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IN THE LARVAE OF THE TOBACCO HORNWORM,
MANDUCA Sexta (L.)

The Ohio State University, Ph.D., 1977
Entomology

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INDUCED ANTIBACTERIAL ACTIVITY AGAINST PSEUDOMONAS AERUGINOSA (SCHROETER) MIGULA IN THE LARVAE OF THE TOBACCO HORNWORM, MANDUCA SEXTA (L.)

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
Frederick Erwin Schreiber, B.S., M.S.

* * * * *

The Ohio State University 1977

Reading Committee: W. Fred Hink John D. Briggs William J. Collins Frank W. Fisk

Approved by

W. Fred Hink Adviser
Department of Entomology
To Ruth, Robert and Mark
I thank my adviser Dr. W. Fred Hink for his assistance and encouragement during the course of this study. I also greatly appreciate his help, some of it above and beyond the call of duty, during the preparation and completion of this dissertation.

The rearing of Manduca required a considerable amount of space and equipment. The Department of Entomology and the individual faculty members involved were more than generous in making available to me what I needed.

A fellow graduate student, Frank Eischen, suggested the use of Manduca sexta as an experimental animal. Having just completed his thesis on Manduca, he also taught me how to rear the animal and gave me the needed equipment.

At the time this research was done, Al Pye was studying the role of phenoloxidase in wax moth immunity. He shared literature, ideas, and equipment, all of which proved very helpful.

Dave Chesemore provided valuable assistance in choosing and interpreting statistical tests.

Ruth, my wife, typed the first drafts of this dissertation. That was probably the least of her sacrifices. Though the gesture is inadequate, I thank her.
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INTRODUCTION AND LITERATURE REVIEW

In the 1950s, 6 different researchers looked for the presence of antibodies in insects using conventional serological techniques that had been developed for the demonstration of antibodies in vertebrate blood. In the earliest study, Bernheimer et al. (1952) immunized the larvae and pupae of 2 lepidopterans and detected no phage inactivation, agglutinins or antitoxins. They did find a nonspecific hemagglutinin, but it did not have the characteristics of antibody. Somewhat later, Krieg (1957a) assayed the hemolymph from larvae of 3 insect species representing 3 orders (Coleoptera, Lepidoptera and Hymenoptera) and was unable to find hemolysins, hemagglutinins, bacteriolysins or agglutinins. He also was unable to demonstrate any immunoglobulins by electrophoresis (Krieg, 1957b). Briggs (1958) using 11 species of lepidopterous larvae, could not find any evidence for agglutinins, precipitins, complement or antitoxins. Finally, Stephens (1959) found no agglutinins, precipitins, or bacteriolysins in the hemolymph of larval Galleria mellonella (Lepidoptera).

The unanimous conclusion of these researchers was that the insects studied did not produce antibodies,
substances which are produced in response to a specific antigen and capable of reacting with that antigen (Herbert and Wilkinson, 1971). This opinion is now generally held to be true for all insects as well as all other invertebrates (Whitcomb et al., 1974). Thus antibodies are now considered as a class of serum proteins called immunoglobulins and to be exclusive to the vertebrates (Herbert and Wilkinson, 1971). The implications of the failure to find antibodies in insects were most clearly voiced by Briggs (1958) who concluded that conventional serological techniques were unlikely to be productive in future studies of insect immunity. He also said that new concepts would have to be sought to explain the unusual immune responses that were found in insects.

In spite of the apparent lack of antibodies, a tolerance to bacterial pathogens could be induced by immunization with attenuated bacteria. Briggs (1958) found this tolerance to be nonspecific, rapidly acquired and of a fairly short duration. He was also able to show a corresponding in vitro antibacterial activity that was stable to autoclaving. A similar, short lived immunity was also found by Stephens (1959) which she suggested was similar to that observed by some early researchers in the 1920s and 30s. In demonstrating this immunity, it was shown that there was antibacterial activity in the cell-free serum and thus some active substance or substances other than
antibody must exist in insect hemolymph (Briggs, 1958; Stephens, 1962a).

The search for immune substances other than antibodies has uncovered several materials previously unknown in insect hemolymph which have in turn led to new concepts of how an insect protects itself from invading pathogens.

Early research on insect immunity in the 1920s and 30s had several shortcomings because very small numbers of insects were used (Stephens, 1963c). Therefore, since recent work has covered most of the same ground but with a more rigorous approach, this review will be limited to about the last 25 years. Earlier work is extensively reviewed by Huff (1940). A startlingly large number of reviews of insect and invertebrate immunity have appeared in the last 15 years which probably give the field one of the highest ratios of review to original research in all of biology. Work has been reviewed by Wagner (1961), Salt (1963, 1967, 1970), Stephens (1963c), Briggs (1964), Heimple and Harshbarger (1965), Chadwick (1967), Carton (1969), Jackson et al. (1969), Tripp (1969, 1971), Alekseyev (1971), Gotz (1973), Lafferty and Crichton (1973), Cooper (1973), Whitcomb et al. (1974) and Maramorosch and Shope (1975) among others.

In reviewing the various substances that have been sought in insects, a comparison will be made with those found in vertebrates. The majority of the descriptions of
the nature and action of vertebrate serum substances came from Herbert and Wilkinson (1971) and Barrett (1974). Other sources will be referred to where used. The literature to be discussed will be divided into 8 sections: agglutinins, opsonins, precipitins, antitoxins, complement, transplant rejection, responses to parasites, and antibacterial responses in normal and immunized animals.

Agglutinins are substances that cause the clumping of particulate suspensions of materials such as red blood cells (hemagglutinins) or bacteria (bacterial agglutinins). The clumps are held together by the agglutinin which forms cross links between the cells. There are 2 general types of agglutinins, antibodies and lectins, which are quite different in nature and, possibly, function. Agglutinating antibodies are either naturally occurring immunoglobulins such as those associated with ABO blood groups or induced immunoglobulins such as those which agglutinate bacteria. Since immunoglobulins are found only in vertebrates (Saunders, 1970), agglutinating antibodies are similarly restricted. Lectins, on the other hand, are not immunoglobulins and have been isolated from lower vertebrates, invertebrates and many plants (Sharon and Lis, 1972). There are also some animal viruses, including insect viruses, which show lectin-like hemagglutinating activity (Reichelderfer, 1974). Sharon and Lis (1972) characterized lectins as being proteins of varying molecular weights that
usually are formed of subunits; many are glycoproteins and require a divalent cation for activity. All lectins agglutinate some type of cell.

It is not known if invertebrate agglutinins are truly defensive molecules being analogous and possibly homologous to vertebrate immunoglobulins or if they are lectins where the serological agglutinating activity may have little or no relation to in vivo function. Renwrantz and Uhlenbruck (1974) and Acton and Weinheimer (1974) are the most recent authors to suggest that invertebrate agglutinins are part of a non-specific defense system in which the agglutinins would function as immobilizing agents or opsonins. While there is some evidence that agglutinins in non-insect invertebrates do have a defensive function in vivo (Pauley, 1974), Sharon and Lis (1972) classed invertebrate agglutinins as lectins along with the plant agglutinins and stated that no function is known for lectins in their source organism.

Hemagglutinins have been found in a variety of insect species. Berheimer (1952) found a hemagglutinin for human erythrocytes in the hemolymph of 10 of 46 larval lepidopterans tested, including Protoparce sexta (=Manduca sexta) the insect used in my study. Some of the hemagglutinins were specific for particular blood types. An induceable hemagglutinin for human erythrocytes found by Bernheimer et al. (1952) in Citheronia regalis larvae
was not considered an antibody because of its low titer and lack of specificity and stability. Feir and Walz (1964) also detected a hemagglutinin for human erythrocytes in the hemolymph of *Oncopeltus fasciatus* which lost its activity after 45 minutes at room temperature, freezing, or heating to 56°C for 10 minutes. Heat labile, non-induceable hemagglutinins have been demonstrated in *Periplaneta americana* hemolymph for erythrocytes of 5 vertebrate species (Scott, 1971a). These hemagglutinins were not gamma globulin (Scott, 1972). Work done by Anderson et al. (1972) showed the presence of a naturally occurring hemagglutinin in the hemolymph of *Blabarus Craniifer* (=*Blaberus craniifer*) which was inactivated by heating at 56°C for one hour but was stable during storage at 0°C or -70°C, and was insensitive to 0.04M ethylenediamine tetraacetate (EDTA). These characteristics indicated the hemagglutinin was not an immunoglobulin. Neither naturally occurring nor induced hemagglutinins could be detected in *Melolontha sp.*, *Bombyx mori* or *Neodiprion sertifer* (Krieg, 1957a).

With one exception, searches for bacterial agglutinins in insects have been unsuccessful. The absence of natural or induced bacterial agglutinins have been reported by Krieg (1957a), Briggs (1958), Stephens (1959), and Gingrich (1964) in insects representing 4 orders. The exceptional results were obtained by Gilliam and Jeter
(1970) with adult worker honey bees, Apis mellifera. A quite specific agglutinin for Bacillus larvae was found 24 hours after immunization with this honey bee pathogen. Since the agglutinins for the same bacterium were also reported in the hemolymph of larvae and adults from infected honey bee colonies (Gary et al., 1948), they merit further study.

In vertebrates, opsonins are either antibodies or complement components that, by binding to the antigen, make it more susceptible to phagocytosis. In non-insect invertebrates some hemagglutinins have been shown to have opsonic activity (Acton and Weinhimer, 1974) but no such activity has been found in insects. Scott (1971b) found no opsonic activity in P. americana serum for vertebrate erythrocytes even though P. americana serum contains a hemagglutinin (Scott, 1971a). Lack of success was also reported by Anderson et al. (1973) who tried to increase phagocytosis of Staphylococcus aureus by B. craniifer hemocytes by preincubating the bacteria in concentrated serum.

Vertebrate precipitins are antibodies that cause the visible precipitation of water soluble antigens by forming large antibody-antigen complexes that are no longer water soluble. Using hemolymph from larvae of 8 Lepidopterous species, Briggs (1958) found non-specific precipitation in both experimental and control tests. The
precipitation occurred in isolated cases and was not reproducible. Stephens (1959), using 4 different techniques, was not able to detect precipitins in larval G. mellonella hemolymph. Also using larval G. mellonella, Bullock and Steinhauer (1970) used an agar well diffusion technique in which the reactants were allowed to diffuse toward each other from separate wells in the agar. Precipitates formed when heat fixed (60°C) or glutathione-treated wax moth sera were diffused against serum from female P. americana and on a few occasions when diffused against normal rabbit or horse sera. It should be noted, however, that the precipitates were not formed as a typical line where the interacting sera met but were formed as a halo encircling the wax moth serum well. This suggests some kind of autoprecipitation rather than a specific protein-protein interaction between the respective sera. Using the Ouchterlony technique, no precipitins could be found in nymphal O. fasciatus; the fluorescent antibody technique also failed to detect any formed antibody (Feir and Schmidt, 1968). The available experimental results, while few in number, suggest that no precipitins occur in insect hemolymph.

As is the case with agglutinins and precipitins, the active agent in toxin-antitoxin reactions in vertebrate serology is antibody. The toxin is neutralized when it combines with specific antibody. While 3 papers note no
evidence for specific antitoxins, one of them does report an inducible response to toxins. Bernheimer et al. (1952) found an inhibition of streptolysin O hemolysis by hemolymph from both control and immunized *Samia cecropia*, but the reaction was inconsistent. After immunization, Briggs (1958) was unable to detect any protection against diphtheria toxin in 2 species of lepidopterous larvae. On the other hand, Kamon and Shulov (1965) reported the presence of an inducible immunity in the locust, *Locusta migratoria migratorioides* against the venom of the scorpion *Leiurus quinquestriatus*. The immunity could be induced by several injections of either bovine serum albumin, amylose or sub-lethal doses of the venom (Kamon, 1965). Precipitin and hemagglutination tests were both negative and immune hemolymph had no ability to neutralize venom before injection into mice. Kamon and Shulov (1965) suggested a cellular defense mechanism was possible because I\(^{131}\)-labeled venom was taken up by the locust pericardial cells immediately after injection.

Vertebrate complement is a naturally occurring, non-inducible serum system consisting of 9 protein components. Complement components interact sequentially (C1, C4, C2, C3, C5, C6, C7, C8, C9) when activated by an antibody antigen reaction to form active enzyme complexes. These complexes form on the outer membrane of Gram negative bacteria and cause the formation of osmotic "holes" which
lead to cell lysis. Thus the system is bactericidal. Some of the complement components are also involved in chemotaxis and opsonization.

The results have been uniformly negative when researchers have sought the complete complement system in insects. Anderson et al. (1972), Briggs (1958), Cappellato and Narpozzi (1960), Conner (1969), and Morgan (1950) were unable to demonstrate complement in the hemolymph of a variety of insect species. However, portions of the complement system or analogous substances have been detected in insects and other invertebrates. This has been accomplished by using alternate methods of activation of complement which do not involve antibody and the first 3 components (Cl, C4, C2). One activating substance is cobra venom factor (CVF) from the venom of the cobra. CVF, acting through a serum factor called C3 proactivator, initiates the cascade reaction without involving the first 3 components. CVF has been used to detect the terminal complement-like activity in the serum of the horseshoe crab and sipunculid worm (Day et al., 1970). But Anderson et al. (1972), using similar techniques, were unable to demonstrate this kind of activity in the hemolymph of the cockroach B. craniifer. They were able to demonstrate the presence of a C3 proactivator in the hemolymph. Considering their findings in invertebrates and the fact that the terminal complement components (C3-9) can also be activated
by bacterial lipopolysaccharides, Day et al. (1970) suggested that C3-9 may be a primitive precursor of the complement system found in vertebrates. The primitive complement system may have been involved in invertebrate inflammatory and phagocytic responses and evolved into the complex vertebrate system by the addition of antibody and C1, C4, and C2. Acton and Weinheimer (1974) have speculated that invertebrate hemagglutinins could be potential activators of the terminal complement components in invertebrates.

The only report of success in finding the terminal components in insects is that of Morgun (1950) who found that the hemolymph of cockroaches, caterpillars and pupal Antheraea pernyi would reconstitute frog complement deprived of the terminal components. Capellato and Narpozzi (1959), however, were unable to detect terminal components in B. mori hemolymph. More work in this area is needed because the presently available evidence is too inconsistent to allow any firm conclusions on the presence of complement in insect hemolymph.

The rejection of transplanted tissue in vertebrates is mediated by lymphocytes and antibodies. Genetically non-identical tissues are rejected mainly by cellular reactions and second transplants are rejected more rapidly because the formation of tissue specific antibodies aids in the rejection process. The relative importance of different
cell types and antibodies in vertebrate transplantation rejection is currently the subject of intensive research. In insects, discussion of the involvement of humoral factors in the recognition of or reaction to foreign tissue at present can only be speculative. It may be that no humoral factors are involved at all because Salt (1970) has interpreted the results of his extensive research to mean that the ability in insects to recognize foreign tissue is mediated by the hemocytes' capacity to recognize surfaces different from the lining of the hemocoel. Transplants between 2 individuals of the same species are not encapsulated by hemocytes unless the connective tissue covering the implant is damaged. A similar principle seems to apply to an insect's own tissues. Tissues degenerating during metamorphosis are not attacked by hemocytes until the surrounding connective tissue breaks down (Salt, 1970). Only circumstantial evidence suggests that humoral factors may be involved. Electrophoresis of hemolymph proteins has revealed that protein patterns are more similar for species within a genus than for species from different genera (Florkin and Jeuniaux, 1964). This correlates with the fact that grafts of uninjured tissue between members of the same species are rarely encapsulated and that sometimes tissues transplanted between species in the same genus are tolerated. Very few inter-generic transplants are accepted (Salt, 1970).
The responses of vertebrates to parasites are similar to their responses to transplanted tissues. However, antibodies seem to have relatively little if any effectiveness because of the size and complexity of the parasite. Generally protection against parasites in vertebrates is rather poor.

Insects often respond to the presence of a parasite in the hemocoel with a cellular reaction. Cells encountering the parasite flatten against it and form a capsule many cells thick with the inner cells often forming a layer of melanin around the parasite. The exception to the general effectiveness of insect reactions occurs with "habitual" or "usual" parasites of a particular host species which either do not provoke a reaction or are apparently not affected by it.

Responses to parasites were reviewed by Salt (1970) who proposed that cellular defense reactions depend on the surface properties of the parasite. He suggested that those parasites that mimic the properties of the host's own connective tissue will not be encapsulated and thus become that host's "usual" parasite. Salt views responses to parasites as being primarily cellular.

Work by Vinson and his colleagues suggests that humoral factors may be involved in resistance to parasitization. The braconid parasite Cardiochiles nigriceps will oviposit in both Heliothis zea and H. virescens but the eggs
survive only in *H. virescens*. Eggs of the parasite in *H. zea* are encapsulated within 24 hours (Lewis and Vinson, 1968). When parasite eggs were removed from the hemocoel of *H. zea* to the hemocoel of *H. virescens*, the normally non-reacting host was then able to encapsulate the eggs (Lewis and Vinson, 1968). The authors concluded that a humoral reaction in *H. zea* altered the parasite surface in order to make it susceptible to encapsulation in *H. virescens*. Electron microscopic evidence for this conclusion (Vinson and Scott, 1974) showed that eggs in *H. zea* lose the outer layer of their chorion which is then replaced by a microflocculate layer before encapsulation occurs. Eggs in *H. virescens* lost the outer chorionic layer more slowly and this layer was replaced by a granular layer which persisted until hatching. Two possible explanations for the difference between *H. zea* and *H. virescens* have been investigated. Brewer et al. (1972) used the immunodiffusion technique to detect a unique protein in *H. zea* hemolymph which they suggested may have an opsonic effect. Brewer and Vinson (1971) found that injections of phenylthiourea or reduced glutathione decreased the ability of *H. zea* to encapsulate parasite eggs or nylon threads. They further noted that *C. nigriceps* eggs which normally became "sticky" in *H. zea* before hemocytes attached did not become "sticky" when *H. zea* received a previous injection of phenylthiourea or
reduced glutathione. Parasite eggs in *H. virescens* were never "sticky" when removed from the hemocoel.

A humoral encapsulation of parasites has recently been shown in dipterous larvae. Poinar and Leutenegger (1971) described encapsulation of a nematode, *Neoaplectana carpocapsae*, by larval *Culex pipiens*. Pigment granules formed within a homogeneous matrix and fused to form a melanin capsule by 5 to 10 hours after entrance of the nematodes into the hemocoel. Blood cells were not seen to participate in the reaction. A similar reaction to 3 species of fungi occurs in *Chironomus* larvae (Gotz and Vey, 1974). The authors suggested that since dipterous larvae generally have few circulating hemocytes, the melanization defense reaction has been shifted from the hemocytes to the serum.

Several serum factors in vertebrates have antibacterial properties. Antibody may function as a bactercidin or, when combined with complement, can cause lysis of Gram negative bacteria. Vertebrate serum also contains lysozyme which is active against Gram positive bacteria.

Several antibacterial substances apparently occur in insect serum. Two have been characterized. One exhibits lysozyme activity; the other is stable to elevated temperatures and low pH. Some authors have concluded that the characterized substances are one and the same while others
suspect a connection between the heat stable factor and the process of melanization. There are also a few reports that suggest other bactericidal substances occur in insects which are distinct from the above 2.

Antibacterial activity has been shown either as an increased tolerance to injected pathogens following immunization or as a serological reaction using hemolymph from immunized or normal insects. Thus induced immunity will be discussed along with antibacterial activity. The discussion will be arranged phylogenetically in the orders Orthoptera, Hemiptera, Coleoptera, Diptera and Lepidoptera.

The immune response in Orthoptera involves either lysozyme activity or a substance which may be a protein. Immunity to *Tetrahymena pyriformis* was induced by injection of the ciliates into male *P. americana* by Seaman and Robert (1968). Those cockroaches that survived the original injection were subsequently resistant to higher doses and produced a factor that immobilized the ciliates in vitro. The activity principle was destroyed by lowering the pH to one for one hour or by heating to 75°C for 20 minutes and could be separated from the hemolymph by ammonium sulfate precipitation. Gel electrophoresis showed that the activity was associated with an acidic protein that occurred in normal roaches which had no activity previous to immunization. The ciliate immobilizing activity of the hemolymph reached maximum levels one day after immunization and then
decreased rapidly. When cockroaches were repeatedly immunized at 3 day intervals, immobilizing activity rose more rapidly and to higher levels with each immunization. This anamnestic type of response has been reported in only one other insect, Locusta migratoria.

Adult L. migratoria can be immunized against a lethal dose of Bacillus thuringiensis by a non-lethal low dose of the live bacterium (D. Hoffmann, 1972). Full protection was achieved by 12 hours and lasted for 15 days. Immunity was also produced by injections of Escherichia coli and with less effectiveness by iron saccharate. This report is very interesting because it is the only report on immunity to B. thuringiensis. A second immunization of L. migratoria produced a higher level of protection than the first. Hoffman and Porte (1973) found that after immunization with either a non-lethal dose of B. thuringiensis or the bacterial culture filtrate, groups of cells in the pericardial tissue differentiated within 12 hours into secretory cells with extensive endoplasmic reticulum and Golgi. The correlation of secretory differentiation in the pericardial cells and the presence of immunity suggests that protein synthesis may be involved. It may also be significant to note that the pericardial tissue of L. migratoria is the insect's hemocytopoietic tissue (J. A. Hoffmann, 1972).
Lysozyme and chitinase activity has been found in the hemolymph of several orthopterans. Malke (1965) found that among 8 species of Blattidae and one species of Acrididae that he tested, all had lysozyme activity in their hemolymph as well as in other tissues. Both lysozyme activity (Powning and Irzykiewicz, 1967) and chitinase activity (Waterhouse et al., 1961) has been noted in *P. americana* hemolymph. Landureau and Jolles (1970) and Landureau et al. (1972) assayed for both enzymes in the tissue culture medium of *P. americana* hemocytes and found the chitinase activity to be much greater than the lysozyme activity. They concluded that the hemocytes produced and released a chitinase.

Whether the hemolymph of orthopterans contain one or both of these enzymes has not been definitely determined. The problem is caused by the fact that each substrate can be hydrolysed at significant rates by both enzymes (Chipman and Sharon, 1969). Since all of the above authors used unpurified preparations, there is no assurance that an assay for one enzyme is not really measuring the activity of the other enzyme or the activity of both. In only one instance has lysozyme, produced by immunization of an insect (*G. mellonella*), been purified and fully characterized (Powning and Davidson, 1973). The fact that injections of egg white lysozyme are highly toxic to *P. americana* suggests that at least in *P. americana* the enzyme is a
chitinase (Daniel and Brooks, 1972; Wharton and Lola, 1969). It would be worthwhile knowing the exact nature of the enzyme in the hemolymph because it may be a significant defense mechanism against Gram positive bacteria or fungi. Ryan and Nichola's (1972) found that 2 Gram positive bacteria, B. thuringienses and Corynebacterium sp. were lysed when injected into the hemocoel of P. americana.

The antibacterial substance in hemipterans has been shown to be highly stable to heat treatment. Frings et al. (1948) found that normal serum of O. fasciatus adults and larvae had antibacterial activity against S. aureus and to a lesser degree against Bacillus subtilis but had no activity against 9 other species of bacteria. They characterized the active component as being water soluble, stable to boiling for 30 minutes but destroyed by longer heating or standing at room temperature for 6 hours. The authors concluded that the active substance was not a protein because of its heat stability and the fact that removal of coagulated protein after boiling did not reduce antibacterial activity.

Ginrich (1964) was unable to detect antibacterial activity against Pseudomonas aeruginosa in normal serum of O. fasciatus but found an induced bacteriolytic factor in immunized adults. The development of the in vitro activity paralleled the increased protection to challenge in the bug. The induced substances migrated with several
electrophoretically separated proteins but when proteins were precipitated by trichloracetic acid, the activity remained in the supernatant. Antibacterial activity was stable to heat (75°C for one hour) and acid and base.

After immunization of adult *O. fasciatus* with *E. coli* lipopolysaccharide, Mortimore (1970) found a high level of protection against *P. aeruginosa* challenge in male bugs only. No immunity could be detected in the females. The author noted that the female bugs used were laying eggs but did not know what effect it may have had on the induction of protection. Kamp (1968) was unable to immunize *Pyrrhocoris apterus* with heat killed suspensions of either *P. aeruginosa* or *Serratia marcescens*.

Although immunity has been demonstrated in coleoptera, the active substance(s) have not been characterized. Induced immunity in *Tenebrio molitor* has been reported by Mortimore (1970). Larvae immunized with *E. coli* lipopolysaccharide and challenged by *P. aeruginosa* or immunized by *S. marcescens* lipopolysaccharide and challenged with *S. marcescens* showed maximum protection 24 hours post immunization. Decreasing levels of protection were detected over the following 4 days. Supralethal doses of x-irradiation, given several hours before immunization, interfered with the development of immunity. It is difficult to interpret this interference because the tissue that produces the antibacterial factor and the mechanism of
production are not known. In contrast to the above results, Ziprin (1970) found that immunization of *T. molitor* with heat killed *P. aeruginosa* potentiated the lethality of the challenge rather than inducing protection.

The immune substances in Diptera have been characterized as being heat stable or as a protein. Landi (1960) found bactericidal activity in the cell free sera from larvae of several species of bot flies and warbles by spreading the serum on a nutrient agar plate seeded with bacteria. Various species of *Sarcina*, *Micrococcus* and *Staphylococcus* were killed while *E. coli*, *P. aeruginosa* and *S. marcescens* among others were not affected by the serum. Boiling the hemolymph did not destroy activity.

Bowman et al. (1972) developed a system with male *Drosophila melanogaster* in which *Aerobacter cloacae* was an effective immunogen but *P. aeruginosa* and *E. coli* were not. Flies immunized with *A. cloacae* were immune to all 3 species of bacteria when challenged 2 to 4 days later, however, one strain of *A. cloacae* grew well when inoculated into immunized flies. The mechanism of protection was apparently not cellular because in vitro bactericidal activity was demonstrated with a cell free homogenate and because induction of immunity was inhibited by cyclohexamide but not colchicine. This last fact also suggests that protein synthesis was involved.
There is an interesting series of reports by Bakula on the mechanism of regulation of symbiotic flora in *Drosophila*. She reported that citric acid extracts of the hemolymph of *D. melanogaster* pupae had antibacterial activity against *Brevibacterium* sp. and *Bacillus* sp.; 2 bacteria isolated from the gut of 2 different strains of *D. melanogaster* (Bakula, 1970). These 2 bacteria were apparently mutualistic for their respective strains of flies. The bacteria showed a striking decrease in numbers during the second 12 hour period in the pupa which corresponds with the period when *in vitro* antibacterial activity was detected (Bakula, 1969, 1970).

Bakula (1971) later isolated antibacterial activity in the lysosomal fraction of homogenates of whole third instar larvae. The antibacterial substance which was extracted with acid-ethanol did not have any lysozyme activity and was trypsin sensitive. The author speculates that during the massive tissue hydrolysis in the early pupa, an antibacterial protein may be released from the lysosomes or some associated cellular fraction into the blood.

Antibacterial activity in Lepidoptera has been attributed to either lysozyme or a heat stable substance which may be related to melanization. Recent work in this order was begun by June Stephens on *G. mellonella*, an insect that continues to be very popular because it is
relatively easy to rear. She has shown both induced immunity and *in vitro* antibacterial activity in this insect. Maximum immunity occurred 12 to 24 hours post immunization and was essentially gone by 72 hours. *In vitro* assays for bactericidal activity showed similar changes in activity (Stephens, 1962a). Although the bacterial immunogen could be prepared by various treatments and still be effective (Stephens, 1963a), non-specific substances such as saline, nutrient broth, egg albumin, glucose and zymosan did not cause the production of bactericidal serum (Stephens, 1962b).

Hemolymph from immunized insects showed an inhibition of melanization with the maximum inhibition corresponding closely with the period of maximum immunity (Stephens, 1962a). A factor that inhibited melanization could be removed from the immune hemolymph by dialysis (Stephens and Marshall, 1962). Dialysis also removed the bactericidal factor and thus the authors concluded that the 2 factors may be the one and the same. Other evidence which supports this conclusion is that a fraction eluted by 0.1N NaOH from an anion exchange resin was both bactericidal and inhibitory of hemolymph tyrosinase. Although no active fraction could be removed from the hemocytes of immunized larvae (Stephens and Marshall, 1962), immunization did cause significant changes in the total hemocyte count (THC). By 20 hours post immunization
the THC dropped to about half its normal level and remained at about this level even after lethal challenge with *P. aeruginosa* (Stephens, 1963b).

Hink and Briggs (1968), building on the work of Stephens, isolated bactericidal factors from both normal and immune *G. mellonella* sera. They found 2 bactericidal fractions by gel chromatography. Factor A with an estimated molecular weight of 7000 a.m.u. occurred in both normal sera and at a higher concentration in immune serum. Factor B with a molecular weight of less than 2000 a.m.u. occurred only in immune serum. The fact that factors A and B did not appear in the same chromatographic peaks as biuret positive materials, ninhydrin positive materials and ultra-violet light (280 nm) absorbing materials suggests that the factor was not a protein or peptide.

Mortimore (1970) also found that *G. mellonella* can be immunized against *P. aeruginosa*. She also showed that 14 Kr of x-ray irradiation received 4 hours before immunization did not interfere with the development of immunity.

The work of Ziprin and Hartman (1971) stands in contrast to virtually all the other work on induced immunity in *G. mellonella*. They found that the bacterin was toxic to most strains of larvae, killing them within 12 to 24 hours. Larvae showed a melanization first along the dorsal median line and then over the entire body. The toxicity in
sensitive strains and the inhibited melanization in resistant strains was shown to be produced by the lysozyme sensitive fraction of the *P. aeruginosa* cell wall.

While many of the above authors suspect that the process of melanization is somehow related to the induction of immunity, Mohrig and Messner (1968) interpret the results of all studies on insect immunity as indicating the presence of lysozyme alone. They consider the coincidence of inhibition of melanization with immunity to be of relatively little consequence. They showed that hemolymph of normal *G. mellonella* lysed *Micrococcus lysodioticus* and that this activity was not decreased after 2 hours incubation with trypsin and only slightly decreased after heating to 100°C at a pH of 4. The lysozyme titer of the hemolymph was increased by a factor of 30 by 24 hours following immunization. Similar increases in lysozyme titer were induced by injection of Ringer's solution, distilled H₂O, methylene blue, Chinese ink, 0.5% Cysteine and normal hemolymph. They suggest that lysozyme may be active on Gram negative bacteria through some kind of synergistic mechanism involving some other hemolymph component. They base their assertion that Stephens and Marshall's (1962) results are attributable to lysozyme on the fact that Stephens and Marshall found the active factor to be heat stable and stable to trypsin treatment. Mohrig *et al.* (1970) explain the results of Hink and Briggs (1968) by
the presence of lysozyme. They found that the ninhydrin and biuret methods Hink and Briggs (1968) used to detect eluted lysozyme were ineffective below a concentration of 500 μg/ml. Thus the active fraction did not also show the presence of protein. Hink and Briggs (1968), however, also used uv absorbance at 280 nm to detect proteins and still did not detect any in the active fraction. Since the molecular weight of lysozyme from various sources ranges from about 14,500 a.m.u. upward (Chipman and Sharon, 1969; Jolles, 1964), Mohrig et al. (1970) reinterpreted Hink and Briggs' (1968) molecular weight determination. They assert that lysozyme would show an abnormally long elution time (and hence an abnormally low molecular weight) on dextran gel columns because the dextran serves as a semi-substrate for the enzyme. However, Hink and Briggs (1968) used Bio-Gel columns which are polyacrylamide and unlikely to serve as a semi substrate for lysozyme.

Chadwick (1970) has objected to Mohrig and Messner's (1968) assertion that lysozyme is responsible for all immunity in insects and presented evidence that suggests lysozyme is not the only active substance in G. mellonella serum. She found that while the increase in lysozyme concentration after immunization approximately paralleled the increase in immunity, the lysozyme activity remained at a high plateau while the protection against P. aeruginosa returned to low levels. She also confirmed that injection
of water or saline increased the lysozyme concentration but found that no protection against *P. aeruginosa* could be demonstrated.

Powning and Davidson (1973) also found that increased lysozyme activity could be induced in *G. mellonella* by immunization, in this case by injection of *M. lysodeikticus*, but no increase in lysozyme activity was induced by sterile saline injections. There may be strain variability in *G. mellonella*’s response to saline injections similar to that shown by Ziprin and Hartman (1971) to injections of heat killed *P. aeruginosa*.

The work of Kamp (1968) is relevant at this point. When a 3 μl suspension of 0.5 or 1.0% lysozyme was injected into *G. mellonella* larvae, the larvae successfully withstood a lethal dose of *P. aeruginosa* 24 hours later but not immediately after the lysozyme dose. A 3.0% lysozyme injection produced no immunity to *P. aeruginosa* either immediately or 24 hours after immunization. This pattern of immunity suggests that lysozyme had induced immunity rather than was itself responsible for the immunity.

A second lepidopteran that has been used to study induced immunity is *B. mori*. The most extensive studies have been done by Kawarabata (1970, 1971) who successfully induced immunity to *P. aeruginosa* in pupae with killed *P. aeruginosa* or live *E. coli*. In vitro antibacterial activity was stable to dialysis, a pH of 2, or 60°C for 15
minutes but not to 70°C for 15 minutes. Chromatography on Sephadex G-100 yielded a fraction that contained both bactericidal and bacteriolytic activities, leading the author to speculate that the active material might be lysozyme. However, in the later study (Kawarabata, 1971), immunized animals showed no increase in lysozyme activity, although a significant increase in *in vitro* bactericidal activity was noted.

It should be noted that Powning and Davidson (1973) did induce increased lysozyme activity in *B. mori* by injecting *M. lysodeikticus* but they did not attempt any correlation with *in vivo* immunity or *in vitro* bactericidal activity. The variability of results on induction of lysozyme activity in *B. mori* may be due to the different strains of animals used or some other unknown factor.

An induced antibacterial response was shown in *B. mori* and five other lepidopteran larvae by Briggs (1958). A low level naturally occurring antibacterial activity was demonstrated *in vitro* in all the larvae except *B. mori*, but a high level of activity could be induced in all the larvae which was stable to autoclaving.

It can be concluded from this review of the literature that the nature of insect immune substances has not been clearly established; but it is apparent that at least one factor in addition to lysozyme does exist. The heat stable antibacterial activity found by many workers may
represent this factor and the concomitant inhibition of melanization found by Stephens (1962a and b) may also be relevant. Thus one of the areas investigated in this research was the relative importance of lysozyme and heat stable factor(s).

A second reason why the nature of insect immune substance is unclear is the difficulty in obtaining a large enough volume of hemolymph so that active substance(s) may be isolated, purified and identified. Given a large enough sample, it should not be difficult for an organic or biological chemist to determine the nature and even the structure of any immune substance. Thus another goal of this study was to establish the presence of inducible immunity in an insect that could provide large quantities of hemolymph.

Induced antibacterial immunity has been observed in detail in only 2 insects, G. mellonella and O. fasciatus, and thus observations should be made of more species. It can then be learned if significant differences exist in abilities of different species to respond to bacteria. Differences in the immune response, if found, also offer the possibility to adding new insights into the immune reaction.

M. sexta was chosen as an experimental animal because it has not yet been used to study insect immunity, yields up to 1 milliliter of hemolymph per larva, has well-established
techniques for continuous rearing and is becoming increasingly popular as a subject for physiological studies. It thus will add another lepidopteran to those already studied and has the potential for adding new insights about the immune response in insects.

To begin this study, the pathogenicity of P. aeruginosa and S. marcescens for M. sexta was determined by finding their respective 48 hour LD_{50}'s. The ability of these two organisms to grow in serum was also demonstrated.

Larvae were immunized with heat killed bacteria and the resulting level of in vivo protection and in vitro antibacterial activity was measured. P. aeruginosa proved to be effective both as an immunogen and as an assay organism while S. marcescens did not give as striking results. Thus all subsequent work was done with P. aeruginosa. The stability of the in vitro antibacterial activity was determined to boiling, extremes of pH and storage at 4°C.

Immunization sometimes produced melanization in the integument and hemolymph. The melanization was shown to be a precondition for in vitro antibacterial activity. Lastly, the lysozyme activity of immunized larvae was measured.
METHODS AND MATERIALS

Rearing

The colony of the tobacco hornworm, *Manduca sexta* (L.), was started from eggs supplied by Dr. D. L. Dahlman of the Department of Entomology, University of Kentucky, Lexington, KY. Rearing procedures and artificial diet were modified from Yamamoto (1968, 1969), D. L. Dahlman (personal comm.), and F. Eischen (personal comm.). Three day old eggs were placed in a shallow filter paper cone surrounded by strips of artificial diet. The cone and diet were placed on the center of a half inch mesh wire rack in a 25 x 30 x 5 cm plastic box (Tri-State Plastics, Henderson, KY). The larvae hatched on the evening of the third day and migrated to the diet. Since newly hatched larvae exhibit positive phototaxis, the boxes were kept in total darkness for the first 4 days to insure that the larvae would not leave the diet. After 4 days, the filter paper cone was removed and the boxes were transferred to an environmental chamber providing a temperature of 30°C and a 15 hour photophase. The 15 hour photophase inhibited pupal diapause (Bell *et al.*, 1975) and thus allowed continuous rearing throughout the year. Diet was added as needed and paper toweling in the bottom of the boxes was
changed when necessary to keep the boxes clean. When fifth instar larvae began to emerge in a box, they were brought into the laboratory (25°C with a 15 hour photophase) for convenient access. Fifth instar larvae were selected as needed and kept in an environmental chamber providing 30°C and a 15 hour photophase during all experiments. All experiments were conducted using fifth instar larvae.

Diseased and malformed larvae were removed from the rearing boxes as they were found. Such animals were infrequent as long as overcrowding was prevented. No disease epizootics occurred in the stock cultures during the course of this study.

The artificial diet was prepared by mixing the first 12 ingredients listed in Table 1 with 900 ml of water in a one gallon Waring blender. Then 2200 ml of agar solution (cooled to 60-65°C) was added and thoroughly mixed. After mixing, the diet was poured into shallow enamel pans and stored in the refrigerator until needed.

Some fifth instar larvae were allowed to continue development to the prepupal or crawler stage and were collected daily to be placed in an artificial pupation chamber (Hoffman and Dickerson, 1968). Four days later, pupae were collected from the chamber and stored. Pupae collected from a 2 week period were placed on the floor of the flight cage where adults emerged. The flight cage was 1.8 x 1.2 x 1.8 m high and was constructed of a wooden
Table 1

Artificial Diet for Tobacco Hornworm Larvae

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>% by Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wesson's salt mixture</td>
<td>36g</td>
<td>0.97</td>
</tr>
<tr>
<td>Sucrose</td>
<td>108g</td>
<td>2.92</td>
</tr>
<tr>
<td>Casein</td>
<td>128g</td>
<td>3.46</td>
</tr>
<tr>
<td>Food yeast</td>
<td>56g</td>
<td>1.5</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>1180g</td>
<td>4.86</td>
</tr>
<tr>
<td>Vitamin diet fortification mixture(^a)</td>
<td>18g</td>
<td>0.49</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>4g</td>
<td>0.11</td>
</tr>
<tr>
<td>Methyl-p-hydroxybenzoate</td>
<td>4g</td>
<td>0.11</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>0.72g</td>
<td>0.02</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>14g</td>
<td>0.38</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td>12g</td>
<td>0.32</td>
</tr>
<tr>
<td>37% formaldehyde</td>
<td>8ml</td>
<td>0.22</td>
</tr>
<tr>
<td>Agar</td>
<td>35g</td>
<td>0.94</td>
</tr>
<tr>
<td>Water</td>
<td>3100ml</td>
<td>83.70</td>
</tr>
</tbody>
</table>

\(^a\)Nutritional Biochemicals Corporation, Cleveland, Ohio.
frame covered with cheese cloth. Ten percent sucrose was provided in artificial flowers for feeding and a tobacco plant was placed at a central point in the cage for oviposition. Eggs were collected daily and stored at 30°C until ready to hatch.

**Bacterial Strains Used**

*Pseudomonas aeruginosa* (Schroeter) Migula had been maintained at the Ohio State University for several years by different workers. The strain used, Pll-1, was originally obtained from Dr. June Stephens Chadwick who has used it for the majority of her work on immunity in *G. mellonella*. A Gram-negative bacillus which produced a red, water-insoluble pigment was isolated from a laboratory strain of *Blatella germanica*. It was presumed to be *Serratia marcescens* Bizio as it is the only species of *Serratia* known to be pathogenic for insects (Steinhaus, 1959). All cultures were grown at 30°C on nutrient agar slants.

**Preparation of Bacterin**

A suspension of heat killed bacteria, used to immunize larvae, is designated as a bacterin. Eighteen hour slants of *P. aeruginosa* were washed with sterile 0.85% NaCl and combined. The bacteria were centrifuged (1000 x g for 15 minutes) and resuspended in saline 3 times. The final suspension was quantitated by plating
0.1 ml of the appropriate dilution on nutrient agar plates and spread with sterile bent glass rods. The suspension was placed in a culture tube sealed with parafilm and heated in a 60°C water bath for one hour. The suspension was diluted to 2x10^6 cells/μl and stored at 4°C. The same procedure was used to prepare *S. marcescens* suspensions. New batches of bacterin were prepared at monthly intervals in order to insure potency (Hink, 1966).

**Immunization and Challenge**

All injections were made using an Isco Micro-applicator (Instrumentation Specialties Co., Lincoln, Nebraska). Using a sterile syringe and 27 gauge needle, CO_2_ anesthetized larvae were injected just under the body wall of the third abdominal segment. All such injections with a bacterin will be referred to as immunizations. Hemolymph usually coagulated and closed the injection site. However, some larvae were observed to bleed and were discarded. After injection, the larvae were placed in the environmental chamber until they were challenged with live *P. aeruginosa* or bled for *in vitro* experiments.

Larvae to be immunized received 1x10^7 heat killed cells in a 5 μl volume. Control animals received 5 μl of sterile 0.85% NaCl (saline).

Doses of live bacteria were obtained from logarithmic phase cultures which were usually 5 to 8 hours old. The bacteria were washed off nutrient agar slants
and diluted to the appropriate concentrations with 0.85% NaCl. The bacteria were quantitated by plating 0.1 ml of the appropriate dilution on nutrient agar plates and spreading with a sterile bent glass rod. If the doses of bacteria were low enough, they were quantitated directly from the syringe by plating a 5 μl drop on a nutrient agar plate, adding 0.1 ml sterile saline and spreading with a sterile bent glass rod.

Since larvae receiving a dose of live bacteria were kept together in the same box, healthy larvae might become diseased by eating food contaminated by dead and dying individuals. To test for this possibility, $10^{10}$ viable bacteria suspended in 0.85% NaCl were spread on the food and wire rack of the experimental box and sterile saline was applied in the control box. Larvae were allowed to feed until the food was nearly consumed and then more food was added so that development might continue to the prepupal stage. This test was done twice using *P. aeruginosa* and once with *S. marcescens* and no mortality was found. Therefore, contamination from diseased and dying larvae was not considered to be a problem. Cannibalism of dead and dying larvae may involve the consumption of a lethal oral dose so generous quantities of diet were supplied in the crispers during all experiments. No cannibalism was noted under these conditions.
All mortality data were taken 48 hours after challenge.

**Agar Well Diffusion Assay**

The 5 antimicrobial compounds in the artificial diet (Table 1) were dissolved in distilled water at the same concentrations used in the diet. Sorbic acid required the addition of NaOH in order to dissolve at a neutral pH. The solutions were sterilized by filtration through 0.45 μm Millipore filters. As modified from Spooner and Sykes (1972) a seed lawn of bacteria was applied to nutrient agar plates and then wells were cut with a sterile #4 cork borer. A 0.1 ml sample of each solution and sterile distilled water controls were pipetted into the wells and the plates were stored right side up at 30°C.

**Preparation of Hemolymph**

With the larva held upside down, the horn was cut off near its base and hemolymph was collected in a small flask by gently squeezing the larva. A range of 0.3 to 1.0 ml of hemolymph was obtained from each larva, depending on its size. Hemolymph was pooled to yield 5 to 10 ml and filtered through a 0.45 μm cellulose ester Millipore filter. A glass fiber prefilter was usually used to trap the larger proteins that tended to clog the membrane. The pooled hemolymph, before filtration, is designated as lymph and after filtration, serum. Serum from immunized larvae
is designated as immune serum, serum from untreated larvae is designated as normal serum, and serum from saline injected larvae is designated as control serum.

For some experiments the flask containing hemolymph was placed in a gently boiling water bath for 5 minutes. A yellow serum and a green, jelly-like coagulum were formed which were separated by filtration through a 0.45 μm Millipore filter with a prefilter. In some experiments the boiled mixture was prefILTERED through #1 Whatman filter paper using suction in a Buchner funnel before Millipore filtration. This removed the coagulum which tended to clog the Millipore filter before filtration was complete. Heated and filtered hemolymph will be referred to as heated serum.

The stability of antibacterial activity to acid and base was tested with the following procedure. Hemolymph was divided into 5 beakers to which equal volumes of 1H HCl, 0.1N HCl, distilled H2O, 0.1N NaOH or 1N NaOH were added. The mixtures were agitated and allowed to remain at room temperature. During this time the pH of each mixture was measured. After 5 minutes all beakers received a neutralizing volume of the appropriate base or acid and the water control received an equal volume of water. The sample diluted with distilled water served as a control for whatever effect dilution may have had on antibacterial activity. After neutralization, the hemolymph (now
diluted to 3 times its original volume) was sterilized by filtration and assayed as in other experiments.

**In Vitro Techniques**

Determination of the growth of *P. aeruginosa* and *S. marcescens* in normal serum were done in sterile plastic depression trays (Linbro Chemical Co., New Haven, Connecticut) using techniques modified from Hink (1955). The trays had 4 columns of 6 depressions, each of which could contain about 2.0 ml. The trays and their plastic covers were washed with 0.1% Hyamine (Rohm and Hass, Philadelphia, PA) and then thoroughly rinsed with double distilled water. The trays and covers were dried in a sterile chamber and simultaneously exposed to radiation from a germicidal ultraviolet light. About 4 hours exposure to the lamp was found to be adequate to insure sterility and no contaminants were ever detected.

Bacterial growth in normal serum was measured as follows. Serum, in volumes of 0.2 ml, was pipetted into a vertical column of depressions. Depressions in the control column contained 0.2 ml sterile saline. Then 0.2 ml of bacterial suspension was added to all the depressions and the tray was gently agitated to insure mixing. Samples of 0.1 ml were taken from one of each of the serum and control depressions immediately and at appropriate times thereafter. Bacterial growth was quantitated by plating 0.1 ml of the appropriate dilution of the sample on
nutrient agar plates. At all other times a sterile cover sheet was kept on the depression tray to minimize evaporation and prevent contamination.

The in vitro assays for bactericidal activity of immune serum were also performed in depression trays. The experimental depressions contained 0.2 ml of immune serum plus 0.1 ml of the appropriate decimal serial dilution of bacteria. Control depressions contained either 0.2 ml of saline or 0.2 ml of control serum plus 0.1 ml of each decimal serial dilution of bacteria. One depression at the bottom of each column which contained serum or saline served as a sterility control. Depression trays were covered with a sterile plastic sheet during incubation. All the depressions were sampled at the same time after 18 to 24 hours of incubation at room temperature (23-25°C). At the end of this incubation period, a 0.1 ml sample was taken from each depression and plated on nutrient agar plates. If quantitation of results was desired, each 0.1 ml sample was spread over the surface of a single petri plate. Completely accurate quantitation was not practical because a significant amount of evaporation usually occurred during the longer incubation periods. Thus, results are stated as the number of bacteria recovered per 0.1 ml of sample volume. When the assay was used to simply determine the presence or absence of viable bacteria,
3 0.1 ml samples were applied to sections of the same nutrient agar plate.

Antibacterial activity was recorded by noting the minimum dilution where no bacteria could be recovered. For example, if the bacteria pipetted into the depressions numbered $6 \times 10^6$, $6 \times 10^5$, $6 \times 10^4$, $6 \times 10^3$, $6 \times 10^2$, and $0$, respectively, and bacteria multiplied in the first 2 depressions while none could be recovered from the next 4, the activity would be recorded as $6 \times 10^4$ bacteria killed. A diagram of this example (Fig. 1) shows columns containing immune and control sera. Typically, bacteria could be recovered from the top 2 depressions of the IMM column and all the depressions in the CON column.

In all the in vitro experiments, the initial number of bacteria was quantitated by plating 0.1 ml of the appropriate bacterial dilution on nutrient agar plates when the experiment was set up. Thus the absolute number of bacteria present was not known until the experiment was over. The growth of *P. aeruginosa* on the nutrient agar slants was consistent enough, however, that the order of magnitude was always assured.

**Lysozyme Assay**

Lysozyme activity was determined by the rate of hydrolysis of freeze dried *Micrococcus lysodeikticus* cells (Calbiochem, Los Angeles, CA) using a procedure modified from Powning and Davidson (1973). *M. lysodeikticus*
Figure 1. Typical arrangement of sera and bacteria in depression tray during antibacterial assays. Depending on the number of treatments or replicates involved, 2 to 8 vertical columns of depressions were used. The number of bacteria added is indicated on the left. Sterility controls at the bottom of the columns contained serum and sterile 0.85% NaCl. IMM, immune serum; CON, control serum.
cells were suspended in a 0.67 M phosphate buffer (pH=6.5) at a concentration of 0.5 mg/ml. Preliminary experiments showed no difference in rate of hydrolysis between hemolymph and serum so hemolymph was used throughout. Activity was assayed by adding 0.1 ml of hemolymph from individual larvae to 4 ml of the cell suspension, mixing with a vortex mixer and then incubating at 30 ± 0.01°C for 5 minutes. The *M. lysodeikticus* cell suspension was preincubated in the water bath to bring it to the proper temperature. At the end of the incubation a 2.5 ml sample was taken and mixed with 0.5 ml of 0.5 M Na$_2$CO$_3$ in a cuvette to stop the reaction and the absorbance at 450 nm was read on a Beckman DU spectrophotometer using a distilled water blank. A mixture of 2.5 ml of *M. lysodeikticus* suspension and 0.5 ml of 0.5 M Na$_2$CO$_3$ was used as a starting point. Activity was calculated in terms of units with each unit being equal to a change in absorbance of 0.001 per minute under the above conditions.
RESULTS AND DISCUSSION

Response of M. sexta to P. aeruginosa and S. marcescens

The LD_{50} of P. aeruginosa and S. marcescens for M. sexta. The response of M. sexta larvae, weighing from 1.0 to 2.5 g to intrahemocoelic injections of P. aeruginosa was measured with doses of from 3.0 \times 10^4 to 4.5 \times 10^5 bacteria. Ten animals were used for each dose. Control larvae received injections of 5 \mu l of saline which caused no mortality.

Figure 2 shows the dose response curve when P. aeruginosa cells were injected into the hemocoel of M. sexta larvae. The data are plotted on a log-probit graph where the dose is on the log scale and the percentage response is transformed to probit mortality (Bliss, 1934a and b). The regression line and the 95% confidence limits were calculated according to the maximum likelihood method (Finney, 1964) using a computer program written by Dr. R. V. Skavaril (Department of Genetics, The Ohio State University). Data from three experiments, each represented by a different symbol, were combined because data from individual experiments did not allow calculation of confidence limits of the LD_{50}. The LD_{50} was equal to 44.
Figure 2. Mortality of fifth instar M. sexta larvae 48 hours after injection of P. aeruginosa. Each point represents 10 larvae. Each kind of symbol represents a separate experiment. The curved lines represent the calculated 95% confidence limits.
110,000 bacteria with 95% confidence limits of 65,000 and 192,000 bacteria. The slope was equal to 1.16 and thus there was a large difference between the $LD_{10}$ and the $LD_{90}$ which were $8.8 \times 10^3$ and $1.4 \times 10^6$ bacteria, respectively.

The response of 3.0 to 5.5 g. larvae to *S. marcescens* was measured by injection of from 4 to 64 bacteria using 20 animals for each dose. Control larvae received injections of saline which caused no mortality.

The $LD_{50}$ for *S. marcescens* was vastly different from that of *P. aeruginosa* (Figure 3). The $LD_{50}$ was 9 bacteria with 95% confidence limits of 4 and 15 bacteria. The slope, equal to 1.27, was similar to that of *P. aeruginosa*.

While the $LD_{50}$ of *S. marcescens* was within the range suggested by previous work, the high $LD_{50}$ of *P. aeruginosa* was totally unexpected. A $LD_{50}$ of no more than 1000 bacteria was expected because the strain of *P. aeruginosa* used (P11-1) is highly pathogenic for adult grasshoppers and wax moth larvae (Stephens, 1962a). Also, injections of *P. aeruginosa* are highly pathogenic in 4 orders of insects: Leipdoptera, Orthoptera, Hemiptera and Coleoptera as can be seen in Table 2.

An interesting analogous situation is reported by Mortimore (1970) for *S. marcescens*. While *S. marcescens* is highly pathogenic for *G. mellonella* ($LD_{50}=40$) it has a very low pathogenicity for *T. molitor* larvae ($LD_{50}=10^6$) and *O. fasciatus* adults ($LD_{50}=10^5$). The bacteria persist in
Figure 3. Mortality of fifth instar M. sexta larvae 48 hours after injection of S. marcescens. Each point represents 20 larvae. The curved lines represent the calculated 95% confidence limits.
<table>
<thead>
<tr>
<th>Insect</th>
<th>Stage</th>
<th>Method of Administration</th>
<th>$LD_{50}$ (Number of Bacteria)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bombyx mori</td>
<td>Larva</td>
<td>Inject</td>
<td>74</td>
<td>Stephens, 1959</td>
</tr>
<tr>
<td>Bombyx mori</td>
<td>Pupa</td>
<td>Inject</td>
<td>2-80</td>
<td>Kawarabata, 1970</td>
</tr>
<tr>
<td>Euxoa ochrogaster</td>
<td>Larva</td>
<td>Inject</td>
<td>124</td>
<td>Stephens, 1959</td>
</tr>
<tr>
<td>Galleria mellonella</td>
<td>Larva</td>
<td>Inject</td>
<td>6-13</td>
<td>Stephens, 1959</td>
</tr>
<tr>
<td>Galleria mellonella</td>
<td>Larva</td>
<td>Inject</td>
<td>52</td>
<td>Hink, 1966</td>
</tr>
<tr>
<td>Galleria mellonella</td>
<td>Larva</td>
<td>Inject</td>
<td>20</td>
<td>Mortimore, 1970</td>
</tr>
<tr>
<td>Melanoplus bivittatus</td>
<td>Adult</td>
<td>Oral</td>
<td>8,000-37,000</td>
<td>Bucher and Stephens, 1957</td>
</tr>
<tr>
<td>Melanoplus bivittatus</td>
<td>Adult</td>
<td>Inject</td>
<td>10-20</td>
<td>Stephens, 1959</td>
</tr>
<tr>
<td>Melanoplus bivittatus</td>
<td>Adult</td>
<td>Inject</td>
<td>10-38</td>
<td>Bucher and Morse, 1963</td>
</tr>
<tr>
<td>Oncopeltus fasciatus</td>
<td>Adult</td>
<td>Inject</td>
<td>60</td>
<td>Gingrich, 1964</td>
</tr>
<tr>
<td>Schistocerca gregaria</td>
<td>?</td>
<td>Inject</td>
<td>27-33</td>
<td>Ashrafi et al., 1965</td>
</tr>
<tr>
<td>Schistocerca gregaria</td>
<td>?</td>
<td>Oral</td>
<td>18,000-24,000</td>
<td>Ashrafi et al., 1965</td>
</tr>
<tr>
<td>Tenebrio molitor</td>
<td>Larva</td>
<td>Inject</td>
<td>5</td>
<td>Mortimore, 1970</td>
</tr>
<tr>
<td>Tenebrio molitor</td>
<td>Larva</td>
<td>Inject</td>
<td>100-1,000</td>
<td>Ziprin, 1970</td>
</tr>
</tbody>
</table>
the hemolymph for 6 weeks in _T. molitor_ but are eliminated from the hemolymph of _O. fasciatus_ when the insects are examined 8 days after infection. Apparently there are 2 different mechanisms of interaction between host and pathogen in these insects, which reduces the pathogenicity of bacterium. Table 3 lists the published LD$_{50}$'s of _S. marcescens_ in various insects.

The slope of the dose-response regression line of _P. aeruginosa_ injected into _M. sexta_ larvae was 1.16. That of _S. marcescens_ was 1.27. The intrahemoloeic LD$_{100}$ of _P. aeruginosa_ is 10 times the LD$_{50}$ in _G. mellonella_ (Stephens, 1959). Similarly, in _B. mori_ the LD$_{50}$ equals 74 bacteria while the LD$_{100}$ equals 1200 bacteria. When _P. aeruginosa_ is injected into grasshoppers, the LD$_{100}$ is 13 to 20 times the LD$_{50}$ (Stephens, 1957). Bucher (1958) has compared the slope of dose-response regression lines of non-toxin producing insect pathogens, toxin producing (crystalliferous) insect pathogens and insecticides. The regression lines of the first group tend to have rather low slopes of around one to 2 while the lines of the second group have slopes ranging from 2 to 3. The regression lines of the insecticides presented have slopes greater than 5. With the exception of the high LD$_{50}$ of _P. aeruginosa_, the 2 bacterial infections in _M. sexta_ larvae seem to be similar to those in other insect species.
<table>
<thead>
<tr>
<th>Insect</th>
<th>Stage</th>
<th>Method of Administration</th>
<th>LD$_{50}$ (Number of Bacteria)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyrthosipon pisum</td>
<td>Nymph</td>
<td>Inject</td>
<td>190</td>
<td>Mackaver and Albright, 1973</td>
</tr>
<tr>
<td>Galleria mellonella</td>
<td>Larva</td>
<td>Inject</td>
<td>40</td>
<td>Stephens, 1959</td>
</tr>
<tr>
<td>Galleria mellonella</td>
<td>Larva</td>
<td>Inject</td>
<td>40</td>
<td>Mortimore, 1970</td>
</tr>
<tr>
<td>Melanoplus bivittatus</td>
<td>Adult</td>
<td>Inject</td>
<td>10-50</td>
<td>Bucher, 1959</td>
</tr>
<tr>
<td>Melanoplus bivittatus</td>
<td>Adult</td>
<td>Oral</td>
<td>300,000-500,000</td>
<td>Bucher, 1959</td>
</tr>
<tr>
<td>Oncopeltus fasciatus</td>
<td>Adult</td>
<td>Inject</td>
<td>100,000</td>
<td>Mortimore, 1970</td>
</tr>
<tr>
<td>Tenebrio molitor</td>
<td>Larva</td>
<td>Inject</td>
<td>4,000,000</td>
<td>Mortimore, 1970</td>
</tr>
</tbody>
</table>
The pathology of infected larvae. *M. sexta* larvae infected with a LD$_{90}$ of *P. aeruginosa* stopped feeding several hours after injection. By 24 hours after injection, the fecal pellets were no longer firm but had become soft and at the time of death had changed to a semifluid excrement. At 24 hours after injection, the turgidity of the larvae was markedly decreased and at 48 hours the dead larvae were completely flaccid. Mortality began at about 24 hours and was usually complete by 48 hours although some moribund individuals sometimes remained beyond 48 hours. Shortly after death, the larvae became light green in color, apparently due to the bacterial pigment pyocyanin being produced by the high numbers of bacteria. Melanization caused the larvae to become black by 24 hours post-mortem. Also by this time, the internal organs were almost entirely lysed. The disease symptoms were delayed somewhat as progressively lower doses of *P. aeruginosa* were used. Smaller larvae died more quickly than larger larvae.

The pathology of *S. marcescens* for *M. sexta* was essentially similar to that of *P. aeruginosa* excepting that it occurred somewhat more rapidly. Also, the initial post-mortem color change was to a red-brown color as the pigment prodigiosin produced by the bacteria is red to orange in color.

White's (1923) classic paper describes the pathology of *Bacillus sphingidis* infections in the tobacco hornworm
Protoparce sexta Johan. (=M. sexta Linn.) and the tomato hornworm P. quinquemaculata Haw. (=Manduca quinquemaculata Haw.). Bucher (1960) in reviewing this work concludes that the bacterium was probably a non-pigmented strain of S. marcescens. The symptoms of the progression of infection are loss of appetite, gradual change of fecal material from solid pellets to watery discharge, watery regurgitation late in the disease and loss of turgidity. With the exception of the regurgitation, these symptoms were commonly seen in this study. The post-mortem changes seen by White are melanization, eventual fragility of the cuticle, hydrolysis of the internal tissue and drying to a friable mass. The conditions used in my experiments did not permit drying to take place, however, all the other post-mortem changes were observed.

The pathogenesis caused by infection of P. aeruginosa and S. marcescens in tobacco hornworms suggested that as these microorganisms multiplied to large numbers, they disrupted the host's metabolism by enzymatic destruction of its tissues. This in turn would suggest that the primary mechanism of pathogenicity of these bacteria was proteolysis by exoenzymes. There is a good correlation between pathogenicity and proteolytic activity for several Pseudomonas species and for S. marcescens (Bucher, 1960). P. aeruginosa releases extracellular proteases (Liu, 1964).
Both proteases and chitinases are released by *S. marcescens* (Monreal and Reese, 1969).

Bucher and Stephens (1957) conclude that the pattern of pathogenicity observed in their study of *P. aeruginosa* infections of grasshoppers suggests that no specific toxin is produced. Lysenko (1963a and b) on the other hand, notes the extreme toxicity of the concentrated filtrate of *P. aeruginosa* broth cultures. When injected into *G. mellonella* larvae, the filtrate produces blackening along with loss of turgor and mobility in 30 to 60 minutes. This toxic fraction from the bacterial culture is a proteolytic exoenzyme (Lysenko, 1964). The manifestations of toxicity can be explained by noting that proteolytic enzymes can activate phenoloxidase enzymes (Ohnishi et al., 1970) and that the melanization produced is highly toxic to insects (Zlotkin et al., 1973). It should also be noted that Lysenko's injections produce melanization within an hour of injection. In contrast, melanization of diseased insects usually takes place near or after death when the number of bacteria is extremely high and consequently the exoenzymes have reached a high titer.

Proteolytic exoenzymes are doubtless a factor in pathogenicity but they are probably toxic in the manner described by Lysenko only when injected in relatively large amounts. If the proteolytic enzyme were injected in amounts consistent with those produced during infection, the enzyme
may then produce effects that mimic the infection. Such a study could be valuable.

A review of the literature reveals no evidence that *P. aeruginosa* and *S. marcescens* have any ability to invade host tissue. They require some trauma to the host tissue in order to establish an infection (Steinhaus, 1959, 1967). In insects, some trauma such as rupture of the intestine (Bucher, 1959) is required in order to establish infection. Thus, in this and many other studies, infection was established by injection into the hemocoel.

Bucher (1960) has classified both *P. aeruginosa* and *S. marcescens* as potential insect pathogens which are characterized as follows. They produce fatal septicemia from relatively low doses when injected into the hemocoel but the host is highly resistant to oral infection as the bacteria do not actively invade the hemocoel from the gut. The pathogens have strong proteolytic exoenzymes and are aerobes. Being aerobes, they multiply freely in the hemolymph but grow poorly if at all in the reducing conditions found in the gut. It can be seen from Tables 2 and 3 that *P. aeruginosa* and *S. marcescens* are usually highly pathogenic only when injected.

As a preliminary study, the food and wire rearing racks of *M. sexta* were contaminated with *P. aeruginosa* or *S. marcescens* and no mortality resulted. This may have been due to a low susceptibility to oral infection but the fact
that the diet contains several antimicrobial compounds may have been important. *P. aeruginosa* also has a very short survival time when allowed to dry in air (Stephens, 1957).

**In vitro growth of *P. aeruginosa* and *S. marcescens** in normal serum. Before trying to demonstrate antibacterial activity in immune *M. sexta* serum, it was necessary to show that bacteria would survive or grow in normal serum. Therefore, depressions containing equal volumes of normal serum and a bacterial suspension were set up. Control depressions contained equal volumes of saline and the bacterial suspension. A 0.1 ml sample was taken from the bacteria-serum and control mixtures at 0, 3, 6, 9, and 12 hours and the results were expressed in terms of the number of bacteria contained in each sample.

The growth of *P. aeruginosa* and *S. marcescens* in vitro, using serum from untreated larvae reflected their pathogenicity in vivo (Figure 4). Both bacteria showed an initial lag phase during which the decrease in numbers of bacteria approximately paralleled that seen in the saline controls. After about 3 hours, the bacteria entered the log phase of growth where *P. aeruginosa* had a generation time of 67 minutes and *S. marcescens* had a generation time of 38 minutes. Thus, *P. aeruginosa* had nearly twice the generation time of *S. marcescens* during the logarithmic phase of growth.
Figure 4. In vitro growth of P. aeruginosa and S. marcescens in normal M. sexta serum at 25°C. Open circles, P. aeruginosa in serum; open triangles, P. aeruginosa in 0.85% NaCl; closed circles, S. marcescens in serum; closed triangles, S. marcescens in 0.85% NaCl.
At first this difference was thought to be an explanation of the wide differences in LD$_{50}$'s between the 2 bacteria in *M. sexta*, but comparison of the *P. aeruginosa* generation time with that found in other insects suggests that this is not so. The log phase generation time of *P. aeruginosa* infections in grasshoppers is 79 minutes (Bucher and Stephens, 1957). The generation time of 87 minutes for *P. aeruginosa* growth in infected wax moth larvae can be calculated from graphs published by Chadwick (1968). The LD$_{50}$ of *P. aeruginosa* in grasshoppers and *G. mellonella* is no more than 53 bacteria (Table 2). Since the generation time of *P. aeruginosa* is similar in the 3 insects but its lethality is widely divergent, other factors are also involved. Other than determining that the antimicrobial compounds in the diet were not responsible for the low pathogenicity of *P. aeruginosa* for *M. sexta*, the search for these factors was not pursued further. The *in vitro* growth rates of the 2 bacteria in *M. sexta* hemolymph established that the bacteria grew normally in normal hemolymph. The typical lag and logarithmic growth phases suggest the absence of any inhibitory principle in normal hemolymph.

The serum used in these experiments was blue in color and melanized quickly after filtration, producing a fine black particulate material as soon as 15 minutes after filtration and becoming black within about an hour. The
serum was usually black by the time it was added to the depression trays. When the depression tray was sampled, a tough, black, sticky film was usually found on the surface of the serum. Millipore filtration always caused melanization in normal serum in this manner.

In contrast to the consistent and rapid melanization of normal serum, normal hemolymph from individual larvae did not always melanize when kept in test tubes at room temperature. Hemolymph thus treated required about 24 hours to turn black and one sample was still blue after 48 hours. The color sequence during melanization was from blue to light green to dark green to black in both normal serum and hemolymph.

The melanization reaction is catalyzed by a single enzyme, phenoloxidase (monophenol monooxygenase, E.C. 1.14.18.1, also called tyrosinase). Only the first 2 steps in the pathway from tyrosine to melanin are catalyzed by the enzyme and the remaining steps occur spontaneously (Figure 5). The formation of melanin from the pathway does not involve polymerization of only the terminal product but rather of at least 3 intermediates and probably products of side reactions (Blois, 1969; Hempel, 1969). Thus melanin is a complex heteropolymer. In addition to reacting with themselves, the quinone intermediates also react with sulfhydryl and aromatic amino groups (Mason and Peterson, 1965). Thus insect melanins along with other
Figure 5. Phenoloxidase catalyzed pathway from tyrosine to melanin. Adapted from Blois (1969).
naturally formed melanins are complexed with proteins (Hackman, 1974).

Phenoloxidase is found in insect integument and hemolymph where it usually occurs as an inactive proenzyme (Hackman, 1974). It may also be sequestered in hemocytes and thus be isolated from its substrate (Rizki and Rizki, 1959). Thus Millipore filtration may have initiated the melanization reaction by activating a proenzyme, by rupturing the hemocytes, or by some other unknown process.

Sensitivity of *P. aeruginosa* and *S. marcescens* to dietary antimicrobial compounds. The artificial diet of the larvae contained five antimicrobial substances: ascorbic acid, 37% formaldehyde, methyl-p-hydroxybenzoate, sorbic acid and streptomycin sulfate. If these substances were absorbed in fairly large amounts into the hemolymph, a sensitivity of *P. aeruginosa* to these compounds may account, in part, for its low pathogenicity. The activity of these compounds against *P. aeruginosa* and *S. marcescens* was assayed by the agar well diffusion assay using the same concentrations as those in the diet. In Table 4 the zones of inhibition are recorded as the average distance from the edge of the well to the edge of bacterial growth. The results show that *P. aeruginosa* was either equally or less sensitive than *S. marcescens* to the antimicrobial compounds in the diet.
Table 4

Inhibition of Bacterial Growth by M. sexta Dietary Antimicrobial Compounds in the Agar Well Diffusion Assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>% in Solution</th>
<th>Width of Zone of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aeruginosa</td>
<td>S. marcescens</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.38</td>
<td>0 mm</td>
</tr>
<tr>
<td>Formalin</td>
<td>0.22</td>
<td>7 mm</td>
</tr>
<tr>
<td>Methyl-p-hydroxybenzoate</td>
<td>0.11</td>
<td>0 mm</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td>0.32</td>
<td>0 mm</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>0.02</td>
<td>5 mm</td>
</tr>
</tbody>
</table>

<sup>a</sup>Approximately a 2 mm zone of noticeably smaller colonies.
P. aeruginosa was sensitive to the formalin and streptomycin sulfate. However its growth rate in normal M. sexta serum was similar to its growth in insects reared on diets not containing antimicrobial compounds. S. marcescens was equally sensitive to streptomycin sulfate and even more sensitive to the formalin than P. aeruginosa. It also showed some sensitivity to the ascorbic acid and methyl-p-hydroxybenzoate. Yet this species grew even more rapidly than P. aeruginosa in normal M. sexta serum. These comparisons strongly suggest that the presence of these antimicrobial compounds in the diet had little or no effect on the bacterial growth in the serum. Thus it is not likely that the presence of these compounds in the insect's diet was a factor in the relative pathogenicity of the bacteria. The compounds may not be absorbed through the gut wall or, if absorbed, may be rapidly metabolized to ineffective substances.

This is the first study of insect immunity using an insect reared on a diet containing antibiotics. Thus the antibiotics might interfere with the search for induced antimicrobial substances in the hemolymph. The above data make the possibility of interference seem unlikely.

**In Vivo and In Vitro Immunity and Melanization**

Integumental melanization occurring with immunization.

When M. sexta larvae (1.5-2.5G) were immunized with a P.
**aeruginosa** bacterin, an integumental melanization developed on the prolegs and lateral abdominal body wall of most larvae. The melanization first developed in the prolegs (where it became evident as soon as 10 minutes after immunization) and sometimes spread to the body wall. The minimum melanization observed consisted of no more than 2 or 3 spots no larger than a spiracle. They occurred on the right side of the third and fourth abdominal segments. If just one proleg melanized, it was on the right side of the third abdominal segment, just ventral to the point where the contents of the needle emptied during immunization.

In other larvae showing greater degrees of melanization after immunization, the darkening always began in the prolegs. There were some animals where only the tips of the prolegs melanized. More commonly, it extended up onto the lateral body wall. When 8 or more prolegs melanized, the rest of the larva usually appeared somewhat darkened and the larva was less turgid than normal. All graduations occurred between the extremes of no melanization and very heavy melanization, although larvae showing a moderate melanization involving one to 6 prolegs were usually the most common. There appeared to be some variability in response depending on the particular preparation of bacterin involved. Some preparations appeared to be more potent than others and often as they aged, they showed a decreasing
ability to provoke melanization in the larvae. Saline injected larvae never developed any melanization.

Depending on the degree of melanization of the larvae, the fecal pellets were considerably darker than their normal light brown color. Shortly after cuticular melanization occurred, the pellets ranged from a dark brown to black color but they became progressively lighter during the 2 or 3 days following immunization. The integumental melanization faded gradually over several days' time. A noticeable fading became apparent about 24 hours after immunization.

Most workers using G. mellonella do not report partial melanization accompanying immunization (Chadwick, 1971; Hink and Briggs, 1968; Powning and Davidson, 1973; Stephens, 1962a and b, 1963a). On the other hand, 2 recent workers report melanization after immunization of G. mellonella larvae. Irregularly distributed melanin deposits form under the epithelium of some immunized larvae (Messner, 1972). Pye (personal comm.) has reported that immunized larvae show a darkening of the hemolymph; the larger the dose of bacterin, the more likely the larvae are to show a "caramel" color as opposed to the normal lighter "honey" color. The fact that the described color changes are rather subtle may explain why others working with G. mellonella have not commented on them. Different strains of G. mellonella also differ widely in their
response to injections of bacterin (Ziprin and Hartman, 1971). Using other insect species, no darkening of the integument or hemolymph after immunization was noted by Briggs (1958), Bowman et al. (1972), Gingrich (1964), Kawarabata (1970, 1971) and Mortimore (1970). Thus the response may be a subtle one that is not easily noticed or the response may be restricted to *M. sexta* and some strains of *G. mellonella*.

**Toxicity of bacterin.** During the entire period of research, about 6 larval deaths seemed to be the result of the immunization procedure. These larvae died after developing extremely heavy melanization. The majority of larvae developed less intense melanization and no mortality occurred that could be ascribed to immunization. If the less intense melanization was not lethal, it still seemed possible that it may be toxic in some manner to the larvae. Thus growth rate was selected as an assay for toxicity of the bacterin. Four groups of 15 larvae were treated as follows. One experimental group received $2 \times 10^7$ heat killed *P. aeruginosa* cells in 5 μl saline. The second experimental group received the normal immunizing dose of $1 \times 10^7$ cells. One control group received 5 μl of sterile saline and the last group received no treatment at all. Larvae were kept at 30°C with a 15 hour photophase and were weighed periodically to the nearest tenth of a gram. Some animals
entered the prepupal stage by 60 hours and at 84 hours all the remaining larvae had developed into prepupae.

Figure 6 shows the mean weight gain for the 4 groups of larvae. Inclusion of the standard deviation data would make the graph unreadable so the complete data are listed in Table 5. Surprisingly, not only did growth retardation occur in the immunized larvae, but also in the saline injected larvae. Injected larvae consistently lagged behind the untreated larvae and entered the prepupal stage about 3 grams lighter. The retardation of growth of the saline injected and immunized larvae may have been partly due to the CO$_2$ anesthesia used for injection; anesthetized larvae required several hours to completely recover during which a reduced feeding rate was noticed. The injection itself may have caused some growth retardation. The design of the experiment did not allow the effect, if any, of the saline injection with or without anesthesia to be distinguished from that of the anesthesia alone. During the first 18 hours after immunization, both groups of immunized larvae gained weight slightly more slowly than saline injected larvae with the larvae receiving the larger immunizing dose showing the greatest retardation. After that, development of immunized larvae approximately paralleled the development of saline injected larvae. The divergence of the 3 curves during the first 18 hours suggests that the bacterin's effect is expressed primarily during this period.
Figure 6. Mean weight gain of immunized, saline injected and untreated fifth instar M. sexta larvae. Fifteen larvae are in each group. Solid triangles, $2 \times 10^7$ cells/larvae; circles, $1 \times 10^7$ cells/larvae; squares, saline injected controls; triangles, untreated controls. Some of the points at zero time are displaced laterally for clarity.
<table>
<thead>
<tr>
<th>Time After Immunization (hrs)</th>
<th>Untreated Controls</th>
<th>Saline Injected Controls</th>
<th>Immunized (10^7 cells)</th>
<th>Immunized (2x10^7 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.28 ± 0.21</td>
<td>2.13 ± 0.14</td>
<td>2.25 ± 0.18</td>
<td>2.13 ± 0.23</td>
</tr>
<tr>
<td>18</td>
<td>4.13 ± 0.45</td>
<td>3.53 ± 0.33</td>
<td>3.05 ± 0.29</td>
<td>2.85 ± 0.43</td>
</tr>
<tr>
<td>24</td>
<td>4.80 ± 0.44</td>
<td>4.13 ± 0.41</td>
<td>3.57 ± 0.38</td>
<td>3.31 ± 0.50</td>
</tr>
<tr>
<td>42</td>
<td>7.51 ± 0.72</td>
<td>6.42 ± 0.82</td>
<td>5.53 ± 0.85</td>
<td>5.11 ± 0.76</td>
</tr>
<tr>
<td>48</td>
<td>8.36 ± 0.72</td>
<td>7.12 ± 0.94</td>
<td>6.18 ± 0.84</td>
<td>5.84 ± 0.92</td>
</tr>
<tr>
<td>60</td>
<td>10.03 ± 1.12</td>
<td>8.63 ± 1.34</td>
<td>7.68 ± 1.27</td>
<td>7.17 ± 1.15</td>
</tr>
<tr>
<td>74</td>
<td>11.47 ± 0.87</td>
<td>9.13 ± 1.26</td>
<td>8.80 ± 1.59</td>
<td>8.49 ± 1.33</td>
</tr>
</tbody>
</table>
Immunization apparently caused a delayed onset of the prepupal stage at the end of the instar. As noted above, the appearance of prepupae occurs on 2 successive evenings with only a portion of the larvae developing into prepupae on the first night. At 60 hours post immunization, 8 of the untreated, 5 of the saline injected, 2 of the immunized larvae receiving $1 \times 10^7$ cells and one of the immunized larvae receiving $2 \times 10^7$ cells had developed into prepupae.

No toxic response similar to that in *G. mellonella* was seen in *M. sexta*. A *P. aeruginosa* bacterin is lethal to several strains of *G. mellonella* (Ziprin and Hartman, 1971). After immunization, a dark line of melanization appears over the dorsal vessel and then the whole larva gradually darkens. Death occurs 12-24 hours after immunization. While some strains consistently show this response, one strain shows it some of the time and another only rarely exhibits the toxic response. Since the dark line pathology of *G. mellonella* should be expressed as a function of dose size, it would have been interesting to see if lower doses of bacterin would have induced immunity without the dark line pathology in the animals used by Ziprin and Hartman (1971). The toxic melanization response resembles that reported by Zlotkin *et al.* (1973) where air activated hemolymph phenoloxidase from tenebrionid beetles is toxic when injected in several other insect species. *G. mellonella*
is killed in 12 hours by this treatment. Dissections of dead *Sarcophaga falculata* reveal that the hemolymph forms a dense black coagulum and thus the authors suggest that the toxic effect is exerted by the oxidizing, denaturing and coagulating effects of the phenoloxidase products.

**In vivo immunity.** *M. sexta* larvae, weighing 1.5 to 2.5 g. were immunized with homologous bacterin or injected with 5 μl of saline, then challenged with a lethal dose of bacteria after 1, 2, 3, 6, 12, 19 and 27 hours.

In larvae immunized with $10^7$ heat killed *P. aeruginosa* cells, resistance to challenge with live bacteria developed as soon as 2 hours after immunization (Table 6 and Figure 7). Resistance was greatest between 6 and 19 hours after immunization and decreased thereafter. The period of maximum immunity coincides with the period when the larvae responded to immunization with a retarded growth rate. The challenge doses ranged from a LD$_{90}$ to a LD$_{95}$ as calculated from Figure 2. This dose range produced 100% mortality in control larvae during the first 6 hours. However, it can be seen in Table 6 that larger larvae (those challenged 12 to 27 hours after immunization) tended to be less susceptible to the challenge dosages. Because of this, the corrected percentage survival has been calculated using the method of Sun and Shepard (1947) and is shown in the final column.
<table>
<thead>
<tr>
<th>Number of Hours After Immunization</th>
<th>Challenge Dose</th>
<th>Percent Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Immunized Larvae</td>
</tr>
<tr>
<td>1</td>
<td>2.0x10^6</td>
<td>0/16 0%</td>
</tr>
<tr>
<td>2</td>
<td>1.8x10^6</td>
<td>4/11 36%</td>
</tr>
<tr>
<td>3</td>
<td>1.4x10^6</td>
<td>8/15 53%</td>
</tr>
<tr>
<td>6</td>
<td>2.3x10^6</td>
<td>15/15 100%</td>
</tr>
<tr>
<td>12</td>
<td>2.8x10^6</td>
<td>13/15 87%</td>
</tr>
<tr>
<td>19</td>
<td>2.0x10^6</td>
<td>12/13 92%</td>
</tr>
<tr>
<td>27</td>
<td>1.5x10^6</td>
<td>11/16 69%</td>
</tr>
</tbody>
</table>
Figure 7. In vivo immunity of M. sexta larvae to challenge with P. aeruginosa and in vitro antibacterial activity of M. sexta immune serum against P. aeruginosa. Larvae were immunized at time zero. Circles and left ordinate, in vivo immunity; triangles and right ordinate, in vitro antibacterial activity.
Experiments were not carried beyond 27 hours because fifth instar larvae complete development in either 4 or 5 days at 30°C. The 1.5 to 2.5 g larvae used for immunization were up to one day old. Thus this 24 hour period added to the 27 hours until challenge and the 48 hour observation period is long enough for some larvae to reach the prepupal or crawler stage. Prepupae are active crawlers and will crawl until exhausted if not placed in a pupation chamber. The complication that this added stress factor would introduce did not allow challenge beyond 27 hours.

Patterns of protection similar to that in M. sexta have been shown in other insects. G. mellonella, immunized with heat killed P. aeruginosa, shows the protective effects of immunization by 4 hours and is most strongly protected from 12 to 24 hours post immunization (Stephens, 1962a). Then the protection drops off rapidly. O. fasciatus adults immunized and challenged with P. aeruginosa show considerable protection after 4 hours and maximum protection from 12 to 96 hours post immunization (Gingrich, 1964). By 120 hours protection essentially disappears. Again with the same bacterium, there is a plateau of maximum immunity in B. mori pupae between 12 and 48 hours. T. molitor larvae immunized with E. coli lipopolysaccharide and challenged with P. aeruginosa show protection, that is first detected at 6 hours, peaks at 12 hours post
immunization and then gradually declines to a low level by 120 hours. Protection against a lethal dose of B. *thuringiensis* occurs by immunization of adult male *L. migratoria* with a non-lethal dose of live bacteria (D. Hoffman, 1972). Some resistance to lethal numbers of bacteria occurs as early as 8 hours and maximum protection, beginning at 12 hours, lasts for 15 days.

The pattern of immunity in *M. sexta* seems to be similar to that found in *G. mellonella*, *B. mori*, *O. fasciatus* and *T. molitor*. The immunity shown in *L. migratoria* may be distinct from that in the other insects because of the relatively long duration of protection and the fact that protection was achieved against infection with a Gram positive bacterium.

Immunization against lethal doses of *S. marcescens* was also attempted using homologous bacterin and a melanization similar to that found with a *P. aeruginosa* bacterin was observed. Difficulty was encountered in obtaining consistent *in vivo* results and the level of immunity did not seem to be as great as with *P. aeruginosa* (Table 7). Preliminary attempts to demonstrate antibacterial activity *in vitro* were unsuccessful so further work was not done with *S. marcescens*.

At least 2 different explanations can be given for the difference between the 2 bacteria. It may be that *S. marcescens* was not as "immunogenic" as *P. aeruginosa* and
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Challenge Dose</th>
<th>Time of Challenge After Immunization (hrs)</th>
<th>48 hr Mortality (number dead/number challenged)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Immunized</td>
</tr>
<tr>
<td>1</td>
<td>68</td>
<td>20</td>
<td>8/20</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>20</td>
<td>13/21</td>
</tr>
<tr>
<td>3</td>
<td>124</td>
<td>15</td>
<td>16/20</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>15</td>
<td>14/20</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
<td>15</td>
<td>14/20</td>
</tr>
</tbody>
</table>

Table 7

Survival of *M. sexta* Larvae Immunized with 1x10^7 *S. marcescens* to Homologous Challenge
did not provoke as strong a response as *P. aeruginosa*. However, both bacterins caused similar responses with respect to melanization and the melanization response will later be shown to be essential before activity develops against *P. aeruginosa*. Immune serum from *M. sexta* immunized with *S. marcescens* was bactericidal for *P. aeruginosa* but not *S. marcescens* in vitro (unpublished results). Thus an alternate explanation may be that *S. marcescens* is not very sensitive to the antibacterial substance(s) produced. This second explanation, of course, assumes that the same antibacterial substance(s) are produced by immunization with both bacteria, an hypothesis recently suggested by Pye (1973).

*T. molitor* can be immunized against *P. aeruginosa* using heat killed homologous bacterin, *E. coli* lipopolysaccharide and live sub-lethal doses of *S. marcescens* (Mortimore, 1970). Similar levels of protection against *P. aeruginosa* in *B. mori* pupae are produced by immunization with heat killed or formalized *P. aeruginosa* or sub LD$_{50}$ doses of *E. coli* (Kawarabata, 1970). *In vitro* bactericidal activity against *E. coli* occur in *B. mori* serum from pupae immunized with formalized bacterins of *P. aeruginosa*, *S. marcescens*, *S. aureus* and *Bacillus sotto* and lower levels of activity are induced by immunization with zymosan and glycol chitin (Kawarabata, 1971). Thus the induction of immunity does not seem to be highly specific.
It may be significant however that a common constituent of all the immunogens is some kind of substituted β(1-4) linked polymer of glucose.

If no great specificity is required for immunization, neither does the antibacterial activity seem to be highly specific. High in vitro antibacterial activity against E. coli, low activity against S. aureus and no activity against S. marcescens and B. scotto is found in serum from B. mori pupae immunized with S. aureus, S. marcescens or B. scotto (Kawarabata, 1970). After the injection of a P. aeruginosa bacterin, Galleria larvae are protected against challenge with P. aeruginosa and to a lesser degree Proteus sp and S. marcescens (Stephens, 1959). In vitro antibacterial activity against E. coli, Micrococcus pyrogenes var aureus and Aerobacter aerogenes but not P. aeruginosa can be found in serum from B. mori larvae immunized with live Salmonella typhosa (Briggs, 1958). Thus it seems that the specificity of the immune response may be at least partly due to the susceptibility of the species or strain of bacterium used for the assay. In light of the responses in other insects, the suggestion that while both P. aeruginosa and S. marcescens provoke a similar response in M. sexta, they differ in their sensitivities to the induced substance(s), seems at least a reasonable hypothesis.
In vitro antibacterial activity and melanization.

*M. sexta* larvae were immunized with a *P. aeruginosa* bacterin or injected with 5 μl of saline just as was done in the previous *in vivo* immunity experiments. However, instead of being challenged, they were bled at various intervals and 0.2 ml volumes of the pooled sera were incubated with 0.1 ml suspensions of *P. aeruginosa*. Decimal serial dilutions containing approximately $10^2$ to $10^6$ live bacteria per 0.1 ml were incubated in each column of serum with the bottom depressions of each column serving as a sterility check. All the depressions were sampled at the same time from 18 to 24 hours after being set up and activity was recorded as the highest concentration where no bacteria were recovered. The incubation period was convenient as experiments set up on one day could be sampled the next. The growth curve of *P. aeruginosa* in normal serum (Figure 4) also suggested that this time period would give surviving bacteria adequate time to multiply to easily detectable numbers.

The results of the *in vitro* assay for antibacterial activity are shown in Figure 7 where the maximum number of bacteria killed at various times are plotted along with the *in vivo* results. There was a lag in the appearance of the *in vitro* antibacterial activity behind the appearance of resistance to challenge by the larvae. Maximum bactericidal activity, however, corresponded quite well
with the 12 hour period where resistance to challenge was also maximal.

A similar correlation between in vitro and in vivo assays is shown by Stephens (1962a) in G. mellonella and by Gingrich (1964) with O. fasciatus. The in vitro assays in these insects show an increase, plateau and decline in activity similar to that found in their in vivo assays. The close correspondence of the 2 kinds of assays suggests that the in vitro serum activity is the active agent in in vivo immunity.

An inducible antibacterial activity is found in the serum of B. mori (as well as several other species of lepidopterous larvae) that appears between 8 and 24 hours after immunization (Briggs, 1958). It is not stated how well it corresponds with the induced immunity in the larvae.

Serum from saline injected larvae never showed antibacterial activity and large numbers of bacteria were always found in the samples. This correlates with the fact that saline injected larvae were not protected against challenge with a lethal dose of P. aeruginosa. Thus the saline solution can be considered relatively inert as far as immunity in M. sexta is concerned.

When injected larvae were bled for in vitro experiments, a color change of the hemolymph correlated with integumental melanization was apparent. Saline injected larvae and immunized larvae with no melanization
or only a few small melanized spots all yielded blue hemolymph similar to that from untreated larvae. However, moderately melanized larvae yielded green hemolymph and the hemolymph from progressively more heavily melanized larvae was yellow-green to brown-green. The brown-green hemolymph was found in those heavily melanized larvae which showed a loss of turgor pressure and was characterized by a greatly reduced viscosity. The reduced viscosity was evident from the way the hemolymph flowed and from the fact that the hemolymph passed through the Millipore filter very easily. Although no quantitative viscosity measurements were made, it could be seen during filtration that green hemolymph from moderately melanized larvae was intermediate in viscosity between blue hemolymph and brown-green hemolymph.

The fact that melanization occurred in immunized M. sexta larvae suggests that it may be significant in the defense against the normally lethal challenge with live P. aeruginosa. Melanization is important to many arthropod's defense against parasites where it occurs along with hemocyte encapsulation (Salt, 1970). Many dipteran larvae have a low titer of circulating hemocytes and thus some respond to parasites with a melanin capsule formed in the absence of cells (Poinar and Leutenegger, 1971; Gotz and Vey, 1974). The hemocytes of the crayfish react to fungal pathogens by releasing phenoloxidase containing
granules; the phenoloxidase becomes activated after coming into contact with the fungus, producing a melanin capsule (Unestam and Nylund, 1972). Some evidence suggests that phenoloxidase may produce products other than melanin in the defense against parasites as in the following example. *Drosophila algonquin* avoids parasitization by *Pseudeucoila bochei* by melanization and hemocyte encapsulation. However, these reactions do not appear until 24 hours after parasitization and many eggs are killed or retarded in development before 24 hours. None of the hosts transferred to a diet containing phenylthiourea at 3 hours post-parasitization make a successful immune reaction while the hosts transferred to a phenylthiourea diet at 21 hours are as successful as the controls (Nappi, 1973). This suggests that hemolymph phenoloxidase is involved in the immune reaction throughout infection and only visibly manifested when the parasites are melanized. Successful formation of melanotic capsules can be inhibited by either glutathion or phenylthiourea in other insects as well (Brewer and Vinson, 1971; Salt, 1956; Vey and Gotz, 1975).

Melanization is also related to antibacterial immunity in larval *G. mellonella*. Hemolymph of immunized larvae does not melanize on exposure to air as does normal hemolymph (Stephens, 1962b). Fractions of immune hemolymph that are bactericidal also inhibit hemolymph phenoloxidase, suggesting that they may be one and the same (Stephens and
Marshall, 1962). Even though immune hemolymph shows a reduced melanization, bacterial cells activate the phenoloxidase of immune serum at a much greater rate than they do of normal serum. This has been used as the basis for the suggestion that antibacterial immunity involves the localized activation of phenoloxidase around bacterial cells producing toxic quinones (Pye, 1974). Quinones are strongly oxidizing compounds (Mason, 1955) and can act as antibiotics (Kavanagh, 1947). Uncontrolled activation of hemolymph phenoloxidase can also be lethal to the insect however (Zlotkin et al., 1973).

No detailed study was made of the changing color and viscosity of hemolymph from melanized larvae. However, it was noted that by the time immunized larvae approached the prepupal stage, the hemolymph had returned to a nearly normal color and viscosity. The gradual return to a nearly normal color by the integument and hemolymph of immunized M. sexta larvae suggests that the melanization products were gradually cleared from the hemolymph and probably excreted. The dark color of the fecal pellets formed by the melanized larvae also supports this suggestion. Since the antibacterial activity was also gradually lost, the active substance(s) may also have been cleared from the hemolymph or excreted.

The pooled hemolymph from saline injected larvae was blue but after filtration it rapidly melanized. The
initiation of melanization by Millipore filtration of saline injected hemolymph was identical to that which occurred in normal hemolymph. In contrast to the reaction that filtration caused in normal and control serum, no change in color occurred after filtration of pooled blue and green hemolymph from immunized insects. It was usually a grass green color and remained that color after filtration. There was thus an inhibition of melanization in immune serum. A similar inhibition of melanization after immunization occurs in G. mellonella (Stephens, 1962b).

Acetone precipitated phenoloxidase preparations from immune hemolymph are much slower in causing melanization than are preparations from normal hemolymph.

Immune serum contains a non-competitive inhibitor of phenoloxidase (Pye, 1974). The inhibition of melanization in M. sexta may also be due to the presence of an inhibitor. The hemolymph may also lack an activator or cofactor or it may contain a reduced concentration of phenoloxidase.

About an hour after bacteria were added to the immune serum in the depression tray, those depressions receiving the larger doses of bacteria would turn black and those depressions receiving lower doses remained green or showed a slight darkening with occasional black spots. After several experiments, it became apparent that the results of the in vitro antibacterial assay could be predicted quite
accurately by the melanization that occurred. If the depression melanized quickly, no antibacterial activity would be found. If melanization occurred slowly over many hours, partial activity would be evident. When no melanization or very slight darkening occurred, only rarely could viable bacteria be recovered from the depression and then they were always few in number.

The pattern of this inverse relationship between melanization and antibacterial activity suggests that the melanization process destroyed the antibacterial activity. Tests for this possibility will be discussed in the next section. The fact that immune serum in depressions with higher numbers of bacteria and no antibacterial activity melanized within an hour just like normal and control serum shows that the absence of melanization after filtration of immune serum is not due to a reduced concentration of phenoloxidase. There are other possibilities remaining. The immune serum was apparently inhibited from melanizing; it lacked an activator or cofactor. Since the higher concentrations of bacteria may have contained a substance that counteracted an inhibitor or had large amounts of an activator, it is not possible to decide about the presence of activators, inhibitors, or cofactors at this point.

When reading the nutrient agar plates, it was observed that those plates with samples from unmelanized depressions (which yielded no viable bacteria) showed
melanization where the blood had been spread. If the plate was relatively dry, the melanization occurred on the surface of the agar. If the plate was relatively new and moist, the serum phenoloxidase diffused into the agar and melanization was noted in the agar. Conversely, the samples from melanized depressions had no melanization on or in the agar.

It is possible that something in the nutrient agar contained an activator or cofactor for the immune serum phenoloxidase in those samples where no activation had yet occurred. It is also possible that an inhibitor diffused through the agar, reaching an ineffective concentration. These two explanations are not mutually exclusive, therefore both events may have taken place. Since no further melanization on the agar occurred with previously melanized samples, it seems that the process of melanization in the depression may have destroyed the serum phenoloxidase. Reaction inactivation of phenoloxidase is frequently noted by those who study the enzyme (Pryor, 1955; Sizer, 1953) and is due to the reaction of some of the enzyme's products at or near the active site (Wood and Ingraham, 1965; Brooks and Dawson, 1966). With the active site altered, the enzyme becomes permanently inactivated.

Four instances have now been shown where treatments increase M. sexta phenoloxidase activity: injection of a bacterin into larvae; Millipore filtration of normal,
saline injected and class I hemolymph; incubation of immune serum with large numbers of *P. aeruginosa* cells; and spreading immune serum on nutrient agar plates. The *in vitro* techniques also involved exposure to air.

In all these instances, the hemolymph was exposed to some form of complex polysaccharide and thus this may be a general method for initiating melanization in *M. sexta*. However, other factors may also be involved because Pye (1974) has found that a variety or relatively pure carbohydrates were usually poor phenoloxidase activators. Complex mixtures such as zymosan and partially disrupted *P. aeruginosa* cells were more effective activators.

Ashida and Ohnishi (1967) list 3 general mechanisms that have been proposed for the activation of insect phenoloxidase: autocatalysis, limited proteolysis by an activator and assembly of subunits. Many hemolymph phenoloxidases are activated by simple exposure to air or extraction with acetone while some of the more unusual activators are 50% methanol, exposure to germicidal uv lamp (Preston and Taylor, 1970) and fatty acids (Heyneman and Vercauteren, 1968). Cuticular extracts also activate hemolymph phenoloxidase (Sin and Thompson, 1971). It is generally accepted that insect phenoloxidase occurs as an inactive proenzyme until it is activated after molting (Ashida, 1971) but the mechanisms, hormonal control, role of hemocytes and other factors are still being explored.
(Chen, 1971; Koepppe and Gilbert, 1974). A great deal will have to be learned about insect phenoloxidases in order to explain the bewildering array of factors that initiate melanization.

In Vitro Antibacterial Activity, Its Stability and Relationship to Melanization

Classification of immunized larvae. In the work up to this point, hemolymph from both melanized and unmelanized immunized larvae had been used for *in vitro* assays. However, the range of melanization responses to immunization was intriguing. In particular, the fact that some immunized larvae had normal integument and blue hemolymph similar to saline injected and normal larvae while most immunized larvae showed some degree of melanization suggested that a relationship might exist between the melanization response and induced immunity. Thus before proceeding further, a system of 3 classes was established on the basis of cuticular and hemolymph melanization. Larvae showing either no melanization or no more than 2 to 3 faint spots about the size of a spiracle were put into class I. These larvae had blue blood. Larvae with one to 6 prolegs or sides of segments melanized and which had green blood were put into class II. Some of the more lightly melanized class II larvae yielded a blue-green hemolymph which frequently melanized after filtration.
Hemolymph from these larvae was considered to be intermediate between class I and II and was not used. This situation occurred more frequently when an old bacterin was used. Larvae were placed in class III when the blood was brown-green and showed melanization on 8 or more prolegs and to a lesser degree over the rest of the body.

Since some of the lightly melanized class II larvae had blue-green blood which melanized after filtration and others had green blood that did not melanize, the melanization of the integument and hemolymph appear to be independent events. In these studies antibacterial activity was correlated with the degree of melanization produced in the hemolymph and not in the integument. What role integumental melanization played in the development of immunity, antibacterial activity and inhibition of further melanization is not known. Since integumental melanization did not always correlate with hemolymph melanization, it may not be significant in the development of these factors.

The correlation between the number of prolegs melanized and the color of blood was not perfect when distinguishing between class II and III. For instance, some larvae with 8 melanized prolegs produced green blood and others produced brown-green blood. As was the case with larvae intermediate between classes I and II when larvae were bled for in vitro experiments hemolymph color was used as the main indication of class and intermediate
animals were avoided. When larvae were assigned to class purely on the basis of cuticular melanization, 1 and 6 prolegs were used as the arbitrary end points for class II.

Larvae of different sizes were found to have different probabilities of melanizing (Figure 8). It can be seen that the larger the larvae, the less intense the melanization reaction. Use was made of this relationship in experiments to produce the maximum number of larvae in the desired class. The range of responses to immunization is a good example of biological variability showing a gaussian curve. The class II larvae occupied the central portion of the curve with the class I and III larvae occupying the two extreme regions. When the dose was held constant and the size of the larvae varied as in Figure 8, the curve was skewed to the left or right. This shows that the response operated on a dose per body weight (number of cells/grams body weight) basis.

**Antibacterial activity of the three classes of hemolymph.** Hemolymph from immunized larvae weighing 1-4 grams was bled into 3 flasks according to class and was assayed for bactericidal activity. The results in Table 8 show that bactericidal activity was found in class II and III larvae only. In addition to not having bactericidal activity, class I serum melanized quickly after filtration. Class I serum was assayed 6 times involving a total of 8
Figure 8. Frequency of classes I, II, and III of cuticular melanization in different size ranges of immunized M. sexta larvae. The number responding in each class is listed within each bar.
<table>
<thead>
<tr>
<th>Number of Bacteria Incubated with Serum</th>
<th>Bacteria Recovered After Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class I</td>
</tr>
<tr>
<td>5.4x10^6</td>
<td>solid growth^a</td>
</tr>
<tr>
<td>5.4x10^5</td>
<td>solid growth^a</td>
</tr>
<tr>
<td>5.4x10^4</td>
<td>solid growth^a</td>
</tr>
<tr>
<td>5.4x10^3</td>
<td>solid growth^a</td>
</tr>
<tr>
<td>5.4x10^2</td>
<td>solid growth^a</td>
</tr>
</tbody>
</table>

^a Serum melanized in depression.

^b Serum melanized on nutrient agar.
replicates and it always melanized. Only once did it show any bactericidal activity, killing 560 bacteria. The accidental inclusion of some class II serum could then cause a low level of antibacterial activity. This is consistent with the fact that in the first series of in vitro antibacterial assays, hemolymph from both class I and II larvae was used. The resulting serum was green; it did not melanize after filtration and showed antibacterial activity. It was obviously fortunate that in those experiments class II larvae predominated or only very low antibacterial activity would have been detected. Class II serum was used for dozens of experiments and as long as the bacterin was less than a month old it was always bactericidal and did not melanize. Class III serum was assayed 5 times in a total of 7 replicates and gave results similar to those found for class II except that the age of bacterin did not seem to be significant.

The size of the larvae was not found to be significant with respect to bactericidal activity. Serum from class II larvae, as large as 7 grams, showed typical bactericidal activity and class I serum from larvae as large as 4.5 grams was without activity.

The fact that antibacterial activity occurred only in the hemolymph of class II and III larvae may be important. It suggests that immunization must produce some minimum in vivo melanization before activity can be
found. This is consistent with the suggestion of Pye and Yendol (1972) that the appearance of antibacterial factors is related to phenoloxidase. The sequence of color changes noted in the melanization of normal hemolymph and normal serum was blue, light green, dark green and black. On the other hand the color of the hemolymph of class II larvae was various shades of green and fits somewhere in the middle of the melanization sequence. Thus the melanization of immunized larvae may more properly be called partial or incomplete melanization and implies that intermediates may be somehow inhibited from completing the pathway to melanization. Since the color of class III hemolymph tended toward brown colors it may also be that the melanization pathway occurring with immunity was different from the one involved in the production of black end products.

Using a 2:1 ratio of immune serum to *P. aeruginosa* suspension, *M. sexta* serum killed a maximum of $5.4 \times 10^5$ bacteria per 0.3 ml total volume ($1.8 \times 10^6$ bacteria/ml at a 2:1 ratio, Table 8). Even larger numbers of *P. aeruginosa* were killed by heated immune serum ($1.3 \times 10^7$ bacteria/ml at a 2:1 ratio, Table 12). A considerably weaker antibacterial activity occurs in *G. mellonella* where immune serum kills $6 \times 10^3$ *P. aeruginosa* in a total volume of 0.04 ml ($3 \times 10^4$ bacteria/ml at a 1:1 ratio) (Stephens, 1962a). Activity
closer to that in *M. sexta* is shown in *G. mellonella* by Hink (1966) where $3.25 \times 10^5$ *P. aeruginosa* are killed in a 0.2 ml mixture ($1.6 \times 10^6$ bacteria/ml at a 1:1 ratio). In a 1:1 mixture totaling 0.1 ml, immune serum from *O. fasciatus* kills $1.4 \times 10^3$ *P. aeruginosa* ($1.4 \times 10^4$ bacteria/ml at a 1:1 ratio) (Gingrich, 1964). Thus, the antibacterial activity of *O. fasciatus* is somewhat less potent than that of *G. mellonella* or *M. sexta* but the author does not indicate whether he tried larger numbers of bacteria. When the ratio of immune serum to bacterial suspension is considered, the antibacterial activity of *M. sexta* immune serum was within the range of activities found in other insects.

**Stability of antibacterial activity.** The stability of the antibacterial activity was assayed after 3 different treatments; class II hemolymph was heated in a boiling water bath, stored at 4°C or mixed with acid or base. After these treatments, the samples were assayed for activity as was done in earlier experiments.

The heat stability of the antibacterial factor was assayed by placing part of a sample of hemolymph in a gently boiling water bath for 5 minutes and leaving the remainder untreated. In the boiled fraction, the proteins precipitated forming a green coagulum which was removed by filtration. Only the supernatant was tested for activity. Table 9 shows that activity was not only maintained but
Table 9

Stability of Antibacterial Activity of Class II Hemolymph to Heating in a Boiling Water Bath for Five Minutes

<table>
<thead>
<tr>
<th>Number Bacteria Added</th>
<th>Untreated Serum</th>
<th>Heated Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5x10^6</td>
<td>solid growth^a</td>
<td>solid growth^a</td>
</tr>
<tr>
<td>1.5x10^5</td>
<td>solid growth^a</td>
<td>0^b</td>
</tr>
<tr>
<td>1.5x10^4</td>
<td>0^b</td>
<td>0^b</td>
</tr>
<tr>
<td>1.5x10^3</td>
<td>0^b</td>
<td>0^b</td>
</tr>
<tr>
<td>1.5x10^2</td>
<td>0^b</td>
<td>0^b</td>
</tr>
<tr>
<td>0</td>
<td>0^b</td>
<td>0^b</td>
</tr>
</tbody>
</table>

^aSerum melanized in depression.

^bSerum melanized on nutrient agar.
also increased by the heat treatment. In 6 other replicates of this experiment, the heated immune serum always had equal or greater antibacterial activity than the unheated immune serum. No melanization ever occurred in the depressions where bacteria multiplied or when the serum was plated on nutrient agar. This suggests that heating denatured the hemolymph phenoloxidase which then may have been removed with the other proteins in the coagulum. The phenoloxidase activity of *Leucophaea maderae* hemolymph is stable to heating at 60°C for 10 minutes. However 65°C causes a partial loss and 72°C a complete loss of activity (Preston and Taylor, 1970). The ability to form melanin is destroyed in other insects by temperatures from 60° to 90°C (Bodine and Allen, 1941) and in sections of human skin by 100°C (Lerner and Fitzpatrick, 1950).

Antibacterial activity of normal *O. fasciatus* hemolymph is stable to boiling for 30 minutes but is destroyed by longer heating (Frings *et al*., 1948). Stability is increased by removal of the lipids by ether extraction. After boiling, the coagulated proteins can be removed without loss of activity. Removal of the coagulum after boiling normal warble or botfly hemolymph results in antibacterial activity in the supernatant equivalent to that in the serum (Landi, 1960). The antibacterial activity in immune *G. mellonella* serum is
also stable to 100°C for 5 minutes (Stephens and Marshall, 1962), while that in *B. mori* survives 121°C for 20 minutes (Briggs, 1958). Antibacterial activity of immune *O. fasciatus* serum survives 75°C for one hour (Gingrich, 1964).

In contrast to the extreme stability of *M. sexta'*s antibacterial activity to treatment with high temperature, activity was lost during storage at 4°C. After an initial assay, serum was kept in a small sterile flask covered with aluminum foil. With one exception, the number of bacteria killed in an assay of stored serum decreased by at least one order of magnitude after 24 or 48 hours storage (Table 10). No antibacterial activity was found in serum stored for 4 or more days. Immunity and antibacterial activity show a steep decline by 27 hours after immunization (Figure 7) so the loss of activity in storage may reflect this trend.

The loss of activity of immune serum is probably not due to enzymatic action because heated class II serum showed greater loss of activity than unheated immune serum. Very few if any enzymes are likely to survive 100°C for 5 minutes. An alternate explanation for the loss of activity may be that the active factor slowly reacts with other serum components and thus becomes inactive. However, one would expect a reaction to be accelerated by the heating of the serum. It has been shown that heating does not reduce antibacterial activity and in some cases increases it. The
Table 10

Stability of Serum Bactericidal Activity During Storage at 4°C

<table>
<thead>
<tr>
<th>Hemolymph Sample</th>
<th>Maximum Number of Bacteria Killed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Hours</td>
</tr>
<tr>
<td>Class II Serum</td>
<td>4.6x10^5</td>
</tr>
<tr>
<td>Class II Serum</td>
<td>5.0x10^3</td>
</tr>
<tr>
<td>Heated Class II Serum</td>
<td>2.9x10^6</td>
</tr>
<tr>
<td>Heated Class II Serum</td>
<td>6.3x10^6</td>
</tr>
<tr>
<td>Class III Serum</td>
<td>2.8x10^4</td>
</tr>
<tr>
<td>Class III Serum</td>
<td>3.8x10^5</td>
</tr>
</tbody>
</table>
same contradiction applies to any explanation involving the deterioration of an unstable compound.

A similar contradiction exists in the case of *G. mellonella* immune serum. Antibacterial activity is stable for at least a month at 4°C while most of it is lost after 24 hours at 37°C. On the other hand, activity is not destroyed by heating at 100°C for 5 minutes (Stephens, 1962a; Stephens and Marshall, 1962). *B. mori* immune serum can be stored for 2 weeks at 5°C without loss of activity (Briggs, 1958). The serum of immune *O. fasciatus* can be stored for 6 months at -20°C without loss of activity (Gingrich, 1964) while a saline extract of normal serum loses most of its activity within 6 hours at room temperature (Frings et al., 1948).

*M. sexta* serum stored at 4°C remained unmelanized for at least one week when some samples began to slowly darken. Immune serum that was stored for longer periods showed a rapid melanization in *in vitro* assays. That is, it melanized in a manner similar to that seen in serum from untreated and saline injected larvae. These data suggest that an inhibitor rather than a lack of activator is responsible for the reduced melanization of immune serum. The pattern of melanization can be explained by the gradual deterioration of a phenoloxidase inhibitor produced by immunization. The gradual resynthesis during storage of an
activator which was depleted during immunization seems a less likely explanation, especially for the heated serum.

Bactericidal material can be isolated from immune *G. mellonella* hemolymph by ion exchange resin and dialysis (Stephens and Marshall, 1962). Both techniques produce a fraction that is both bactericidal and inhibitory of phenoloxidase. Thus in *G. mellonella*, the melanization inhibitor and bactericidal factor may be one and the same. The source of the inhibitor was not shown but a heat stable, dialysable phenoloxidase inhibitor can be isolated from the serum and fat body of *A. pernyi* (Evans, 1968).

Nutrient agar plates used for these experiments as well as others were kept up to a week after sampling and showed no bacterial growth where none had been detected originally. It thus can be concluded that immune serum was bactericidal and not just bacteriostatic. There are 2 reasons why this seems a reasonable conclusion. First, considering the stability of the active substances in storage at 4°C, it is doubtful that any active material was left after one week at room temperature. Second, it seems likely that if any active substances remained, they would have diffused into the agar within a week, leaving an ineffective concentration in the area of the bacterial cells. Immune serum is bactericidal in *G. mellonella* (Stephens, 1962a) and *B. mori* (Kawarabata, 1970). No intact cells can be detected in areas where antibacterial
activity has killed *P. aeruginosa* and thus Gingrich (1964) concluded that the active substance(s) in *O. fasciatus* are bacteriolytic.

Table 11 shows the antibacterial activity of acid and base treated hemolymph and the pH produced by the treatment. Antibacterial activity was stable to the 0.1N HCl treatment and indeed showed greater activity than the water control. Treatment with 1 N HCl destroyed all antibacterial activity. Antibacterial activity was largely destroyed by both treatments with base. The 1.0 N NaOH treatment resulted in partial activity against 190 bacteria while the 0.1 N NaOH treated hemolymph showed partial activity against 1900 bacteria. The numbers of bacteria found on the nutrient agar plates were estimates accurate to within about 10%. Lower than expected antibacterial activity occurred in the distilled water treated hemolymph. It can be seen that some activity was detected against 1.9x10⁴ cells.

Some explanation for the relatively low activity in the control may lie in the fact that addition of the distilled water caused the hemolymph to turn smokey gray in color. It may be that the partial melanization was responsible for the reduced activity. The *in vitro* assay showed complete melanization only in the top 2 depressions where heavy growth of *P. aeruginosa* was detected. When samples from the *in vitro* assay were plated on the nutrient
Table 11
Stability of Class II Serum Antibacterial Activity to Treatment with Equal Volumes of Acid or Base

<table>
<thead>
<tr>
<th>Number Bacteria Added</th>
<th>1N HCl pH=1.0</th>
<th>0.1N HCl pH=3.2</th>
<th>H₂O pH=6.0</th>
<th>0.1N NaOH pH=9.5</th>
<th>1N NaOH pH=11.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9x10⁶</td>
<td>solid growth</td>
<td>solid growth</td>
<td>solid growth</td>
<td>solid growth</td>
<td>--</td>
</tr>
<tr>
<td>1.9x10⁵</td>
<td>solid growth</td>
<td>0</td>
<td>solid growth</td>
<td>solid growth</td>
<td>solid growth</td>
</tr>
<tr>
<td>1.9x10⁴</td>
<td>solid growth</td>
<td>0</td>
<td>500ᵃᵇ</td>
<td>solid growth</td>
<td>solid growth</td>
</tr>
<tr>
<td>1.9x10³</td>
<td>solid growth</td>
<td>0</td>
<td>0ᵃᵇ</td>
<td>400ᵃ</td>
<td>solid growth</td>
</tr>
<tr>
<td>1.9x10²</td>
<td>solid growth</td>
<td>0</td>
<td>0ᵃᵇ</td>
<td>0ᵃ</td>
<td>1000</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0ᵃᵇ</td>
<td>0ᵃ</td>
<td>0</td>
</tr>
</tbody>
</table>

ᵃSerum melanized in depression.
ᵇSerum melanized on nutrient agar.
agar, melanization occurred with the samples from the bottom 4 depressions. This is an example of the inverse relationship between degrees of melanization in the in vitro assay and the amount of antibacterial activity shown. The degree of in vitro melanization that occurred seemed to determine how much activity remained. It may be that the intermediates in melanization pathway reacted with the antibacterial substance(s). Alternatively, melanization intermediates may themselves be the antibacterial substance(s) and be used up as melanization occurs.

The 0.1 N HCl treated hemolymph showed no melanization in the depressions or on the nutrient agar plates. The serum phenoloxidase was apparently destroyed by this treatment. Thus in this case and in the case of the boiling of immune hemolymph, it can be seen that no active phenoloxidase need be present in the in vitro assay for antibacterial activity to occur. The 1.0 N HCl treatment apparently destroyed both serum phenoloxidase and the antibacterial substance(s). Melanization began immediately upon treatment with 0.1 N NaOH and the hemolymph was black by the time the in vitro assay was set up. The melanization was not complete however in that only fine black particulate material was produced and not a thick sticky substance.

The pH stability of antibacterial factors in the hemolymph of other insects is generally greater than that of
M. sexta. The immune hemolymph of O. fasciatus, when mixed with an equal volume of 1 N HCl or NaOH for one hour shows no loss of activity (Gingrich, 1964). The antibacterial activity of immune B. mori hemolymph is also stable to 0.1 N HCl or NaOH for 2 hours (Briggs, 1958). Normal hemolymph of B. mori has antibacterial activity against E. coli which is stable to pH 2 and is reduced by two thirds by pH 10 for 30 minutes (Kawarabata, 1970). On the other hand, column chromatography fractionation of immune B. mori hemolymph yields an antibacterial fraction that is fully stable to treatment with a pH of 3 or 10.

Studies on the relationship between in vitro melanization and antibacterial activity. In vitro assays for antibacterial activity showed an inverse relationship between activity and melanization. If the depression melanized quickly, no antibacterial activity was found. When no melanization or only very slight darkening occurred, strong antibacterial activity was found. Intermediate cases were occasionally found where melanization occurred slowly and some but not all of the bacteria were killed. The pattern of this relationship suggested that the melanization process destroyed the antibacterial activity. Since the melanization and loss of activity always occurred in depressions where large numbers of living P. aeruginosa were added, it was possible that the bacteria
cells or some substance associated with them was initiating the melanization.

To test for the effects of large numbers of bacterial cells, bacterin (10^7 cells in 5 μl of saline) was added to the *in vitro* assays along with immune sera and live *P. aeruginosa* suspensions. Table 12 shows the effect of adding bacterin to class II and heated class II sera. The bacterin induced melanization in the unheated class II sera and caused a concomitant loss in antibacterial activity. On the other hand the addition of bacterin had no effect on the activity of heated class II serum. The lower than normal activity of the untreated class II serum is an example of the reduced potency of an aging bacterin. The extreme difference in the antibacterial activities between the untreated class II and heated class II sera is very interesting. Heated class II sera often had somewhat greater activity than untreated class II sera but the difference had never been of this magnitude. These results suggest that the induction of antibacterial substance(s) and the inhibition of *in vitro* melanization may be separate, though related responses to immunization.

Since heating apparently destroys phenoloxidase but not the antibacterial activity, this experiment showed that the bacterial suspension acts on the melanizing enzyme and not on the substance(s) responsible for antibacterial activity. The action of the bacterial suspension may be
Table 12
Effect of Adding Bacterin to Depression on \textit{In Vitro} Melanization and Bactericidal Activity

<table>
<thead>
<tr>
<th>Number Bacteria Added</th>
<th>Class II Serum</th>
<th>Heated Class II Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Bacterin Added</td>
</tr>
<tr>
<td>$3.8 \times 10^6$</td>
<td>solid growth$^a$</td>
<td>solid growth$^a$</td>
</tr>
<tr>
<td>$3.8 \times 10^5$</td>
<td>solid growth$^a$</td>
<td>solid growth$^a$</td>
</tr>
<tr>
<td>$3.8 \times 10^4$</td>
<td>1-2000$^b$</td>
<td>solid growth$^a$</td>
</tr>
<tr>
<td>$3.8 \times 10^3$</td>
<td>1$^b$</td>
<td>solid growth$^a$</td>
</tr>
<tr>
<td>$3.8 \times 10^2$</td>
<td>0$^b$</td>
<td>90$^a$</td>
</tr>
<tr>
<td>0</td>
<td>0$^b$</td>
<td>0$^a$</td>
</tr>
</tbody>
</table>

$^a$Serum melanized in depression.

$^b$Serum melanized on nutrient agar.
simple activation of the phenoloxidase or it may be through adsorption of an inhibitor by the cells. The addition of zymosam, an insoluble carbohydrate from yeast cells, causes rapid melanization and loss of antibacterial activity in immune *G. mellonella* serum (Stephens, 1962a). The results were interpreted to mean that the zymosam adsorbs the antibacterial activity from the serum, however it seems equally plausible to suggest that the antibacterial activity of *G. mellonella* and *M. sexta* was destroyed by reacting with the highly reactive polyphenolic intermediates of the melanization reaction. Zymosam does in fact act as a potent activator of *G. mellonella* phenoloxidase (Pye, 1974).

Since it has been shown that *in vitro* melanization of class II serum destroys antibacterial activity, it may be that the *in vitro* melanization of class I and control sera masked any activity they might have. If the melanization could be inhibited, antibacterial activity might be found. Tests for antibacterial activity in these sera made use of the fact that heating class II serum caused the loss of ability to melanize but left the antibacterial activity intact and could be expected to do the same in the class I and control sera.

Pooled hemolymph from saline injected and class I larvae was divided into two fractions. The first fraction of each was sterilized by Millipore filtration and the
second fraction was heated in a $100^\circ$C water bath for 5 minutes and then filter sterilized. Table 13 shows that untreated saline injected and class I sera melanized in the usual manner and had no antibacterial activity. The heated fractions also showed no antibacterial activity. Thus there was no antibacterial activity in these sera that was being masked by \textit{in vitro} melanization.

Two conclusions can be made. First, the correspondence between partial melanization of the immunized larvae and induced immunity has been confirmed. Saline injected and class I immunized larvae did not develop partial melanization and their serum showed no antibacterial activity, even when \textit{in vitro} melanization was prevented. Apparently, some "immunized" larvae did not respond to the immunizing dose and thus were physiologically similar to the saline controls. Secondly, that saline injections did not induce \textit{in vitro} antibacterial activity in \textit{M. sexta} was also confirmed. This was not unexpected since saline injected larvae showed no resistance to challenge in earlier experiments.

To test for the possibility that tyrosine may cause the melanization of immune serum, 0.1 ml of 0.0001 M tyrosine was added to class II serum. The tyrosine solution was also added to heated class II serum as a check for any non-specific interaction with the antibacterial factor(s). Table 14 shows that the addition of tyrosine to the
Table 13

Effect of Heating on Melanization and Antibacterial Activity of Serum from Saline Injected and Class I Larvae

<table>
<thead>
<tr>
<th>Number Bacteria Added</th>
<th>Saline Injected Serum</th>
<th>Class I Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Heated</td>
</tr>
<tr>
<td>1.8x10^6</td>
<td>solid growth</td>
<td>solid growth</td>
</tr>
<tr>
<td>1.8x10^5</td>
<td>solid growth</td>
<td>solid growth</td>
</tr>
<tr>
<td>1.8x10^4</td>
<td>solid growth</td>
<td>solid growth</td>
</tr>
<tr>
<td>1.8x10^3</td>
<td>solid growth</td>
<td>solid growth</td>
</tr>
<tr>
<td>1.8x10^2</td>
<td>solid growth</td>
<td>solid growth</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*aSerum melanized in depression.*
Table 14

The Effect of Adding 0.001 M Tyrosine on In Vitro Melanization and Antibacterial Activity

<table>
<thead>
<tr>
<th>Number Bacteria Added</th>
<th>Class II Serum Untreated</th>
<th>Class II Serum 0.1 ml Tyrosine</th>
<th>Heated Class II Serum Untreated</th>
<th>Heated Class II Serum 0.1 ml Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2x10^6</td>
<td>solid growth</td>
<td>solid growth</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1.2x10^5</td>
<td>solid growth</td>
<td>solid growth</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1.2x10^4</td>
<td>25^b</td>
<td>0^b</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1.2x10^3</td>
<td>0^b</td>
<td>0^b</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.2x10^2</td>
<td>0^b</td>
<td>0^b</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0^b</td>
<td>0^b</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

^a Serum melanized in depression.
^b Serum melanized on nutrient agar.
serum-bacteria mixtures had no effect on melanization or antibacterial activity. Thus the melanization of immune *M. sexta* serum was due to the bacterial cells or some soluble substance other than tyrosine in the bacterial suspension. Melanization of immune serum can be caused by the addition of tyrosine in *G. mellonella* (Stephens, 1962b).

**Hemolymph Lysozyme Activity**

Groups of larvae weighing 1.5 to 3.0 g received one of the following treatments. The first group was immunized with $10^7$ heat killed *P. aeruginosa* cells. One control group received 5 μl of 0.85% saline and the second received no treatment at all. At various intervals after injection the larvae were removed from the environmental chamber and bled for lysozyme assays. Since an assay of 5 larvae took about 40 minutes, the mean time of sampling was used for all the larvae in that sample.

The level of lysozyme found in whole hemolymph of the 3 groups of larvae is shown in Figure 9. Solid points represent the activity from individual larvae and empty points the mean activity at the indicated time. Only class II larvae were used from the immunized group.

Lysozyme activity in hemolymph of immunized animals rose rapidly during the first 15 hours after immunization and then maintained a plateau for the rest of the fifth instar up until shortly before the development of the prepupa. Both the untreated controls and the saline
Figure 9. Hemolymph lysozyme activity. Solid points represent the activity of individual larvae and the empty points the mean activity. Class II hemolymph (circles); saline injected control hemolymph (squares); and untreated control hemolymph (triangles). Some points when crowded are slightly displaced laterally for clarity.
injected larvae showed a gradual increase in lysozyme activity during the instar. The highest level of activity in the saline injected and untreated controls was about two thirds the level maintained by the immunized larvae. All 3 groups showed a wider range of activity as development proceeded.

Since only class II larvae were used in the above experiment the activity of heated serum and the other 2 classes remained to be determined. Three assays of heated sera showed a complete absence of activity and Table 15 lists the lysozyme activity of saline injected and class I, II, and III immunized larvae during the period 15 to 18 hours after immunization.

The Mann-Whitney U Test (Zar, 1974) showed that the activity of class I, II, and III were significantly different from the saline injected control (p=0.05). The test showed no significant difference between classes I, II, and III. It can be seen that the activity of class III hemolymph very well may show a significant difference from classes I and II if the sample size were larger. Such a lower activity would not be surprising, however, because the extensive melanization in the hemolymph may denature some of the enzyme. Mixing phenoloxidase with its substrate and lysozyme causes inactivation of the lysozyme (Sizer, 1953) possibly by a mechanism similar to that involved in the
Table 15

Units of Lysozyme Activity of Hemolymph from Saline Injected Larvae and Class I, II, and III Immunized Larvae

<table>
<thead>
<tr>
<th></th>
<th>Saline Injected</th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units of lysozyme activity of individual larvae</td>
<td>45 86 97 71</td>
<td>49 87 87 83</td>
<td>34 97 93 88</td>
<td>36 102 95 86</td>
</tr>
<tr>
<td></td>
<td>36 102 95</td>
<td>50 97 94 86</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>Mean</td>
<td>43 94 92 81</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s.d.</td>
<td>7 7 4 9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
reaction inactivation of the phenoloxidase itself (Wood and Ingraham, 1965; Brooks and Dawson, 1966).

The assays for lysozyme activity produced 3 items of evidence that some factor other than lysozyme was responsible for induced immunity in *M. sexta*. First, the lysozyme activity and the immune response of immunized larvae did not correspond with each other. While lysozyme activity peaked at 16 hours after immunization, resistance to challenge and *in vitro* antibacterial activity reached their maximum values within 6 hours. Also the level of lysozyme activity maintained a plateau while the *in vivo* and *in vitro* assays showed a decrease after about 18 hours post immunization. The time scales in Figures 7 and 9 are identical to facilitate comparison. Second, while serum from class I, II, and III larvae all had similarly elevated lysozyme activities after immunization, antibacterial activity was absent from class I serum. Third, boiling hemolymph for 5 minutes destroyed all lysozyme activity but did not affect antibacterial activity. It is thus quite certain that lysozyme activity cannot be the primary agent of immunity of *M. sexta*.

Chadwick (1970) found that the levels of immunity and lysozyme activity do not correspond in *G. mellonella*. Just as in *M. sexta*, the lysozyme activity of the serum remains high while immunity to *P. aeruginosa* decreases. Her results differ from those found in *M. sexta* in that she
found that saline injections induce increased lysozyme activity levels. The lysozyme activity levels of saline injected *M. sexta* were similar to those of untreated larvae.

Mohrig and Messner (1968) propose that lysozyme is the active agent in insect immunity because the rise in lysozyme activity parallels the rise in antibacterial activity and because lysozyme and antibacterial substances are stable to heat and acid treatment. However, in the literature review for this study, it has already been shown that there are numerous difficulties with their interpretation. The results of this study also make it difficult to accept Mohrig and Messner's view.

In order to explain the antibacterial activity of immune hemolymph against Gram-negative organisms normally not lysed by lysozyme (Salton, 1957) Mohrig and Messner (1968) suggested that other agents in the hemolymph may act synergistically with lysozyme to increase its normally weak effect on these bacteria. They suggested antibiotics, detergents, chelating agents or trypsin may be present to make lysozyme an effective lytic agent against Gram-negative bacteria. *P. aeruginosa* is not lysed by lysozyme because apparently the lysozyme sensitive component in the cell wall is not the only component responsible for maintaining cell wall integrity (Carson and Eagon, 1966; Eagon and Carson, 1965). Other agents such as EDTA or tris-(hydroxymethyl)aminomethane must be added to a lysozyme
solution in order to achieve lysis (Cox and Eagon, 1968). Thus the synergistic agents suggested by Mohrig and Messner (1968) are necessary if lysozyme is to be the primary agent in insect immunity but they remain to be demonstrated.
GENERAL DISCUSSION AND CONCLUSIONS

It has been suggested that quinones produced by phenoloxidase may be a primitive, non-specific defense mechanism in insects (Cottrell, 1964; Hackman, 1974; Taylor, 1964). Localized melanization accompanying hemocytic encapsulation of metazoan parasites is well known (Salt, 1970) and may also occur in the absence of hemocytes (Poinar and Leutenegger, 1971). It has also been shown that preventing melanization reduces the incidence of successful encapsulation (Nappi, 1973). Hemolymph phenoloxidase of G. mellonella occurs as a latent proenzyme and is activated by P. aeruginosa cells; the activation by P. aeruginosa is much more pronounced in immune serum than it is in normal serum (Pye, 1974). This work with M. sexta's response to P. aeruginosa provides further evidence that the insect antibacterial defense mechanism involves phenoloxidase. This evidence can be divided into 4 major categories: the correlation in timing between melanization and induced immunity, the toxicity of the melanization reaction for the larvae, the requirement for in vivo melanization before in vitro antibacterial activity occurred and the lack of correlation between lysozyme activity and immunity.
Melanization appeared in the integument of *M. sexta* as soon as 10 minutes after injection and increased in intensity during the next several hours. The larvae began to show immunity by 2 hours after injection and *in vitro* antibacterial activity by 3 hours. Both immunity and antibacterial activity peaked between 6 and 19 hours and then began to decline. Larvae showed their maximum melanization in the integument and hemolymph during this period. Immunized larvae kept until the end of the instar returned to a near normal coloration, having lost most of the melanin in both the integument and hemolymph.

The toxicity caused by the immunization of *M. sexta* larvae was expressed as a retarded growth rate. The toxicity symptoms can be explained by noting that the melanization pathway involves various quinones as intermediates and these quinones are highly reactive and toxic (Mason, 1955; Zlotkin et al., 1973). Melanization can also inactivate a wide variety of proteins (Sizer, 1953). The growth rate of the larvae was retarded during the first 18 hours after which it returned to normal. The change in growth rate at 18 hours suggests that a decrease in toxic substances occurred at this time.

Immunized larvae which were separated into three classes on the basis of the degree of integumental and hemolymph melanization showed that only those that melanized had serum with antibacterial activity. Unmelanized larvae
had serum with characteristics similar to that from control or untreated larvae. The relationships are summarized in Table 16. The requirement that partial melanization precede the appearance of antibacterial activity suggests that the active agent in immunity may be intermediates in the melanization reaction. Intermediates would be excreted in the feces and this would lead to a loss of immunity and antibacterial activity. The increased larval growth rate that occurred at about 18 hours also suggests that immunity may be related to toxic intermediates of melanization.

Serum from class II and III larvae did not melanize when sterilized by Millipore filtration (Table 16). Therefore it may be that the partial melanization in these larvae produced an inhibitor of phenoloxidase. It is possible that the inhibitor and antibacterial substance(s) are one and the same as has been suggested for G. mellonella (Stephens and Marshall, 1962) but no evidence for or against this hypothesis was obtained in M. sexta.

Immunization caused an increase in lysozyme activity in all 3 classes of larvae. This uniform response contrasts markedly with the range of melanization reactions and antibacterial activities that were encountered. The increase in lysozyme activity after immunization did not correspond in time with the changes in immunity or antibacterial activity. Lysozyme activity, but not
Table 16
Bactericidal Activity of Serum in Relation to Melanization

<table>
<thead>
<tr>
<th>Serum</th>
<th>Cuticular Melanization</th>
<th>Color of Blood</th>
<th>In Vitro Melanization After Filtration</th>
<th>Antibacterial Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>None</td>
<td>Blue</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Saline inj.</td>
<td>None</td>
<td>Blue</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Class I Immunized</td>
<td>Up to 2 spots</td>
<td>Blue</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Class II Immunized</td>
<td>1 to 6 prolegs</td>
<td>Green</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Class III Immunized</td>
<td>8 or more prolegs</td>
<td>Brown-green</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
antibacterial activity, was destroyed by heating. Thus it is unlikely that lysozyme activity plays a significant role in *M. sexta's* immunity to *P. aeruginosa*. However, it should be pointed out that there is a double response to immunization in all three lepidopterans that have been studied. Immunization causes the formation of heat stable antibacterial material and an increase in lysozyme activity in *M. sexta*, *G. mellonella* (Chadwick, 1970), and *B. mori* (Briggs, 1958; Powning and Davidson, 1973). Thus it would be desirable if an explanation for the role of lysozyme activity in immunity could be made. Otherwise we are left with the conclusion that increases in lysozyme activity following immunization may simply be an experimental artifact. Pye (1974) has suggested the hypothesis that lysozyme acts on the bacterial cell wall but does not kill the bacterium. Rather lysozyme activity exposes a site on the bacterium that activates prophenoloxidase to a form that produces bactericidal substances. Thus lysozyme would not need to be present once the phenoloxidase had produced bactericidal substances. The results in this study are consistent with Pye's suggestion.

The experimental conditions used in these studies are completely artificial and of course are not found under field conditions. However, it is possible to extrapolate the experimental results to field conditions. It may be that infected larvae respond with melanization localized
around the infection and the quinones produced by the melanization reaction would be toxic to the invading pathogen. A similar, more detailed hypothesis has been proposed by Pye (1974) for the antibacterial defense mechanism in *G. mellonella*.

Three reasons were given in the introduction for studying immunity in *M. sexta*. The first was to establish a system suitable for the eventual isolation of the active substance(s) involved in insect immunity. Isolation may be possible using *M. sexta* serum if the problem of the stability of the activity can be overcome. The second reason for studying *M. sexta* was the opinion that new insights might be gained by studying an animal no one had used before for the study of immunity and this seems to have been justified. The most frequently used animal for insect immunity studies is *G. mellonella*. When the larvae of this species are immunized, a slight darkening of the hemolymph occurs and apparently all larvae respond similarly (Pye, personal comm.). *M. sexta* on the other hand shows extensive and obvious melanization and the range of melanization responses varies widely. Without these differences from *G. mellonella*, the new correlations made in this study would have been impossible. The results obtained have strengthened the hypothesis that phenoloxidase is involved in insect defense against microorganisms. The third reason for this study was to assess the significance
of lysozyme activity in *M. sexta*'s immunity. It seems that lysozyme activity does not act directly to kill *P. aeruginosa*. It may act indirectly, however (Pye, 1974), and may play an as yet undetermined role against Gram positive bacteria. No suggestion for the role lysozyme plays can be made from the results obtained in *M. sexta*.

Briggs (1958) has suggested that since insects lack specific immunoglobins, new concepts would have to be sought to explain insect immunity. One concept that should be considered is that of enzyme mediated immunity since this study along with others points toward the involvement of 2 enzymes: phenoloxidase and possibly lysozyme or chitinase. If such a concept were used, it might prove fruitful in explaining the complexities of insect immunity and emphasizing the distinctions between insect and vertebrate immunity.
SUMMARY

The LD$_{50}$ of *Pseudomonas aeruginosa* (Schroeter) for *Manduca sexta* (L.) was $1.1 \times 10^5$ cells while the LD$_{50}$ of *Serratia marcescens* Bizio was 9 cells. The *in vitro* generation times of these bacteria in serum (cell free hemolymph) were 67 minutes and 38 minutes, respectively.

Larvae immunized with $10^7$ heat killed *P. aeruginosa* survived a normally lethal challenge of the same bacterium. Protection peaked between 6 and 19 hours after immunization and declined thereafter. *In vitro* assays of serum from immunized larvae showed a similar pattern of antibacterial activity. It was concluded that the *in vitro* antibacterial activity was probably also the active agent in *in vivo* immunity. *In vivo* and *in vitro* immunity against *S. marcescens* was weak and inconsistent so work with this bacterium was discontinued.

Immunized larvae developed a spectrum of melanization reactions in the hemolymph and integument and were placed in 3 classes based on the degree of melanization. Class I larvae had no more than 2 small spots of integumental melanization and blue hemolymph. Larvae with 2 to 6 melanized prolegs and green hemolymph were placed in class II while those with 8 to 10 melanized prolegs and
brown-green hemolymph were placed in class III. In vitro antibacterial activity was found only in class II and III sera. This activity was stable to 100°C for 5 minutes but decreased considerably after acid and base treatments and 24 to 48 hours storage. The sera from normal, control and class I insects melanized after Millipore filtration. Sera from class II and III insects did not melanize after filtration. Lysozyme activity was found in normal larvae and was increased by immunization. All 3 classes had similarly elevated lysozyme activities (Mann-Whitney U Test, p=0.05). It was concluded that lysozyme contributed little to the protection against P. aeruginosa and that the heat stable in vitro activity associated with melanization was probably the primary agent in in vivo immunity. Since the heat stable substance(s) were found only in the immunized larvae that had melanized, it was suggested that the insect's phenoloxidase may be involved in their production.


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