

Insect Immunity

II. Simultaneous Induction of Antibacterial Activity and Selective Synthesis of Some Hemolymph Proteins in Diapausing Pupae of *Hyalophora cecropia* and *Samia cynthia*

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We have previously shown that pupae of the giant silkworm *Samia cynthia* have a humoral antibacterial activity, which was induced by viable, nonpathogenic gram-negative bacteria (H. G. Boman et al., 1974). We show here that this activity was formed simultaneously with a selective incorporation of amino acids into eight polypeptide chains characterized by their electrophoretic behavior. If actinomycin D or cycloheximide were given at an early time, no antibacterial activity was found. If the inhibitors were given at the time of maximum activity, there was no effect with actinomycin D but a rapid decrease of the activity in the case of cycloheximide. The results imply that the messenger ribonucleic acid was stable, but that at least one protein component was turning over. Hemolymph from immunized pupae of another giant silkworm, *Hyalophora cecropia*, was fractionated by ammonium sulfate precipitation. This procedure, together with the isotope distribution after co-electrophoresis in polyacrylamide gels, was used for comparing the response to injury and to different infections. Almost identical polypeptide patterns were obtained as a response to an infection with either viable *Enterobacter cloacae* or *Bacillus subtilis*. These patterns differed both qualitatively and quantitatively from the injury effect created by an injection as such. There was only a low antibacterial activity in each of the four fractions obtained by ammonium sulfate precipitation. However, a combination of three fractions restored a high killing activity. Fractionation of hemolymph from untreated pupae provided evidence for at least one preexisting factor which stimulated the killing of *Escherichia coli*. The osmotic pressure of the bacteria contributed to the antibacterial activity towards *E. coli*, but not towards *B. subtilis*. The killing of *E. coli* was inhibited by lipid A and, to a lesser extent, by an inhibitor of proteolytic enzymes. The similarities and differences with the mammalian complement system are discussed.

All living organisms depend on defense systems which protect them against pathogenic microorganisms and maintain their balance with the natural flora. Such systems always have a specificity which prevents the host from "self"-destruction. In higher vertebrates the specificity of both cellular and humoral defense systems is carried by different classes of immunoglobulins. However, it is generally accepted that invertebrates lack immunoglobulins (1, 11, 13), and their defense system must therefore depend on other recognition mechanisms.

Compared to mammals little is known about immunity systems in insects (11, 19, 34). However, some of the humoral defense mechanisms studied in different invertebrates have shown a superficial resemblance to the complement system (3, 8, 14). The antibacterial activity in pu-

pae of *Samia cynthia* was recently shown to be due to an inducible multicomponent system (3, 4). We demonstrate here that this activity in *S. cynthia* and the closely related silkworm, *Hyalophora cecropia*, was induced simultaneously with the selective synthesis of certain hemolymph proteins identified by their electrophoretic behavior. The killing activity and the proteins were fractionated and characterized by reconstitution and inhibition experiments.

The synthesis of hemolymph proteins in *H. cecropia* has been studied previously (22, 27, 32), but in no case were questions asked about the role of these proteins in the immune response. However, every injection of a pupa inevitably also creates a cuticle injury. Earlier studies of protein synthesis after injury (22, 32, 35) are therefore relevant to our present character-

ization of proteins formed as a response to the injection of viable bacteria.

MATERIALS AND METHODS

Bacterial strains. The test organism used in our standard assay for antibacterial activity was *Escherichia coli* K-12, strain D31, a penicillin- and streptomycin-resistant mutant with a defective lipopolysaccharide (LPS) (2, 3). *Bacillus subtilis* strain Bs11 is a streptomycin-resistant mutant isolated from a wild-type strain kept in our stock collection. Most pupae were immunized with *Enterobacter cloacae* strain β 12, a nalidixic acid-resistant mutant (3).

Standard assay for antibacterial activity. The normal reaction mixture contained 1% hemolymph and 5×10^5 to 10×10^9 viable cells of *E. coli* strain D31, but we used 5% hemolymph to measure the killing of *B. subtilis*. The volume of the reaction mixture was about 0.1 ml, and the buffer was always 0.1 M potassium phosphate, pH 6.4, with 2×10^{-3} M dithiothreitol. Incubation was carried out at room temperature, and at different times 5- μ l samples were withdrawn and the number of viable bacteria was determined by plating. We earlier used the initial rate as a measure of the antibacterial activity (3). With some exceptions, we here used the result of a 3-min reaction as a measure of the antibacterial activity. All such data were obtained as interpolations from time curves with a minimum of three points. Other details were as before (3).

Insects and their vaccination. The induction experiments with *S. cynthia* were performed with diapausing pupae obtained from American sources, whereas for the fractionation experiments we used *S. cynthia* pupae of French or Italian origin. The antibacterial activity induced in the latter group was usually poorer, and they did not seem to have a firm diapause. Pupae of *H. cecropia* were obtained from an American dealer. All pupae were stored in a refrigerator at 8 C, and during the experiments they were kept at 25 C and a relative humidity of 60 to 80%.

Pupae were normally vaccinated by injecting viable cells of *E. cloacae* strain β 12 (3) into the thorax, for *S. cynthia* about 5×10^5 cells in 50 μ l and for *H. cecropia* about 10^6 cells in 50 μ l. Control animals were given an equal volume of "W-saline," a salt solution physiological to *Lepidoptera* (33). Small samples of hemolymph were taken up directly in capillary pipettes, whereas extensive bleeding was according to Schneiderman (30) or as described below, when phenylthiourea was omitted.

Ammonium sulfate fractionation of hemolymph. Except for the bleeding, all following steps were carried out at 0 to 4 C. From a 2- to 4-mm incision on the forewing, the hemolymph was collected directly into a 0.5-ml Carlsberg pipette and layered under 1 ml of ice cold 0.1 M potassium phosphate buffer, pH 7.4. The hemolymph volume obtained from two pupae of *H. cecropia* was usually 3.1 to 3.5 ml. Water was then added to a total volume of 10 ml, the sample was mixed, and 2.20 g of solid ammonium sulfate was added to give 38% saturation at 0 C (9). After 5 to 10 min of stirring, the

precipitate was collected by centrifugation at 23,700 $\times g$ for 15 min. The sediments at this and subsequent steps were washed by 2 to 3 ml of an ammonium sulfate solution of the corresponding saturation. The respective supernatants were added back to the main part of the preparation, and the total volumes were adjusted by the respective washing solutions. As the second step, 1.13 g of solid ammonium sulfate was added to 15 ml of supernatant, thereby raising the concentration to 50% saturation. For the third and fourth steps to 62 and 75% saturation, 1.60 and 2.25 g of ammonium sulfate were added to 20 and 25 ml of supernatants, respectively. Each precipitate was dissolved in 1 ml of 0.05 M potassium phosphate buffer, pH 7.4. Nonsoluble residues occurred only at the first step and were removed by a final centrifugation. Fractions were stored in aliquots at -80 C.

Analytical methods. Polyacrylamide slab gel electrophoresis was performed using apparatus GE-4 of Pharmacia Fine Chemicals, Uppsala, Sweden. The gel concentration was 7.5%. All electrophoresis buffers were 0.1 M sodium phosphate containing 0.1% sodium dodecyl sulfate. The pH of the electrode buffer was 6.4 and the pH of the gel buffer was 7.2. Staining and other details of the procedure were as recommended by Maizel (18). For the determination of the radioactivity in double-labeled samples, the gel was cut into 2-mm slices, dried, and combusted in a Packard sample oxidizer, model 306. The $^3\text{H}_2\text{O}$ and $^{14}\text{CO}_2$ separated were counted in the scintillation liquids recommended for the oxidizer but using a Nuclear-Chicago Mark II liquid scintillation counter. External standard was used to check that the quenching was constant. The overall efficiency for ^{14}C was about 55% and for ^3H it was around 30%. All radioactivity data are given without any corrections.

Lysozyme was determined using a suspension of freeze-dried cells of *Micrococcus lysodeikticus* with an optical density of 570 nm of 0.3 to 0.5. The sample to be assayed was added to a final concentration of 1% on a volume basis. The mixture was incubated at 37 C for 30 min. The unit of activity used corresponds to a decrease of 0.001 in optical density at 570 nm. Assays for phenoloxidase and for proteins were those used by Pye (24).

Materials. Lipid A and core polysaccharide from LPS of *E. coli* strain D21 were prepared as before (2). Synthetic glucolipid, *N*-lauroyl-D-glucosamine (26), was kindly donated by A. Nowotny. Zymosan, actinomycin D, and cycloheximide were from Sigma Chemical Co. Trasylol, a polypeptide inhibitor of proteolytic enzymes, was from Bayer UK Limited, and 1-phenyl-2-thiourea was no. 1569 from Eastman Kodak Co. [^{14}C]leucine (384 mCi/mmol) was from The Radiochemical Centre, Amersham, and [^3H]leucine (81.3 Ci/mmol) was from New England Nuclear Corp. Agar plates, with the respective antibiotics and other media, were those used previously (3).

RESULTS

Induction kinetics of the antibacterial activity. We previously demonstrated that in pu-

pae of *S. cynthia* an injection of a gram-negative bacterium induced a cell-free antibacterial activity not detectable in hemolymph of untreated animals (3). We have now extended this work to include also a gram-positive bacterium, and we first studied the time course during which immunity was developed. Figure 1 shows three typical induction experiments using as vaccine viable *E. coli*, *E. cloacae*, and *B. subtilis*. In each case there was a lag period of about 10 h before any immunity reaction was detected. After this time the antibacterial activity began to increase, and the optimum was reached after 2 to 4 days. There were significant quantitative differences in the immune response; with *E. cloacae* a high level of activity was obtained, whereas with *E. coli* and *B. subtilis* the response was weaker. We have previously reported that a sterile salt solution (W-saline) can give a varying immune response (3), and in this particular experiment the activity was very weak.

Our earlier finding that the antibacterial activity is sensitive to pretreatment with trypsin (3) suggested that one or more of the components were proteins. We therefore followed the incorporation of amino acids into hemolymph proteins after an injection of either viable *E. cloacae*, *B. subtilis*, or W-saline. Figure 2 shows that for amino acid incorporation there was a lag similar to the one found for the antibacterial activity (cf. Fig. 1). After the lag period of 12 h incorporation increased until day 4, when the specific activity usually reached its maximum.

Figure 2 shows that the injury created by the

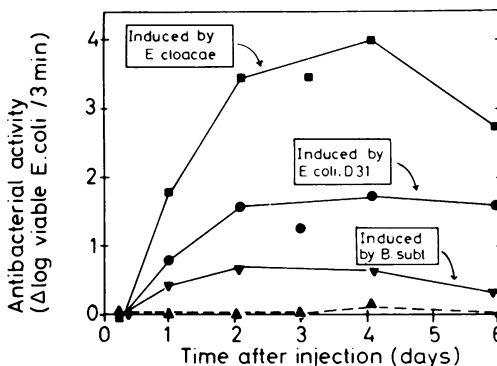


FIG. 1. Capacity of different bacteria to induce antibacterial activity in *S. cynthia*. Pupae were injected with *B. subtilis* strain Bs11 (▼), *E. coli* K-12, strain D31 (●), and *E. cloacae* strain β 12 (■). A control pupa was injected with W-saline (▲). Samples of hemolymph were withdrawn at different times after the injection, and the antibacterial activity was determined as described in Materials and Methods.

injection of W-saline also induced the incorporation of [3 H]leucine, but at a somewhat lower rate than that provoked by the bacteria. The lag period and the rate of protein synthesis are in agreement with the known characteristics for diapausing pupae and different from those of developing animals (32).

With *E. cloacae* the magnitude and duration of the response was rather constant, but with *E. coli* and *B. subtilis* it fluctuated between different pupae. In all cases, the duration was longer than reported for larvae of *Galleria mellonella* (5). Rhythmic variations like the one observed on day 3 have sometimes been recorded, but their cause is not understood.

Induction kinetics after inhibition of ribonucleic acid and protein synthesis. Earlier experiments showed that pretreatment of *S. cynthia* pupae with actinomycin D completely blocked the formation of the antibacterial activity (4). We have now performed a series of experiments where actinomycin D was given at different times during the lag period that follows an infection. The left part of Fig. 3 shows that when actinomycin D was given at zero time or 5 h after the vaccination hardly any

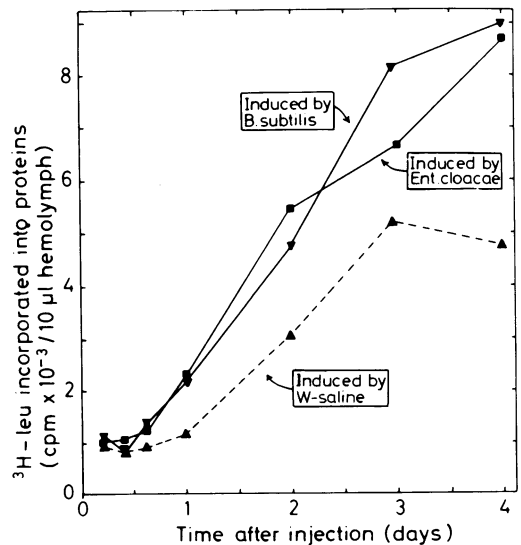


FIG. 2. Incorporation of [3 H]leucine into hemolymph proteins. At zero time *S. cynthia* pupae were injected with 50 μ l containing 50 μ Ci of [3 H]leucine and the bacteria indicated: *E. cloacae* (■); *B. subtilis* (▼); and a sterile salt solution, W-saline (▲). At different times 10- μ l samples of hemolymph were withdrawn and added to 200 μ l of buffer. To this mixture was added 0.8 ml of 10% trichloroacetic acid with 4 mg of cold leucine per ml. The precipitate was collected on a filter, washed with acetone, dried, and counted.

antibacterial activity could be demonstrated in the hemolymph. When actinomycin D was given near the end of the lag period, there was a significant antibacterial activity, which, however, declined very rapidly. In another experiment, actinomycin D was given on day 3, when the antibacterial activity has reached its maximum. The right part of Fig. 3 shows that in this case actinomycin D had no clear effect on the antibacterial activity.

We performed a similar set of experiments with cycloheximide, an inhibitor of protein synthesis in eukaryotic cells. The results in the left part of Fig. 4 show that cycloheximide given during the lag period effectively blocked the

formation of the antibacterial activity. If cycloheximide was given when the immunity had reached its plateau, there was a rapid drop in the antibacterial activity (Fig. 4, right). A comparison of Fig. 3 and 4 shows that cycloheximide was a more potent inhibitor than actinomycin D.

Effects of actinomycin D and cycloheximide on infected and uninfected *Drosophila* were previously examined (B. Rasmuson and H. G. Boman, unpublished data). These studies showed that actinomycin D alone was highly toxic. Neither cycloheximide nor *E. coli* alone affected the flies, but given together the bacteria killed the flies within 24 h.

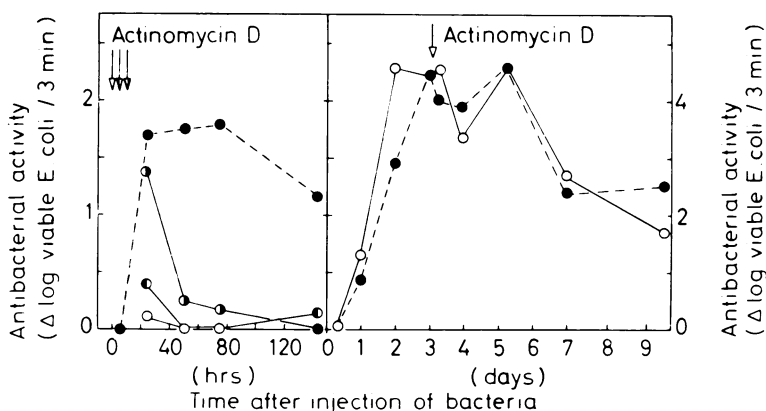


FIG. 3. Effect of actinomycin D (25 μg in 50 μl) on the induction kinetics of the antibacterial activity in pupae of *S. Cynthia*. Samples of hemolymph were withdrawn at different times after injection of the bacteria, and the antibacterial activity was determined. (Left) Pupae injected with actinomycin D, as indicated by arrows at 0 time (○), after 5 h (●), and after 10 h (◐). Control pupa without actinomycin (●). The bacteria used as vaccine were *E. coli* strain D31. (Right) Pupa injected with actinomycin (○) at day 3 (as indicated by arrow). The control pupa (●) received an injection of sterile W-saline at the same time. *E. cloacae* strain B12 was used as vaccine.

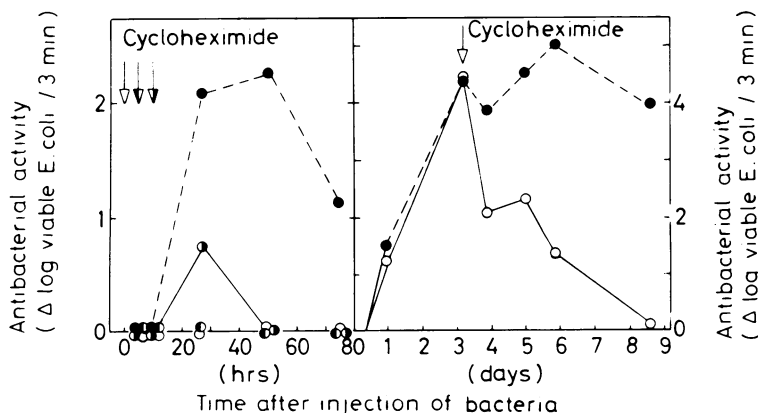


FIG. 4. Effects of cycloheximide (50 μg in 50 μl) on the induction kinetics of the antibacterial activity in pupae of *S. Cynthia*. The experiment was performed as in Fig. 3, using the same bacteria for the left and right portions of the figure and the same symbols for the injections of the inhibitor.

Comparison of proteins induced in *S. cynthia* and *H. cecropia*. We demonstrate in Fig. 2 that injection of diapausing pupae of *S. cynthia* with either viable bacteria or a sterile salt solution induced the incorporation of amino acid into hemolymph proteins. To characterize the protein synthesized, we performed sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole and fractionated hemolymph. At this stage of our investigation a shortage of *S. cynthia* pupae forced us to change to *H. cecropia*, which has the advantage of being two to three times larger. We therefore first compared pupae of *S. cynthia* and *H. cecropia* with respect to the hemolymph proteins labeled after an injection with viable *E. cloacae*. The pupa of *H. cecropia* was simultaneously given [^{14}C]leucine while the pupa of *S. cynthia* was given [^3H]leucine. The hemolymph of both pupae was collected on day 3 and examined by co-electrophoresis. Figure 5 shows that the label was distributed into two larger and four smaller peaks. These bands may represent polypeptide chains rather than proteins. However, for simplicity, they will hereafter be referred to as "proteins" and tentatively labeled as follows: P1 slices 4-7, P2 slices 11-13, P3 slices 14-17, P4 slices 17-20, P5 slices 25-30, P6 slices 30-33, P7 slices 33-35, and P8 slices 35-38. The last

three peaks are often poorly separated, and the evidence for their different identities comes from later experiments. Using the above designation, Fig. 5 illustrates that there are basic similarities in the protein pattern of *H. cecropia* and *S. cynthia*. Minor differences were found in the amounts of proteins P2, P3, P7, and P8. In these two hemolymphs there was a high antibacterial activity in the *H. cecropia* sample but a very poor activity in the *S. cynthia* sample.

Fractionation of hemolymph by ammonium sulfate precipitations. Since the killing of bacteria so far is the simplest known function of the immune systems of *H. cecropia* or *S. cynthia* and since an isolated component may be without any measurable activity, all fractionation attempts that are based on a function will have to be assayed by reconstitutions. We first tried fractionations on different anion and cation exchangers. No combination of any of the chromatographic fractions so obtained restored more than a minor fraction of the killing activity towards *E. coli*. However, using columns of sulfoethyl cellulose, some of the killing activity towards *B. subtilis* was recovered together with the lysozyme (referred to as "crude lysozyme").

More promising results were obtained by consecutive ammonium sulfate precipitations (for

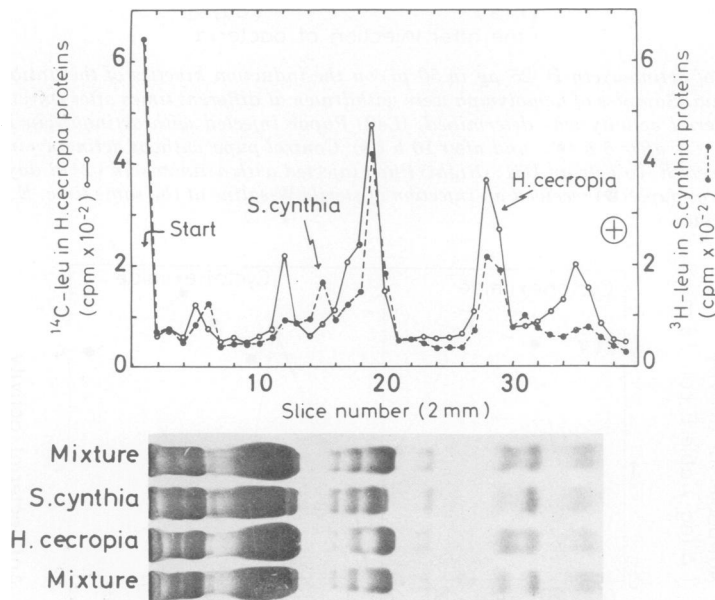


FIG. 5. Co-electrophoresis experiment of hemolymph proteins from *S. cynthia*, labeled with 45 μCi of [^3H]leucine (\bullet), and *H. cecropia*, labeled with 33 μCi of [^{14}C]leucine (\circ). The immunity reaction was induced by viable cells of *E. cloacae*. (Upper) Isotope distribution obtained after cutting the slab into 2-mm slices and burning each slice in a sample oxidizer. (Lower) Photograph of the slab gel electrophoresis showing the two hemolymph samples separate and in mixture. The tracker dye, bromophenol blue, was located in slices 39-40.

details see Material and Methods). By trial and error adjustment of the ammonium sulfate concentrations, we arrived at four fractions designated A, B, C, and D, defined as indicated in Table 1. Each of these fractions showed only a slight killing activity towards *E. coli*. Most of the lysozyme and some of the killing activity against *B. subtilis* were found in fraction D. However, a combination of fractions A, B, and D gave a high antibacterial activity against *E. coli*. When fraction D was replaced by "crude lysozyme" there was some killing activity against *B. subtilis*, but nothing against *E. coli*. The precipitation behavior of phenoloxidase and lysozyme was in close agreement with earlier findings for the same enzymes from larvae of *G. mellonella* (24).

Preliminary experiments showed that in reconstitution experiments the ratio between the components added was of significant importance both for the initial rate of the reaction and for the killing capacity. This is illustrated in Fig. 6, which shows that the initial rate was increased when fraction A+B were constant and the amount of fraction D was increased. The reversed experiment, when D was constant and A+B increased, yielded a higher maximal reaction rate. Therefore, in further experiments we used an excess of A+B.

We also fractionated hemolymph from five European pupae of *S. cynthia* immunized by *E. cloacae*. The killing activity towards *E. coli* was low for both unfractionated and reconstituted hemolymph (fractions A, B, and D combined), and this inefficiency could be traced to fraction D. The active fractions A and B from *S. cynthia* were interchangeable with those from *H. cecropia*. Fractionation and reconstitution experiments were, however, sometime difficult to reproduce to the accuracy of the assay. The

reason for this is not understood, but may in part depend on the "sticky" nature of the proteins and in part on inaccuracy in the fractionation with solid ammonium sulfate.

Are any immune components present in an untreated pupa? Hemolymph from an untreated pupa has no measurable antibacterial activity (3). However, since the killing activity is due to a multicomponent system, one or more components could still be present in the hemolymph of an untreated animal. To obtain evi-

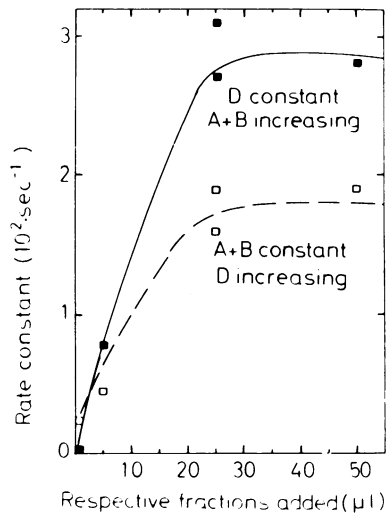


FIG. 6. Dependence of fractions A+B, in combination, and D for obtaining maximum initial rate of the antibacterial reaction. Two experiments are shown: (i) when a mixture of A+B was constant and an increasing amount of D was added (□), and (ii) when D was constant and increasing amounts of a mixture of A+B was added (■). Reaction volume was 0.1 ml, and the concentration of the constant fraction was 0.5%.

TABLE 1. Ammonium sulfate fractionation of hemolymph from pupae of *H. cecropia*^a

Ammonium sulfate fractionation		Labeled proteins								Phenoloxidase (units/ml of sample)	Lysozyme (units)	Killing of:	
Fraction	"Cut" as % of saturation	P1	P2	P3	P4	P5	P6	P7	P8			<i>E. coli</i>	<i>B. subtilis</i>
A	0-38	—	—	—	—	L	—	—	S	8,300	0	0.6	<0.01
B	38-50	S	S	S	S	S	S	—	—	2,300	0	0.7	<0.01
C	50-62	S	S	S	S	—	?	S	—	0	20	0.9	<0.01
D	62-75	—	—	S	L	—	S	S	—	0	61	0.7	0.3
A + B + D		S	S	S	L	L	S	S	S	—	—	3.7	0.7

^a Hemolymph was fractionated by adding solid ammonium sulfate as indicated. The precipitates were washed and dissolved as described in Materials and Methods. The activities for phenoloxidase and lysozyme are given in units, defined in reference 24 and in Materials and Methods. Antibacterial activity is given as the log of the number of viable cells at zero time minus the log of the number of viable cells at 3 min. The concentration of each fraction was 1 and 5% in the killing of *E. coli* and *B. subtilis*, respectively. Evidence for the distribution of the labeled proteins were from Fig. 9 and 11, where (L) indicates a large peak and (S) a small peak. The protein concentration in fractions A through D varied between 8 and 25 mg/ml and was always highest in fraction C.

dence for the possible existence of such a component, hemolymph from an untreated pupa of *H. cecropia* was fractionated by ammonium sulfate according to the same procedure as used for immunized animals (see Table 1). Each of the fractions obtained (designated A_c, B_c, D_c, and C for control) were assayed for their ability to stimulate the complementary fractions from an immunized pupa. The results in Fig. 7 show that A_c could stimulate the antibacterial activity of fractions B and D in combination. The effect was smaller than the one obtained by a similar amount of fraction A from an immunized pupa. No stimulatory effects were detected when fractions B_c or D_c replaced the corresponding fractions from an immunized pupa. The results obtained are consistent with the interpretation that fraction A_c contains at least one preexisting immune component.

Are the same proteins induced by W-saline, *B. subtilis*, and *E. cloacae*? This question was answered by a series of co-electrophoresis experiments with whole and fractionated hemolymph labeled with [¹⁴C]leucine in the case of *E. cloacae* and [³H]leucine in the cases of W-saline or *B. subtilis*. The results from a comparison of the responses towards W-saline and *E. cloacae* are given in Fig. 8 and 9. The former shows the isotope distribution after co-electro-

phoresis of whole hemolymph, below which is a photograph of the stained slab with five samples. The uppermost sample of the slab corresponds to isotope distribution given in the upper part of Fig. 8. The four lower samples show co-electrophoresis experiments with the respective ammonium sulfate fractions for which the isotope distributions are given in Fig. 9. Together these figures show that proteins P1, P4, P5, P7, and P8 were present in reduced amounts or were absent in the injury response caused by the injection of W-saline. On the other hand, the main component in the injury response, protein P3, was only a minor fraction in the response obtained with *E. cloacae*. The injury effects here recorded seem to be much smaller than those reported after perfusion (22) or after larger injuries to the integument (35).

Figures 10 and 11 show a similar comparison of the response obtained after induction with *E. cloacae* and *B. subtilis*. In this case there was a general agreement in the isotope patterns obtained. However, there were lower levels of the antibacterial activity and of proteins P1, P7, and P8 in the response to *B. subtilis*.

The photographs of the slabs (Fig. 8 and 10) and the distribution of isotopes (Figs. 9 and 11) show that the ammonium sulfate fractionations overlap in their protein patterns. Some proteins like P5 and P8 (in fraction A) and proteins P4 and P7 (in fraction D) were clearly separated, but the amounts recovered in the respective fractions fluctuated from one experiment to another. It should also be emphasized that we often obtained labeled precipitates when we added sodium dodecyl sulfate to hemolymph or fractions A through D. Despite various efforts we could not prevent the formation of radioactive precipitates in the starting zone. Disregarding these difficulties, we indicate in Table 1 how the different labeled proteins distributed themselves in typical ammonium sulfate precipitations (cf. Fig. 9 and 11).

The labeled protein pattern obtained as response to an infection with *E. cloacae* was confirmed using [³⁵S]methionine. In this case almost 30% of the injected label was incorporated into proteins, compared to about 3% for [³H]leucine. We assume this difference to be due to the pool size of the respective amino acids, giving differences in isotope dilution. We also performed co-electrophoresis experiments with mixtures of ³⁵S- and ³H-labeled proteins. However, since ³⁵S cannot be handled by the oxidizer and since the slices gave a variable and heavy quenching, no safe conclusions could be drawn from this set of experiments.

Properties of reconstituted systems. As emphasized before (3) the use of viable bacteria

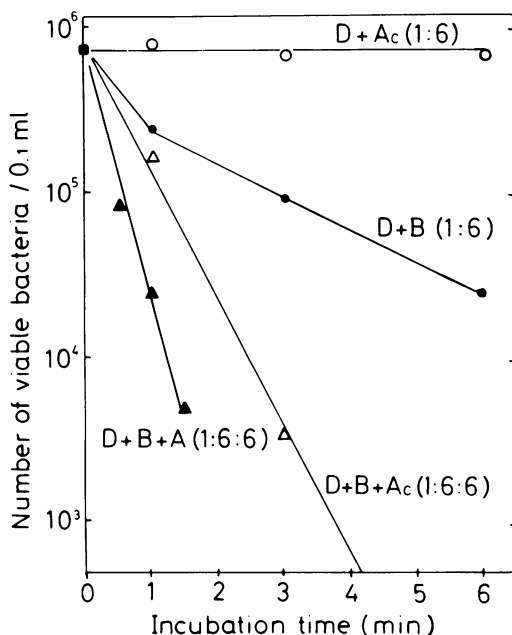


FIG. 7. Stimulation of the antibacterial activity in fractions D+B by fraction A, prepared from either an untreated pupa (A_c) or an immunized pupa (A). Ratios indicate percentage of final reaction volume.

in the assay limits the number of inhibitors that can be tested. However, we have with a reconstituted system investigated phenylthiourea, a known inhibitor of phenoloxidase, as

well as some compounds which were of interest for a comparison with mammalian complement. Table 2 shows that there was neither an effect of phenylthiourea nor from low concentra-

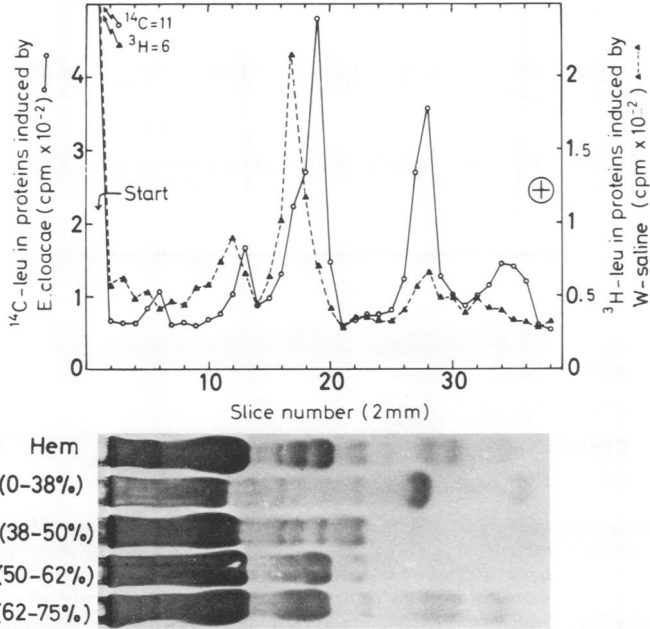


FIG. 8. Co-electrophoresis of hemolymph proteins from two pupae of *H. cecropia*, one injected with 90 μ Ci of [3 H]leucine in 100 μ l of W-saline (\blacktriangle) and the other injected with 33 μ Ci of [14 C]leucine and viable *E. cloacae* in 100 μ l of W-saline (\circ). (Upper) Isotope distribution for a hemolymph mixture; (lower) hemolymph mixture and the four fractions obtained after ammonium sulfate precipitation to the saturation levels indicated. The isotope distribution of the latter samples are given in Fig. 9.

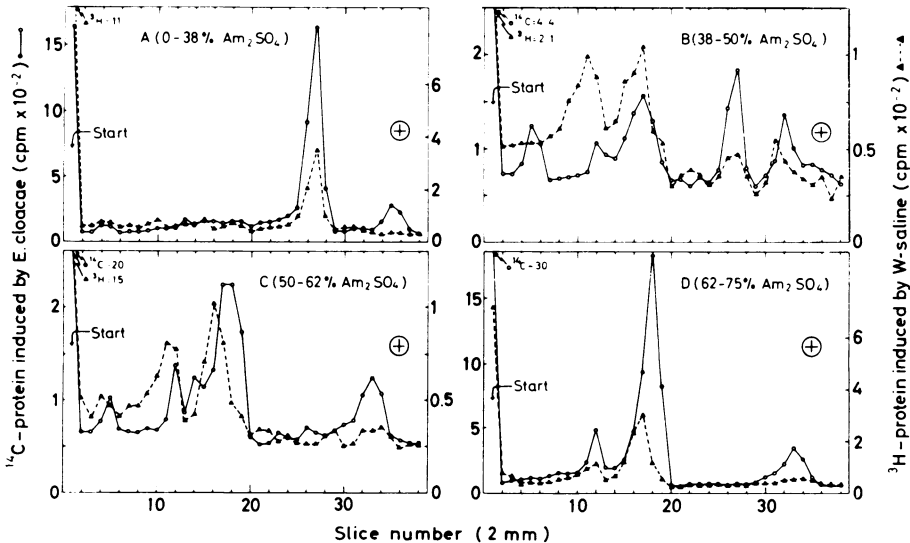


FIG. 9. Isotope distribution obtained after co-electrophoresis of the four ammonium sulfate precipitations shown in the photograph of the slab gel shown in Fig. 8. The pupa injected with [14 C]leucine shows the response to *E. cloacae* (\circ); the one with [3 H]leucine shows the injury effect from an injection as such (\blacktriangle). Note that the scales for the two isotopes are different.

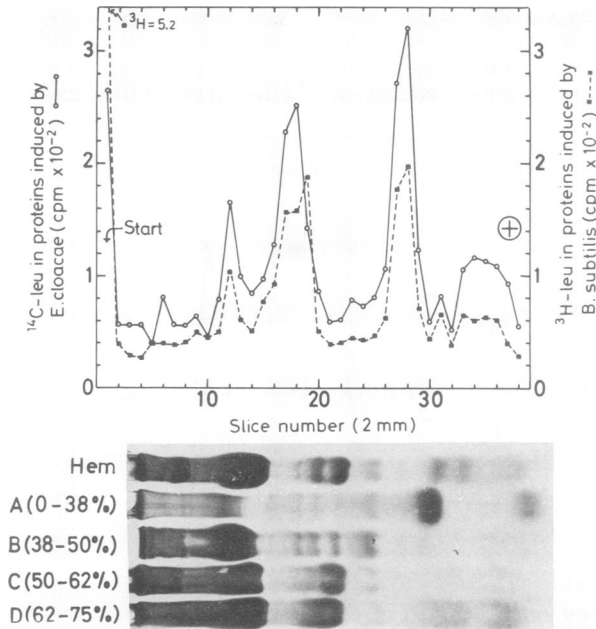


FIG. 10. Co-electrophoresis experiments of hemolymph samples from two pupae of *H. cecropia*, one injected with 90 μ Ci of [3 H]leucine and viable *B. subtilis* in 100 μ l of W-saline (■) and the other injected with 33 μ Ci of [14 C]leucine and viable *E. cloacae* in 100 μ l of W-saline (○). (Upper) Isotope distribution in the sample of mixed hemolymph shown in the lower part of the figure. The rest of the photograph shows the electrophoresis pattern obtained with the four ammonium sulfate precipitations obtained from the hemolymph mixture. The isotope distribution of these samples are given in Fig. 11.

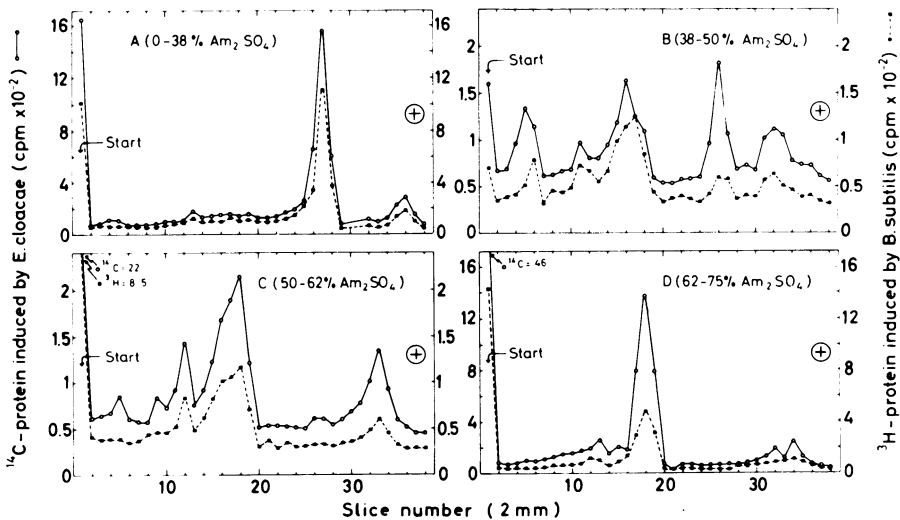


FIG. 11. Isotope distribution obtained after co-electrophoresis of the four ammonium sulfate precipitations shown in Fig. 10. The pupa injected with [14 C]leucine shows the response to *E. cloacae* (○), and the one with [3 H]leucine shows the response to *B. subtilis* (■).

tions of zymosan or the polysaccharide part of the LPS. The best inhibitor was lipid A, in agreement with earlier findings for whole hemolymph (4). There were some effects with the

proteolytic inhibitor Trasylol and with synthetic *N*-lauroyl-D-glucosamine, despite difficulties in preparing a water solution of the latter compound (26). The inhibition obtained by the

higher concentration of core polysaccharide may well be due to contaminating traces of lipid A (2). We also obtained inhibition with several commercial antisera. However, the re-

sults could not easily be interpreted and we therefore now abstain from reporting the details.

To characterize the system further, we compared the killing of *E. coli* in the presence and absence of 20% sucrose. As control we performed the same experiments with *B. subtilis*, which is known to have the same type of mu-rein skeleton as *E. coli* (29). The results obtained in Fig. 12 (left) suggest that the osmotic pressure inside *E. coli* contributed to the killing rate. The killing of *B. subtilis* was facilitated by sucrose. Compared to whole hemolymph, the reconstituted system had only a low activity against *B. subtilis*. A possible explanation is the fact that a main part of the lysozyme remained in the supernatant after the last precipitation with ammonium sulfate.

TABLE 2. Inhibition of reconstituted antibacterial activity^a

Addition	Relative killing of D31 after:	
	1 min	5 min
1-Phenyl-2-thiourea (10 ⁻³ M)	1.0	0.7
Zymosan (10 µg/ml)	1.0	0.6
Zymosan (100 µg/ml)	0.3	0.1
Trasyolol (100 µg/ml)	0.4	0.07
Polysaccharide from D21 LPS (10 µg/ml)	0.8	0.8
Polysaccharide from D21 LPS (50 µg/ml)	0.4	0.06
Lipid A from D21 LPS (10 µg/ml)	0.2	0.002
<i>N</i> -lauroyl-D-glucosamine (100 µg/ml)	0.5	0.08

^a All additions were preincubated with fractions A+B+D (4:4:1) for 90 min at room temperature before the addition of test bacteria. *N*-lauroyl-D-glucosamine was solubilized by ultrasound (26). Relative killing was defined as the ratio between the number of viable bacteria in the control and the number of viable bacteria in the treated sample using standard assay conditions.

DISCUSSION

Induction of the antibacterial activity. In our studies of the induction kinetics (Fig. 1-4) we always found that a lag period of about 10 to 12 h was required between the injection of bacteria and the first appearance of a measurable antibacterial activity or a rise in the amino acid incorporation. If either actinomycin D or cycloheximide was added during the lag period, the antibacterial activity was drastically inhibited

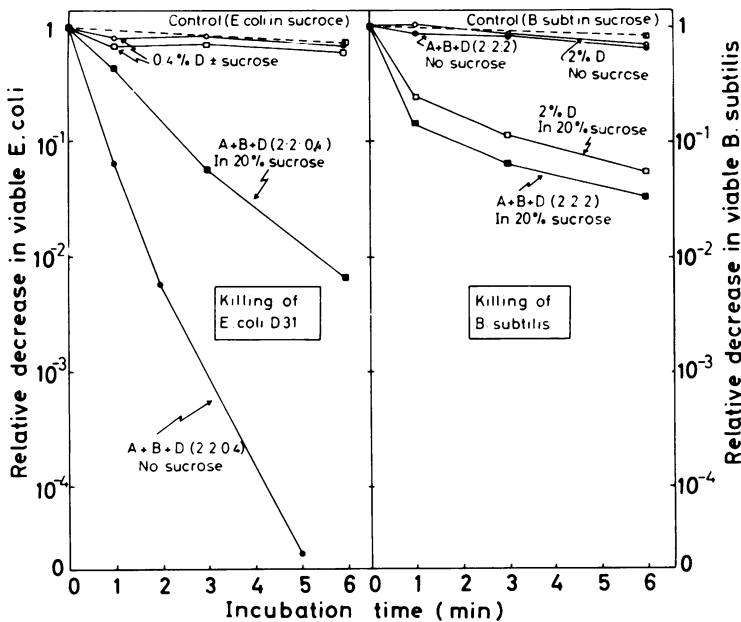


FIG. 12. Effects of sucrose on reconstituted antibacterial activity towards *E. coli* strain D31 (left) and *B. subtilis* (right). Symbols: Bacteria in 20% sucrose (squares); without sucrose (circles). With *E. coli*, A+B+D was used concentrations of 2,2, and 0.4%, respectively; with *B. subtilis* the concentrations were 2% for all fractions.

(left parts of Fig. 3-4). These findings suggest that ribonucleic acid and protein synthesis are required for the expression of the antibacterial activity.

When cycloheximide was injected into a pupa in which the maximum antibacterial activity was expressed (Fig. 4, right), there was a rapid drop in the antibacterial activity. This finding implies a rapid turnover of one or more of the proteins involved in the antibacterial activity. Such a fast inactivation could have a biological function in protecting the animal against self-destruction.

If actinomycin D was added at the time when the antibacterial activity reached its maximum (Fig. 3, right), no inhibition was observed. Since one or more proteins are turning over, this experiment indirectly suggests that their messenger ribonucleic acid was stable. This interpretation is in accordance with results obtained with other systems for selective protein synthesis in eukaryotic cells (15, 28).

Products synthesized during induction. Our results show that we have a simultaneous induction of the killing activity towards *E. coli* and the selective synthesis of at least eight different polypeptides in the hemolymph. It is tempting to believe that these two phenomena are linked. If so, reconstitution experiments (Table 1) imply that all of the components could be needed and that the presence of P7 and P8 may be correlated with a high antibacterial activity. We have also considered the possibility that P3 is a precursor of P4 (see Fig. 8 and 9), and that the injury effect obtained by the injection of a sterile salt solution represents intermediary forms of proteins, which by further processing are converted into molecules with antibacterial activity. However, these suggestions are based only on correlations and conclusive experiments will have to be performed with purified components. Finally, it should be emphasized that the diapause presumably is a necessary condition for obtaining the selective synthesis of only the immunoproteins which we have observed here.

Irrespective of the limitation discussed above, our present data would permit the following conclusions. (i) Induction by a gram-negative and a gram-positive bacteria (*E. cloacae* and *B. subtilis*, respectively) induced almost identical polypeptide patterns in spite of the evidence for two independent killing mechanisms against these two types of bacteria (3, 4). (ii) The fact that the response is the same makes it very unlikely that the labeled polypeptides should be of bacterial origin. (iii) There are clear qualitative as well as quantitative differences between the five components la-

beled as the result of an injury (injection of a sterile salt solution) and the eight components obtained simultaneously with an effective antibacterial reaction. (iv) Since the outer surface layers of *E. cloacae* and *B. subtilis* are very different and there is no LPS in *B. subtilis*, it is highly unlikely that any surface components, especially any part of LPS (6), function as *specific* inducers for the immune response. (v) Normal hemolymph from an untreated pupa of *H. cecropia* contained at least one immune component in fraction A. (vi) Conclusions (i) and (iv) make biological sense if the natural function of the immune system would be to protect the animal against a general sepsis, e.g., as the result of histolysis of the gut during metamorphosis.

Nature of the antibacterial reaction. Insects can respond to infections as well as to injury with both cellular and humoral reactions (3, 5, 7, 12, 19, 25, 34, 35). For the latter system several authors have emphasized the importance of phenoloxidase (12, 19) and lysozyme (20, 23), especially for the defense against fungi and bacteria, respectively. Since phenylthiourea is a powerful inhibitor of phenoloxidase (30) and since our antibacterial reaction was unaffected by this agent (Table 2), the enzyme cannot directly contribute to the killing reaction studied here.

It is more difficult to evaluate the role of lysozyme. This enzyme may well be a main agent for the killing of gram-positive bacteria, but reconstitution experiments (Table 1; Fig. 12) imply that other factors are involved. In the killing of *E. coli* "crude lysozyme" could not substitute for fraction D. Moreover, preliminary findings indicate that lysozyme can be separated from most of the labeled proteins in fraction D (T. Rasmuson, unpublished data). Therefore, at present we can not fully evaluate the role of the lysozyme in either of the antibacterial reactions studied.

Day et al. (8) first emphasized the complement-like nature of the humoral defense system in invertebrates, but some hesitations were expressed in a later report (14). In our previous study (3) the single-hit kinetics, the multicomponent nature, and inhibition by LPS provided evidence for a superficial resemblance to the complement system. This claim is now substantiated by the following results. (i) The induction of the antibacterial activity is paralleled by selective synthesis of eight polypeptide chains, which by correlation are linked to the killing activity. Kolb and Müller-Eberhard (16) have recently shown that in human complement the membrane-attacking complex is composed of seven polypeptide chains. (ii) The antibacterial

activity was inhibited by lipid A but not by the polysaccharide part of the LPS molecule. Lipid A is known to have an anticomplementary effect even when the intact LPS was inactive (17). (iii) The osmotic pressure inside the bacteria contributed to the killing of *E. coli* in the same way as found for human complement (10, 21, 31). (iv) Compared to *E. coli*, *B. subtilis* was considerably more resistant to the antibacterial activity in insects. The same difference was earlier found for human complement (21).

From the above properties we conclude that the antibacterial activity in pupae of giant silkworms is of a complement-like nature. However, a comparison with mammalian complement reveals the following differences. (i) The insect system is inducible, whereas mammalian systems normally seem to be constitutive. (ii) Cycloheximide experiments indicate a rapid turnover of the insect system, whereas we do not know of any report of a fast turnover of complement in mammals. (iii) When measured with the same test organism, the insect system seems to be 100 to 1,000 times more effective in the killing of *E. coli*. These differences can be explained if complement occurred before immunoglobulins during the evolution of immunity. Then the lack of immunoglobulins in insects may have increased the selection pressure on their complement-like systems.

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LITERATURE CITED

- Bernheimer, A. W., E. Caspari, and A. D. Kaiser. 1952. Studies on antibody formation in caterpillars. *J. Exp. Zool.* 119:23-35.
- Boman, H. G., and D. A. Monner. 1975. Characterization of lipopolysaccharides from *Escherichia coli* K-12 mutants. *J. Bacteriol.* 121:455-464.
- Boman, H. G., I. Nilsson-Faye, K. Paul, and T. Rasmuson, Jr. 1974. Insect immunity. I. Characteristics of an inducible cell-free antibacterial reaction in hemolymph of *Samia cynthia* pupae. *Infect. Immun.* 10:136-145.
- Boman, H. G., I. Nilsson-Faye, and T. Rasmuson, Jr. 1974. Why is insect immunity interesting?, p. 103-114. *In* Dietmar Richter (ed.), Lipmann symposium: energy, biosynthesis and regulation in molecular biology. Walter de Gruyter Verlag, Berlin.
- Chadwick, J. S. 1968. Some aspects of immune responses in insects. *In Vitro* 3:120-128.
- Chadwick, J. S. 1971. An assessment of the ability of individual moieties of *Pseudomonas aeruginosa* endotoxin to induce immunity in larvae of *Galleria mellonella*. *J. Invertebr. Pathol.* 17:299-300.
- Cherbas, L. 1973. The induction of an injury reaction in cultured haemocytes from Saturniid pupae. *J. Insect Physiol.* 19:2011-2023.
- Day, N. K. B., H. Gewurz, R. Johannsen, J. Finstad, and R. A. Good. 1970. Complement and complement-like activity in lower vertebrates and invertebrates. *J. Exp. Med.* 132:941-950.
- di Jeso, F. 1968. Ammonium sulfate concentration conversion nomograph for 0°. *J. Biol. Chem.* 243:2022-2023.
- Donaldson, D. M., R. R. Roberts, H. S. Larsen, and J. G. Tew. 1974. Interrelationship between serum beta-lysin, lysozyme, and the antibody-complement system in killing *Escherichia coli*. *Infect. Immun.* 10:657-666.
- Götz, P. 1973. Immunreaktionen bei Insekten. *Naturwiss. Rundsch.* 26:367-375.
- Götz, P., and A. Vey. 1974. Humoral encapsulation in Diptera (Insecta): defence reactions of *Chironomus* larvae against fungi. *Parasitology* 68:193-205.
- Hildeman, W. H. 1974. Some new concepts in immunological phylogeny. *Nature (London)* 250:116-120.
- Johannsen, R., R. S. Anderson, R. A. Good, and N. K. Day. 1973. A comparative study of the bactericidal activity of horseshoe crab (*Limulus polyphemus*) hemolymph and vertebrate serum. *J. Invertebr. Pathol.* 22:372-376.
- Kafatos, F. C. 1972. mRNA stability and cellular differentiation. *Acta Endocrin.* 168(Suppl.):319-345.
- Kolb, W. P., and H. J. Müller-Eberhard. 1975. The membrane attack mechanism of complement. Isolation and subunit composition of the C5b-9 complex. *J. Exp. Med.* 141:724-735.
- Lüderitz, O., C. Galanos, V. Lehmann, M. Nurminen, E. T. Rietschel, G. Rosenfelder, M. Simon, and O. Westphal. 1973. Lipid A: chemical structure and biological activity. *J. Infect. Dis.* 128(Suppl.):S17-S29.
- Maizel, J. V., Jr. 1969. Acrylamide gel electrophoresis of proteins and nucleic acids, p. 334-362. *In* K. Habel and N. P. Salzman (ed.), *Fundamental techniques in virology*. Academic Press Inc., New York.
- Messner, B. 1974. Einige Aspekte der Abwehrmechanismen bei wirbellosen Tieren. *Biol. Rundsch.* 12:272-275.
- Mohrig, W., and B. Messner. 1968. Immunreaktionen bei Insekten. I. Lysozym als grundlegender antibakterieller Faktor im humoralen Abwehrmechanismus der Insekten. *Biol. Rundsch.* 8:439-470.
- Müller-Eberhard, H. J. 1968. Chemistry and reaction mechanisms of complement. *Adv. Immunol.* 8:1-80.
- Patel, N. G., and H. A. Schneiderman. 1969. The effects of perfusion on the synthesis and release of blood proteins by diapausing pupae of the Cecropia silkworm. *J. Insect. Physiol.* 15:643-660.
- Powning, R. F., and W. J. Davidson. 1973. Studies on insect bacteriolytic enzymes. I. Lysozyme in haemolymph of *Galleria mellonella* and *Bombyx mori*. *Comp. Biochem. Physiol.* B 45:669-686.
- Pye, A. E. 1974. Microbial activation of prophenoloxidase from immune insect larvae. *Nature (London)* 251:610-613.
- Ratcliffe, N. A., and A. F. Rowley. 1974. *In vitro* phagocytosis of bacteria by insect blood cells. *Nature (London)* 252:391-392.
- Rosenstreich, D. L., J. Asselineau, S. E. Mergenhagen, and A. Nowotny. 1974. A synthetic glycolipid with B-cell mitogenic activity. *J. Exp. Med.* 140:1404-1409.
- Ruh, M. F., J. H. Willis, and M. P. Hollowell. 1972. Blood protein synthesis in pupae of the silkworm, *Hyalophora cecropia*. *J. Insect Physiol.* 18:151-160.
- Schimke, R. T., R. E. Rhoads, R. Palacios, and D. Sullivan. 1973. Ovalbumin mRNA, complementary DNA and hormone regulation in chick oviduct. *Acta Endocrinol.* 180(Suppl.):357-379.
- Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* 36:407-477.
- Schneiderman, H. A. 1967. Insect surgery, p. 753-766. *In* F. H. Wilt and N. K. Wessels (ed.), *Methods in developmental biology*. Thomas Y. Crowell Co., New York.

31. Schultz, D. R. 1971. The complement system. S. Karger, Basel.
32. Telfer, W. H., and C. M. Williams. 1960. The effects of diapause, development, and injury on the incorporation of radioactive glycine into the blood proteins of the *Cecropia* silkworm. *J. Insect Physiol.* 5:61-72.
33. Weevers, R. de G. 1966. A lepidopteran saline: effects of inorganic cation concentrations on sensory, reflex and motor responses in a herbivorous insect. *J. Exp. Biol.* 44:163-175.
34. Whitcomb, R. F., M. Shapiro, and R. R. Granados. 1974. Insect defence mechanisms against microorganisms and parasitoids, p. 447-535. *In* M. Rockstein (ed.), *The physiology of insecta*, vol. V, 2nd ed. Academic Press Inc., New York.
35. Wyatt, G. R. 1962. Biochemistry of diapause, development, and injury in silkworm pupae, p. 23-41. *In* *Insect physiology*. Proceeding of the 23rd Biology Colloquium. Oregon State University Press, Corvallis.