty. 1973. Microbiology. 2nd ed., p. 642 and p. 653. Harper and Row, Maryland.

Fournier, J. and D. Gouju. 1967. Equations for absorption of carbon dioxide by three ethylamine and ethylenediamine. C. R. Acad. Sci., Paris, Ser. C. 264:502-503.

Heathcote, J. G. and K. Jones. 1965. The rapid resolution of complex mixtures of the naturally occurring amino acids by two-dimensional thin-layer chromatography on cellulose powder. Biochem. J., 97:15p.

Herbst, E. J., E. B. Glinos and L. H. Amundsen. 1955. An analysis of the putrescine requirement of Hemophilus parainfluenzae. J. Biol. Chem., 214:175-184.

Herbst, E. J. and A. S. Sion. 1970. Polyamine changes during development of Drosophila melanogaster. Fed. Proc., 29: 1563-1567.

Liao, T. H., G. W. Robinson and J. Salinkow. 1973. Use of narrow-bore column in amino acids analysis. Anal. Chem., 45:2286-2288.

Martin, W., M. J. Pelczar and P. A. Hansen. 1952. Putrescine as a growth requirement for Neisseria. Science 116:483-484.

Provasoli, L. and A. D'Agostina. 1962. Vitamine requirements of Artemia salina in aseptic culture. Am. Zool., 2:439.

Sadtler. 1973-1975. The Sadtler Standard Spectra. Sadtler Research Lab., Inc., Philadelphia.

Sneath, P. H. 1955. Putrescine as an essential growth factor for a mutant of Aspergillus nidulans. Nature Lond., 175:818.

Tabor, H., C. Tabor and S. M. Rosenthal. 1961. The biochemistry of the polyamines: spermidine and spermine. Ann. Rev. Biochem., 30:579-604.

Tabor, H. and C. Tabor. 1964. Spermidine, spermine and related amines. Pharmacol. Rev., 16:245-300.

Weiland, R. and O. Trass. 1971a. Absorption of carbon dioxide in ethylene-diamine solutions. I. Absorption kinetics and equilibrium. Can. J. Chem. Eng., 49:767-772.

Weiland, R. and O. Trass. 1971b. Absorption of carbon dioxide in ethylene-diamine solutions. II. Pilot-plant study of absorption and regeneration. Can. J. Chem. Eng., 49:773-781.

VITA

Peter Yee-Kong Cheung

Candidate for the Degree of

Doctor of Philosophy

Thesis: A STUDY OF SOME PARAMETERS RELATING TO MYCOCIDAL ACTIVITY OF BEAUVERIA BASSIANA FOR THE CORN EARWORM HELIOTHIS ZEA

Major Field: Microbiology

Biographical:

Personal Data: Born in Sawtow, China, July 17, 1950, the son of Mr. and Mrs. Cheung Chei Chwin.

Education: Attended primary school in Hong Kong; graduated from LingNan Middle School in 1970; received the Bachelor of Science degree from Oklahoma Baptist University in May, 1974; received the Master of Science degree at Oklahoma State University in May, 1977; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in May, 1981.

Professional Experience: Laboratory assistant, Biology Department, Oklahoma Baptist University, 1971-1974; graduate teaching assistant, Microbiology Department, Oklahoma State University, 1975-1976; research assistant, Entomology Department, Oklahoma State University, Summer, 1976; graduate research assistant, School of Biological Sciences, Oklahoma State University, 1976-1981.

Professional Affiliations: Member of the American Society for Microbiology; member of Sigma Xi.