# IMMUNOLOGICAL STUDIES OF INSECT METAMORPHOSIS

## I. Qualitative and Quantitative Description of the Blood Antigens of the Cecropia Silkworm

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The net result of the complete metamorphosis of an insect is the formation of a highly complex and specialized adult from an equally complex and specialized larva. The processes here include the destruction of certain larval tissues, the rejuvenation and remolding of others into adult tissues, and the direct production of adult tissues from imaginal discs. This reshaping of the organism is presumably a manifestation of more fundamental alterations involving the enzymes, structural proteins, and other macromolecules of the insect. A knowledge of such changes is therefore important to an understanding of metamorphosis. We have studied these changes by immunological techniques, in the course of which the macromolecules were identified by their antigenic properties. The present report is concerned with changes in the blood antigens of the *Cecropia* silkworm during its metamorphosis.

Immunological techniques have often been used in the study of embryonic development, as is evident in the reviews of Cooper (4) and Ten Cate and Van Doorenmaalen (19). We have been able to find only a single report, however, of an immunological investigation of insect metamorphosis; namely, Avrech and Heronimus' briefly described study of the honeybee (1). Though several reports on the embryonic development of vertebrates have been concerned specifically with blood antigens (14, 4), we are unaware of any previous investigation of the blood antigens of invertebrates during either embryonic or postembryonic development.

Little is known about the chemical identity or the functional significance of antigens whose appearance and disappearance during development have been reported. Several exceptions include the studies of the lens protein (19) and of hemoglobin (6). The blood antigens of the *Cecropia* silkworm were chosen as the subject of the present study with the hope that they would eventually submit to physiological analysis and thereby become recognizable as functionally significant entities, rather than simply as "blood antigens."

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*Cecropia* blood, like most biological fluids, is a complex mixture of antigens. Consequently, when injected into a rabbit, it invariably evokes the production of antibodies which react with at least several different blood antigens. In the study of such complex antigen-antibody systems, the usual precipitin techniques are of limited use. However, Oudin (11) has developed a method for resolving the total reaction into its component antigen-antibody reactions. This technique has already been applied to some extent by Cooper (5), Telfer and Williams (17, 18), and Spar (16) to the study of antigens in development.

When an antigen is permitted to diffuse from a solution into an agar gel containing a homologous antiserum, it reacts with antibody to form a band of precipitate which appears initially at the interface between the antigen solution and the antiserum-agar and then advances slowly into the antiserum-agar. As described by Oudin (11) and by Munoz and Becker (9), reactions of this type can be studied conveniently in small-bore glass tubes which have been sealed at one end. The antiserum is mixed with melted agar and the mixture is pipetted into the tube. After the antiserum-agar has cooled and solidified, it is overlayered by an antigen solution. The leading edge of the band of precipitate is sharply defined and, in the case of rabbit antiserum, represents the farthest point that the antigen has reached in its diffusion into the antiserum-agar. Oudin demonstrated that the distance travelled by a band of precipitate, measured as the distance from the surface of the agar to the leading edge of the band, varies linearly with the square root of time, so that

$$h = k\sqrt{t} \tag{1}$$

in which $h$ is the distance travelled by the band, $t$ is the time elapsed since the antigen solution was added to the antiserum-agar, and $k$ is a coefficient which describes the rate of advance of the particular band of precipitate in question.

In view of the linear relation between $h$ and $\sqrt{t}$, Oudin concluded that the antigen undergoes free diffusion through the antiserum-agar, the precipitation of antigen by antibody having, within certain limits, no detectable effect on the diffusion of that antigen which has not yet reached the leading edge of the band of precipitate. Therefore, as would be anticipated from physical laws governing diffusion, the rate of advance of the leading edge of a band of precipitate, as expressed by the coefficient $k$, varies with the initial concentration of the antigen in the overlying solution (12) and with the diffusion coefficient of the antigen (2). $k$ is also influenced by the concentration of antibody in the antiserum-agar (12) because the antibody acts, in effect, as a barrier to the diffusion of antigen at the leading edge of the band of precipitate. Assuming constancy of all physical conditions and other non-specific factors, these relationships can be simply expressed as follows:—

$$k = h/\sqrt{t} = f(G, A, D) \tag{2}$$

in which $G$ is the antigen concentration, $A$ is the antibody concentration, and $D$ is the diffusion coefficient of the antigen.

In equation (2), $h$ and $t$ are measurable, and $A$ can be held constant experimentally. When $D$ is constant, as is presumably the case when one considers a single species of

antigen, $k$ becomes a measure of the concentration of antigen. For this reason, the antiserum-agar technique appears to be adaptable to the measurement of antigen concentrations, a possibility which was initially suggested by Oudin (12).

In a reaction more complex than a single antigen-antibody system, as many bands are formed as there are antigen-antibody precipitates in the antiserum-agar. Different bands normally vary considerably in their density (opacity) and in their rates of advance through the antiserum-agar.

Qualitative comparisons of the antigenic content of different solutions can be made by counting the number of bands appearing when each solution is added to a tube of the same type of antiserum-agar. However, this procedure does not eliminate the possibility that two bands may advance through the antiserum-agar at identical rates and thus appear as a single band of precipitate, or that a band of very low density may advance at a slower rate than a band of high density and therefore be obscured (11). Furthermore, if each of two solutions contains an antigen which the other does not contain, these solutions could cause the appearance of the same number of bands of precipitate despite their qualitative difference. Therefore, adequate qualitative comparisons of different antigen solutions have to include absorption tests or mutual dilution tests (described below) which permit one to locate and identify the corresponding bands of precipitate in different tubes.

## *Materials and Methods*

*1. The Cecropia Silkworm.*—The life cycle of our experimental animal, *Platysamia cecropia*, begins with a 2-week period of embryonic development. After hatching from the egg, the caterpillar goes through four larval moults and attains a weight of 10 to 15 gm. Three weeks after the last larval moult, the mature caterpillar stops feeding and spins a cocoon, within which it transforms into a pupa and begins a period of diapause. The diapausing pupa is the overwintering stage of the insect. After a suitable period of chilling, the pupa terminates diapause and develops into an adult moth (20).

*2. Preparation of Antigen Solutions.*—Saline extracts of entire silkworms were used for the immunization of rabbits. The *in vitro* tests were performed with antigen solutions consisting of cell-free blood samples from animals at particular morphological stages of metamorphosis. Since both *Cecropia* extracts and blood normally blacken on exposure to air, precautions were taken to prevent this reaction. Blackening is presumably due to the presence of tyrosinase and its substrate, and it has been demonstrated that tyrosinase-catalyzed oxidations alter the physiological properties of certain proteins (15).

(*a*) *Preparation of Extracts.*—Animals at particular stages of metamorphosis were extracted in the presence of cyanide in order to prevent blackening. The extractions were made in a solution containing 0.015 M KCN and 0.135 M NaCl, the pH being adjusted to 7.3 by the addition of 0.15 M $KH_2PO_4$. Whole animals from which the midgut and its contents had been removed were added to approximately twice their weight of extraction medium. The mixture was homogenized in a Waring blendor and centrifuged at 10,000 *g*. The supernatant liquid was dialyzed at 1.5°C. against six changes of the extraction medium and then centrifuged again at 10,000 *g*. The ex-

tract was finally dialyzed against 0.15 M NaCl to eliminate the cyanide in preparation for injection into rabbits. The final extract, which did not blacken on exposure to air, was stored at −20°C., and thawed just prior to injection.

(*b*) *Collection and Treatment of Blood.*—Blood was collected under mineral oil in order to prevent its contact with the air, centrifuged to remove the cells, and stored at −20°C. Since larval blood, unlike that of pupae and adults, undergoes rapid gelation and cannot be handled in pipettes, extra precaution had to be taken in its collection. The larvae were chilled at 1.5°C. for 30 to 45 minutes and then bled at the low temperature. Clotting occurs slowly during the subsequent centrifugation but, under these conditions, a considerable volume of serum is unclotted and remains so indefinitely.

*3. Preparation of Antisera.*—Nine rabbit antisera were prepared. Three rabbits were injected with saline extracts of mature fifth instar larvae, three with extracts of diapausing pupae, and three with extracts of adult moths. Two ml. of extract were used for each injection. The schedule of injections and preparation of the antisera were performed according to the presensitizing technique described by Leone (8). The resulting antisera, like the antigen solutions, were stored at −20°C., a treatment which had no demonstrable effect on either the density or rate of advance of any band of precipitate observed in antiserum-agar tests.

*4. Antiserum-Agar Tests.*—Glass tubes 6 to 7 cm. in length and 3 mm. in inside diameter were lined with agar by being filled and emptied with a 1.5 per cent agar solution, and dried in a desiccator. The antiserum-agar was prepared by mixing the antiserum with melted agar, sodium merthiolate, and 0.15 M NaCl at 45°C. to yield final concentrations of 33 per cent rabbit antiserum, 0.25 per cent washed agar, and 1:10,000 parts of sodium merthiolate. The merthiolate and agar stock solutions contained 0.15 M NaCl. The bottom 2 to 3 cm. of the tubes were filled with antiserum-agar and the tubes were placed at room temperature until the agar had solidified. The antigen solution under examination was then layered over the antiserum-agar and covered, in turn, by a layer of mineral oil. The tubes were stored at 25°C.

The distance travelled by each band of precipitate within the antiserum-agar was recorded at approximately 20, 48, and 96 to 120 hours after the antigen solution was brought into contact with the antiserum-agar. The rate of advance of the band, $k$ (Equation 1), was calculated for each of the three measurements. If $k$ was constant within the limits of error of the measurements, the $k$ value of the final measurement was accepted for comparison with other tubes. Variations in $k$ beyond the limits of accuracy of the measurements were rarely observed and then only in tubes in which melanin formation or bacterial growth had occurred. Table I records a typical example of the measurements and calculations.

*5. Absorption Tests.*—For the purpose of aiding the qualitative comparison between different antigen solutions, a series of absorption tests was performed. 0.4 ml. of an appropriate dilution of antigen solution was added to 0.3 ml. of antiserum. After standing at 1 to 2°C. overnight, the mixture was centrifuged and the supernatant decanted from the antigen-antibody precipitate. The supernatant was then heated to 45°C. and mixed with enough of a 1.5 per cent solution of agar and a 1:2,000 solution of sodium merthiolate to yield a final mixture containing 0.25 per cent agar and 33 per cent antiserum. The "absorbed antiserum-agar," thus produced, was then pi-

petted into glass tubes, allowed to solidify, and overlayered by appropriate antigen solutions in order to test for the presence of residual antibodies.

*6. Identification of Bands of Precipitate.*—Since each blood sample invariably produced several bands of precipitate when layered over antiserum-agar, it was frequently necessary to use special methods for identifying the bands. Preliminary identifications could often be made by noting the relative densities of the several bands in a tube. As Oudin (11) demonstrated, the density of a band does not vary significantly with antigen concentration or with time, but does vary with the concentration of antibody in the antiserum-agar. Therefore, when constant antibody concentration is assured, a particular antigen always produces a band of characteristic density.

TABLE I

*The Measurement and Calculation of the k Values of Three Bands of Precipitate Which Appeared in the Antiserum-Agar Reaction of Pupal Blood with Adult Antiserum a*

| Band | Time | Distance travelled | $k$ |
|---|---|---|---|
| | *hrs.* | *cm.* | *cm. hr.*$^{-1/2}$ |
| 1 | 20 | 0.58 | 0.130 |
| 1 | 48 | 0.93 | 0.134 |
| 1 | 119 | 1.44 | 0.132 |
| 2 | 20 | 0.49 | 0.110 |
| 2 | 48 | 0.79 | 0.114 |
| 2 | 119 | 1.23 | 0.113 |
| 3 | 20 | 0.40 | 0.090 |
| 3 | 48 | 0.64 | 0.092 |
| 3 | 119 | 1.00 | 0.092 |

A more precise method for identifying bands of precipitate utilizes the mutual dilution of the two antigen solutions as suggested by Oudin (11). In such studies, a series of tubes was prepared, each containing antiserum-agar of the same composition, and to each was added one of the following solutions: antigen solution "A," antigen solution "B," and a series of mixtures of antigen solutions "A" and "B," the proportions being varied to yield a graded series ranging from pure solution "A," through an equal mixture of the two, to pure solution "B." In this manner, a gradual transition was obtained between the $k$ values of the corresponding bands in the two solutions under comparison, and any two bands which showed such a transition on mutual dilution were attributed to the same antigen.

In an analogous manner, the bands of precipitate produced by different antisera in reactions with the same antigen solution were identified. Aliquots of the antigen solution were layered over a series of antiserum-agar columns, one containing antiserum "A," another containing antiserum "B," and the others containing a series of intermediate mixtures of the two. In all tubes, the *total* antiserum concentration in the agar was 33 per cent by volume. A band of precipitate whose $k$ value showed a

gradual shift from the antiserum "A" tube to the antiserum "B" tube was then interpreted as being due to the precipitation of the same antigen by both antisera.

## RESULTS

*1. Description of the Bands of Precipitate and Their Corresponding Antigens.* —The maximum number of bands which we observed in any one reaction tube was nine (Table II). We therefore conclude that the cell-free blood of *Cecropia* contains at least nine substances which are antigenic to rabbits. When *Cecropia* blood was layered over normal rabbit serum, no bands of precipitate appeared.

TABLE II

*The Number of Bands of Precipitate Appearing in Antiserum-Agar Reactions between Nine Rabbit Antisera and Blood Obtained from Animals at Three Stages of Metamorphosis*

| Antiserum | No. of bands appearing when antiserum-agar is overlayered by | | |
|---|---|---|---|
| | Larval blood | Pupal blood | Adult blood |
| Larval *a* | 3 | 3 | 3 |
| Larval *b* | 5 | 4 | 6 |
| Larval *c* | 5 | 5 | 6 |
| Pupal *a* | 8 | 9 | 7 |
| Pupal *b* | 4 | 5 | 5 |
| Pupal *c* | 7 | 8 | 7 |
| Adult *a* | 4 | 6 | 4 |
| Adult *b* | 4 | 5 | 4 |
| Adult *c* | 4 | 4 | 3 |

Several tests were performed on pupal blood in order to ascertain the nature of the blood antigens. The samples of blood on which the tests had been performed were reacted in individual antiserum-agar tubes with adult antisera *a* and *c*, pupal antiserum *b*, and larval antiserum *c*. All the antigens reacting with these antisera were non-dialyzable, labile at 75°C., and precipitable with 75 per cent saturated ammonium sulfate. Presumably therefore, all these antigens are proteins.

Several antisera precipitated an antigen containing a yellow chromatic group. When this antigen-antibody precipitate was washed three times with cold saline, dissolved in dilute acetic acid, and analyzed spectrophotometrically (7), two absorption peaks were evident in the visual spectrum: one at 468 mμ and a slightly lesser one at 480 mμ. Chefurka and Williams (3) have demonstrated the presence in *Cecropia* blood of a globulin fraction containing α-carotene and taraxanthin. We therefore conclude that at least one of the antigens dealt with in this study is a carotenoid protein.

An antiserum such as pupal antiserum *b* which, as recorded in Table II,

produces nine bands of precipitate when reacted with pupal blood, is too complicated for use in studies such as those described below. Serious difficulties are encountered in identifying the bands of precipitate when a large number of bands occur in a single tube. Most of our studies were therefore performed with simpler antisera. The optimal antiserum is one which produces, not only a small number of bands, but also bands that vary considerably in density, since, as previously noted, the density of a band is an aid in its identification. Three antisera possessing these properties were selected as the principal reagents used in the work that follows.

*Adult Antiserum a* showed a maximum of six bands when reacted with pupal blood. Three were ignored because of their low density, but the remaining three were of sufficiently high and dissimilar densities to facilitate their identification. We have numbered the latter three according to the rapidity of their advance through the antiserum-agar. The fastest band had an intermediate density; it will be designated as band 1 and the corresponding antigen as antigen 1. Band 2 was characterized by an intermediate rate of advance and the lowest density of the three. Band 3 was the slowest of the three and had the highest density. Its leading edge coincided with the limit of diffusion of the yellow color of the blood and, since this antiserum precipitated an antigen with absorption maxima at 468 and 480 m$\mu$, band 3 is undoubtedly caused by the precipitation of a carotenoid protein.

*Adult Antiserum c* reacted with pupal blood to form the same bands 1, 2, and 3 as did adult antiserum *a*. This fact was shown by the mutual dilution of adult antisera *a* and *c* (Fig. 1). The gradual shift of the three bands in question from their *k* values in adult antiserum *a* to their *k* values in adult antiserum *c* indicates that both antisera form the same three bands. This, in turn, means that they precipitate the same three antigens. Band 2 often was not visible in the reaction between pupal blood and adult antiserum *c*, probably because band 1 was denser with this antiserum than with adult antiserum *a* and thus more effectively obscured band 2.

*Larval antiserum c* reacted with pupal blood to form five bands of precipitate. Two were of low opacity and were not studied, while three were conspicuous and easily distinguished from each other. Band 4 possessed the fastest rate of advance and the lowest density. Band 5 was distinguished by an intermediate rate of advance and an intermediate density; its rate of advance often closely paralleled that of band 4. Band 6 had the slowest rate of advance and the highest density. The possibility existed that either band 1, 2, or 3 could have been identical with band 4, 5, or 6. However, the mutual dilution of adult antiserum *a* with larval antiserum *c* disproved this possibility and gave assurance that six different bands and their corresponding antigens were being dealt with. As recorded in Fig. 1, no gradual shift in the rate of advance of a band was observed from the tube containing adult antiserum *a*, through the intermediate mixtures, to the tube containing larval antiserum *c*. In fact, all six bands appeared simultaneously in the tube containing equal amounts of the two antisera.

In summary, we find that the antigens of cell-free *Cecropia* blood comprise a rather complex system. By the antiserum-agar technique, a minimum of

nine blood antigens was demonstrated, all of which had the properties of proteins in being non-dialyzable, labile at 75°C., and salted out by 75 per cent saturated ammonium sulfate. One antigen was identified as a carotenoid protein. On the basis of the fact that Oudin (10) observed nine bands of precipitate in an antiserum-agar study of horse serum, it can be stated that *Cecropia* blood, as a protein solution, is comparable in complexity to mammalian serum.

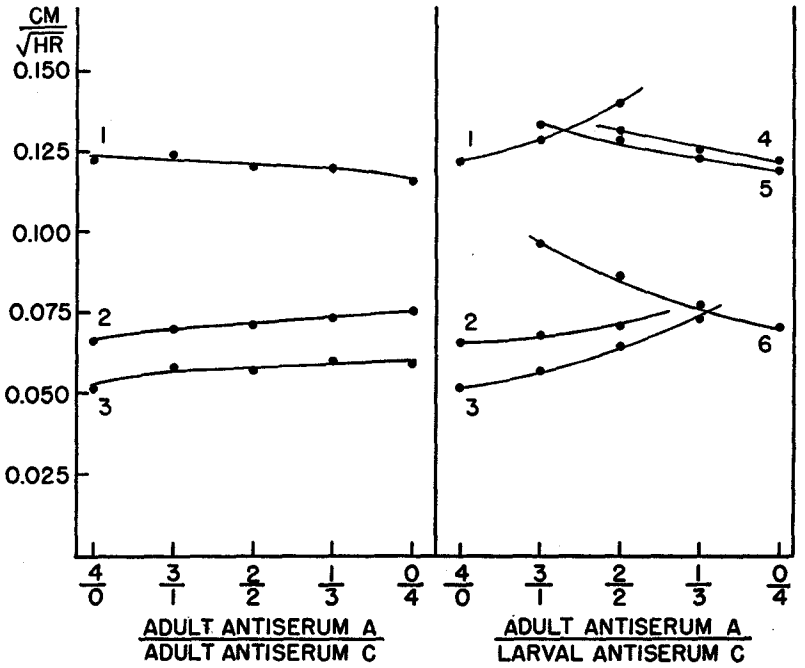


FIG. 1. A comparison by means of mutual dilution tests of the bands of precipitate produced in antiserum-agar by different antisera. The $k$ values on the $y$ axis are those of the bands of precipitate produced in the reactions of a constant concentration of pupal blood with graded mixtures of the antisera.

From among the total of nine antisera, three were selected which had properties desirable for the studies which follow. Between them, these three antisera reacted with six different pupal blood antigens to form six conspicuous and readily distinguishable bands of precipitate. The bands and their corresponding antigens have been designated by the numbers one through six. The following sections of this paper consider the behavior of these six blood antigens during metamorphosis.

*2. Qualitative Changes Occurring in the Blood Antigens during Metamorphosis.*—Table II lists the number of bands observed when late fifth instar blood, diapausing pupal blood, and adult blood reacted in antiserum-agar

tubes with each of the nine antisera. It appears that considerable qualitative similarity exists between the bloods at these stages of metamorphosis. In order to determine the extent of these similarities, a series of absorption tests was undertaken.

TABLE III

*The Number of Bands of Precipitate Appearing in Antiserum-Agar Reactions between Cecropia Blood and Antisera Which Had Been Absorbed with Blood from Animals at Several Stages of Metamorphosis*

| Antiserum | Absorbing antigen solution | Dilution of absorbing antigen solution | No. of bands when supernatant reacted with | | |
|---|---|---|---|---|---|
| | | | Late 5th instar blood | Pupal blood | Adult blood |
| Adult *a* | Late fifth instar blood | 1:1 | 0 | 0 | 0 |
| | | 1:4 | 0 | 0 | 0 |
| | | 1:16 | 0 | 0 | 0 |
| | Pupal blood | 1:1 | 0 | 0 | 0 |
| | | 1:4 | 0 | 0 | 0 |
| | | 1:16 | 0 | 0 | 0 |
| | Adult blood | 1:1 | 0 | 0 | 0 |
| | | 1:4 | 0 | 0 | 0 |
| | | 1:16 | 0 | 0 | 0 |
| Larval *c* | Late fifth instar blood | 1:1 | 0 | 0 | 0 |
| | | 1:4 | 0 | 1 | 1 |
| | | 1:16 | 0 | 1 | 1 |
| | Pupal blood | 1:1 | 0 | 0 | 0 |
| | | 1:4 | 0 | 0 | 0 |
| | | 1:16 | 0 | 1 | 0 |
| | Adult blood | 1:1 | 1 | 1 | 0 |
| | | 1:4 | 1 | 1 | 0 |
| | | 1:16 | 2 | 2 | 0 |

Adult antiserum *a* and larval antiserum *c* were absorbed with three different dilutions of late fifth instar blood, of diapausing pupal blood, and of adult blood, respectively. Each of the bloods was a pool obtained by bleeding a number of individuals. From each reaction mixture, the supernatant was removed and solidified with agar in glass tubes. The "absorbed antiserum-agar," thus prepared, was then tested for the presence of residual antibodies by being overlayered with larval, pupal, and adult bloods.

The results listed in Table III indicate that absorption of adult antiserum *a* by any of the three types of blood caused the loss of all its antibodies, as indicated by its subsequent inability to form bands of precipitate in anti-

serum-agar tests. The same was true with larval antiserum *c*, except when this antiserum was absorbed with adult blood. In the latter case, the absorbed antiserum retained the capacity to form a single band when overlayered by pupal and larval bloods. It is thus apparent that adult blood is deficient in one antigen which is present in pupal and fifth instar bloods. The identity of this antigen was determined as follows:—

When larval antiserum *c* was absorbed by adult blood, solidified, and then overlayered by pupal blood, the single band which appeared had the *k* value and the density recorded in Table IV. This table also records the *k* values and the densities of bands 4, 5, and 6 as they appeared when unabsorbed larval antiserum *c* was reacted in an antiserum-agar test with pupal blood.

TABLE IV

*A Comparison of the Antiserum-Agar Reactions of Pupal Blood with Unabsorbed Larval Antiserum c and with Larval Antiserum c That Had Been Absorbed with Adult Blood*

| Absorbing antigen solution | Dilution of absorbing antigen solution | No. of bands | Band characteristics | | Band No. |
|---|---|---|---|---|---|
| | | | *k* value | Density | |
| | | | $cm.hr.^{-1/2}$ | | |
| None | — | 3 | 0.122 | Low | 4 |
| | | | 0.121 | Medium | 5 |
| | | | 0.092 | High | 6 |
| Adult blood | 1:1 | 1 | 0.093 | High | |
| | 1:4 | 1 | 0.094 | High | |
| | 1:16 | 2 | 0.096 | High | |
| | | | 0.125 | Low | |

Since both of these tests were performed with the same final concentration of antiserum in the agar layer and with aliquots from the same pool of pupal blood as the overlying antigen solution, one would expect the single band produced by the absorbed antiserum to have the same *k* value and density as its counterpart in the unabsorbed antiserum-agar. According to this criterion, the data in Table IV indicate that it is antigen 6 which is undetectable in adult blood.

A second series of absorption tests was designed to study larval blood at an earlier stage. For this purpose, the blood of fourteen mature fourth instar larvae was collected and pooled. As recorded in Table V, absorption of adult antiserum *c* with fourth instar blood eliminated its capacity to form any bands of precipitate when overlayered by pupal blood. In the case of larval antiserum *c*, however, the absorbed antiserum retained the capacity to form a single band of precipitate when reacted with pupal blood. This band of pre-

cipitate had the same $k$ value and density as band 6 in the reaction between pupal blood and unabsorbed antiserum. Therefore, it can be concluded that fourth instar blood, like adult blood, does not contain detectable amounts of antigen 6.

In this connection, it should be noted that 0.4 ml. of undiluted adult or fourth instar blood was unable to deplete 0.3 ml. of the antiserum of the antibodies which produce band 6, while 0.4 ml. of a 1:100 dilution of pupal blood was able to do so. Therefore, if this antigen is actually present in adult or

TABLE V

*The Number and Characteristics of the Bands of Precipitate Produced in the Antiserum-Agar Reactions of Cecropia Blood with Unabsorbed Antisera, and with Antisera That Have Been Absorbed with Fourth Instar Blood*

| Absorbing antigen solution | Reacting antigen solution | No. of bands | Band characteristics | | Band No. |
|---|---|---|---|---|---|
| | | | $k$ value | Density | |
| | | | $cm.\ hr.^{-1/2}$ | | |
| Adult antiserum *c* | | | | | |
| Fourth instar blood | Fourth instar blood | 0 | | | |
| | Pupal blood | 0 | | | |
| Larval antiserum *c* | | | | | |
| Fourth instar blood | Fourth instar blood | 0 | | | |
| | Pupal blood | 1 | 0.077 | High | |
| None | Pupal blood | 3 | 0.122 | Low | 4 |
| | | | 0.121 | Medium | 5 |
| | | | 0.074 | High | 6 |

fourth instar blood, its concentration is less than one hundredth of that in pupal blood.

Another fact which emerges from these absorption tests concerns the constancy during metamorphosis of each antigen's immunological specificity. Fourth instar, fifth instar, pupal, and adult bloods were apparently able to precipitate all the antibodies responsible for the formation of bands 1 through 5, since the antisera lost all their capacities to form bands of precipitate when absorbed with any of the four types of blood, with the exception of the cases cited concerning band 6. Therefore, in so far as their reactions with these antisera are concerned, antigens 1 through 5 appear to be constant in their immunological specificity throughout metamorphosis. The same is true of antigen 6 during the late larval and pupal periods when it is present.

From these experiments we learn that five of the six antigens are present in

the blood in readily detectable concentrations at all stages of metamorphosis. Antigen 6, however, is undetectable in fourth instar blood, appears in the blood during the fifth instar, persists during the pupal stage, and then disappears or falls to an undetectable concentration in adult blood. The transitory existence of this antigen is of special interest since it is the only qualitative change we have found in the six blood antigens during metamorphosis.

*3. Changes in the k Values of the Bands of Precipitate during Metamorphosis.* —In the course of the preceding qualitative studies, it was observed that the rates of advance of the six bands of precipitate under consideration varied considerably with the stage of metamorphosis from which the blood was derived. An investigation was undertaken for the purpose of describing the magnitude and timing of these changes and of ascertaining the extent to which they are caused by concentration changes in the six antigens. A series of mutual dilution studies was performed on blood from eight stages of metamorphosis, permitting us to identify the bands of precipitate at all eight stages and thus to determine their $k$ values.

The eight stages studied were as follows: (1) early fifth instar within 2 days after the last larval moult; (2) the late fifth instar within a few days prior to spinning; (3) the diapausing pupal stage immediately after being placed at 25°C. after storage at 5°C. ("chilled diapausing 1" on subsequent charts); (4) the diapausing pupal stage 1 week after being placed at 25°C. from 5°C. ("chilled diapausing 2" on subsequent charts); (5) the 1st, (6) 6th, and (7) 14th days of adult development; and (8) the adult stage just before emergence from the pupal cuticle. The pooled blood of four to twelve animals was collected at each of the eight stages. For the study of antigens 1, 2, and 3, the various blood mixtures were reacted in antiserum-agar tests with adult antiserum *a*, while antigens 4, 5, and 6 were studied with larval antiserum *c*.

The blood of chilled pupae was chosen as the reference blood with which all other samples were compared because, as described above, the particular bands of precipitate produced by the reaction of pupal blood with adult antiserum *a* and with larval antiserum *c* were, by definition, bands 1 through 6.

The results of the mutual dilution tests are presented in Fig. 2. The graphs show that in most cases there is no doubt about the identification of the bands of precipitate produced by the blood samples being compared with pupal blood. Some possibility of incorrect identification exists in those cases in which two bands have almost identical $k$ values (as in Graphs 2 and 9 to 12), or where the $k$ values of all three bands are extremely low (as in Graph 13). However, in such cases, the error in $k$ value resulting from the incorrect identification of a band would have been insignificant compared to the magnitude of the $k$ value changes under consideration. The results of these tests are plotted in Figs. 3 and 4.

The large quantities of antisera and antigen solutions required for mutual dilution tests made it desirable to obtain as much information as possible

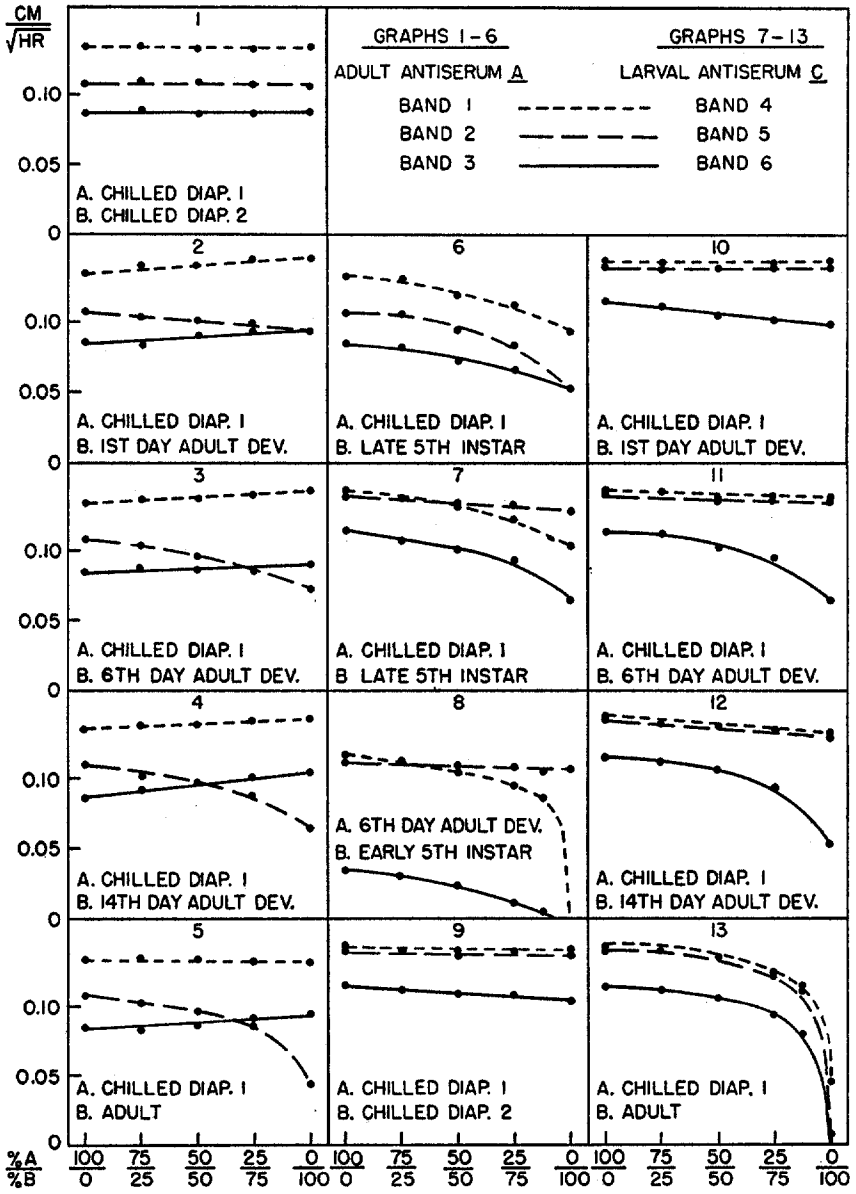


FIG. 2. Identification by means of mutual dilution tests of the bands of precipitate produced in antiserum-agar by blood samples from animals at different stages of metamorphosis. Each tube contained a standard concentration of the antiserum listed on the figure.

without this rather elaborate procedure. As noted above, under the condition of constant antiserum concentration observed in this study, each band of

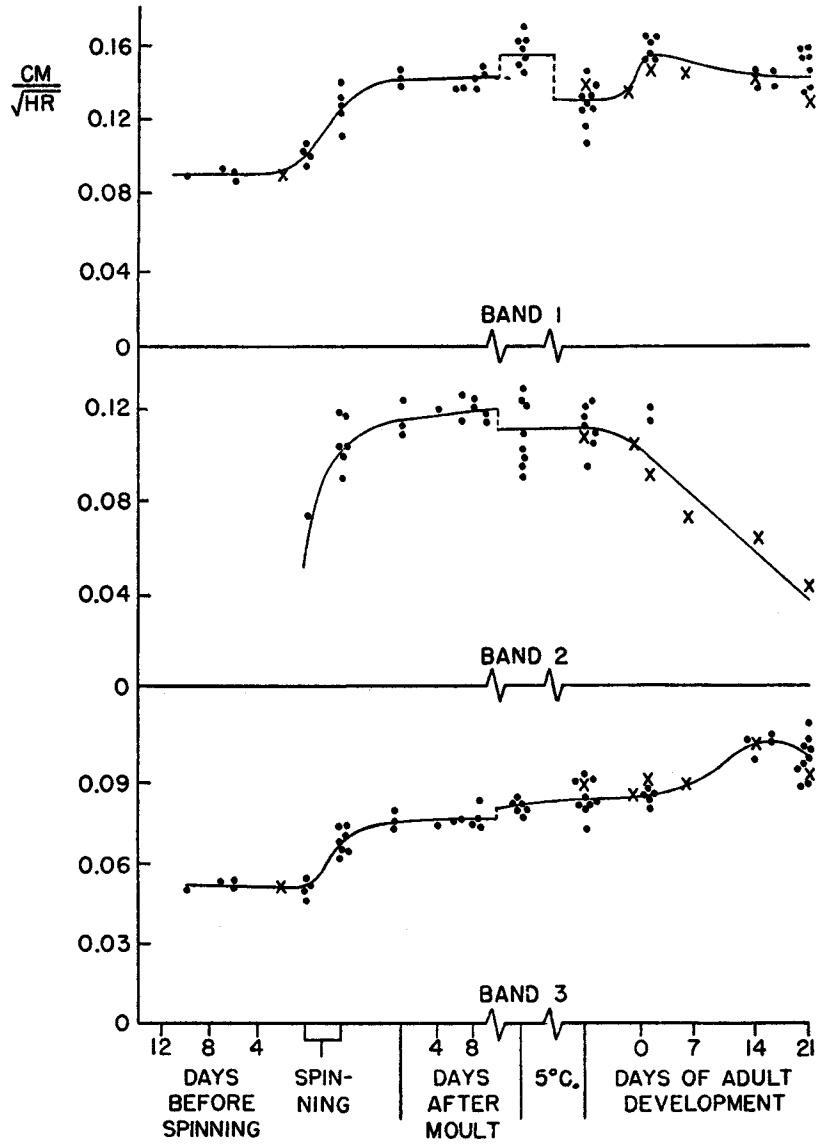


FIG. 3. The $k$ values of bands 1, 2, and 3 which were produced in antiserum-agar reactions between a standard concentration of adult antiserum $a$ and blood from animals at various stages of metamorphosis. The bands were identified by mutual dilution tests (×) or by density (●).

precipitate possesses a characteristic density which can serve as an aid to its identification. The antisera used in this study were selected for the particular reason that each produced three bands of precipitate of readily distinguishable

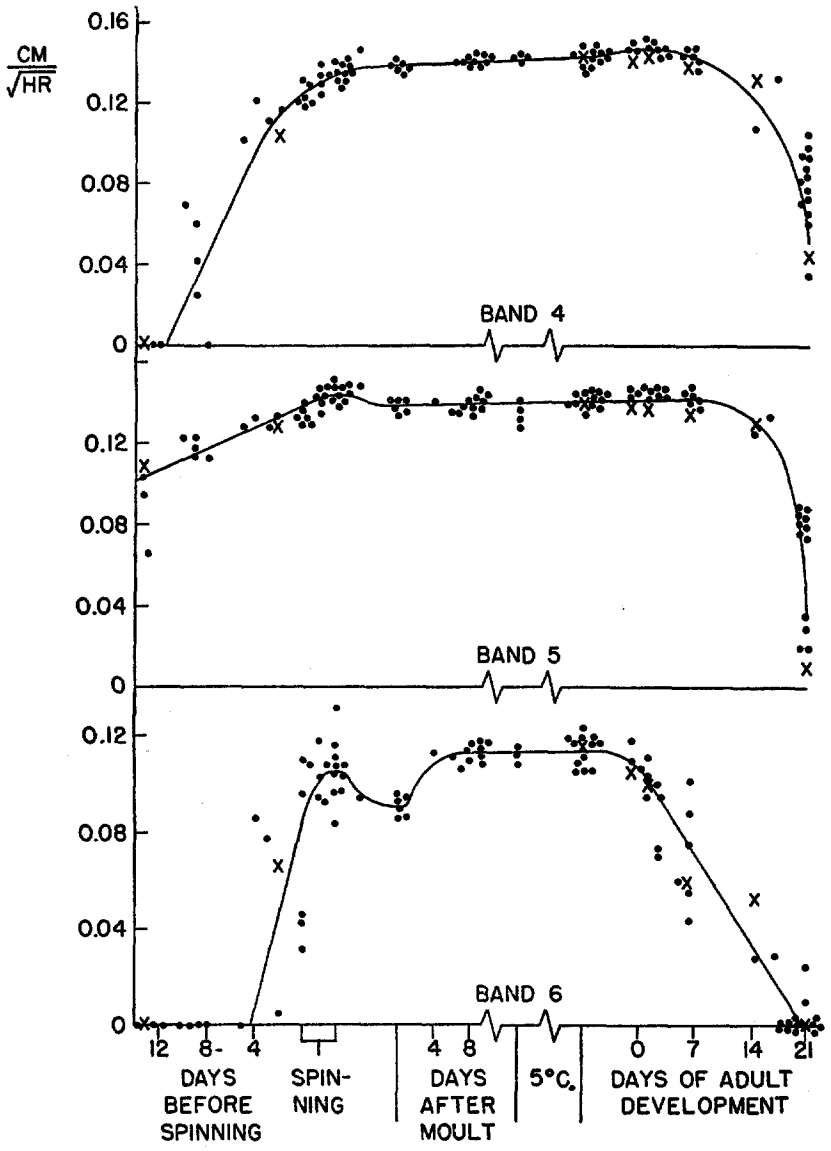


FIG. 4. The $k$ values of bands 4, 5, and 6 which were produced in antiserum-agar reactions between a standard concentration of larval antiserum $c$ and blood from animals at various stages of metamorphosis. The bands were identified by mutual dilution tests (×) or by density (●).

density. Consequently, it was possible to react any sample of *Cecropia* blood with any of these antisera and to identify the bands of precipitate without the aid of mutual dilution tests.

Figs. 3 and 4 show the $k$ values obtained from a series of tests in which the bands were identified by their density. These data were obtained from antiserum-agar tests on 0.1 ml. samples of blood from individual animals at various stages of metamorphosis. From two to five samples of blood were drawn from each animal at different times during its development. Neither the form nor the timing of development of the animals was altered by bleeding. Justification for the identification of the bands of precipitate in terms of their density is seen in the close agreement between the data obtained in this manner and those obtained by mutual dilution tests.

It appears in Figs. 3 and 4 that every one of the six bands of precipitate undergoes systematic variations in its $k$ value in close correlation with the morphological changes of the silkworm's metamorphosis.

*4. Interpretation of the k Value Changes during Metamorphosis.*—It would be of inestimable assistance in the study of the synthesis and function of the blood antigens if the changes in $k$ value, observed in the preceding section, could be attributed to corresponding changes in the concentrations of the blood antigens during metamorphosis. Let us first accept this assumption and consider how the $k$ value may then be directly converted into units of relative antigen concentration. The validity of the assumption itself will then be examined in detail.

The procedure used in converting $k$ values into units of relative antigen concentration will be described using antigen 6 as an example. A standard curve in which the $k$ value of band 6 is plotted as a function of the relative concentration of antigen 6 was constructed in the following manner. A sample of blood was selected which, when layered over the appropriate antiserum-agar, produced a high $k$ value for band 6. The high $k$ value was presumed to be caused by a correspondingly high concentration of antigen 6 in that particular blood sample. The blood was then serially diluted and the dilutions were layered over antiserum-agar columns of constant composition. The $k$ value of band 6 was determined for each dilution and plotted as a function of the relative concentration of antigen 6 (1/dilution of the blood). Fig. 5 records the standard curve thus constructed. The latter was then used to transform the $k$ values of band 6, as read from the curve in Fig. 4, into units of relative antigen concentrations. This procedure was repeated for each of the six antigens with the results shown in Fig. 6.

Unfortunately, a number of complicating factors interfere with the validity of this interpretation of the $k$ value changes. A consideration of equation (2) shows that, for $k$ to vary with antigen concentration alone, there must be constancy of the antibody concentration, of the diffusion coefficient of the antigen, and of all factors which could non-specifically affect the $k$ value of any band of precipitate. The bearing of these and other factors on our experimental results will now be considered.

(*a*) *Antibody Concentration.*—Since antiserum concentration was an experimental constant, the concentration of antibody in the antiserum-agar was presumably constant also. However, if the specificity of an antigen, and thus its capacity to react with antibodies, underwent alterations during metamorphosis, the concentration of antibodies capable of reacting with that antigen in antiserum-agar would be altered correspondingly, even though a standard amount of total antibodies was present in the antiserum-agar.

The absorption tests described above suggest that the six antigens under consideration possess a constant immunological specificity with regard to the antisera used here. Therefore, it seems safe to assume that the concentrations

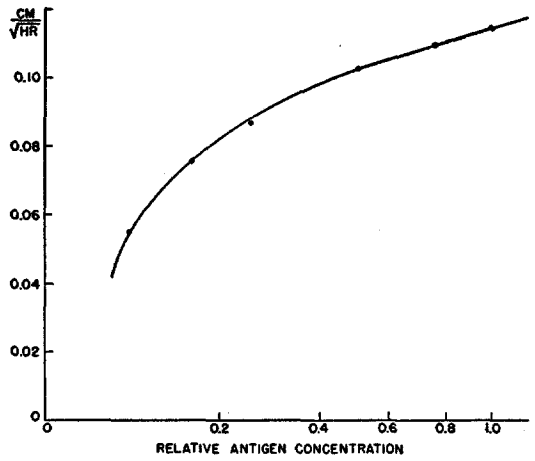


FIG. 5. The *k* values of band 6 in the antiserum-agar reactions between various dilutions of pupal blood and a standard concentration of larval antiserum *c*.

of the antibodies capable of reacting with each antigen were constant in these tests.

(*b*) *Non-Specific Factors.*—Oudin (13) states that the *k* value of a band of precipitate in antiserum-agar is sometimes increased to a significant degree by the presence of protein contaminants in the overlying antigen solution, an effect for which no satisfactory explanation has been proposed. Since *Cecropia* blood contains a considerable number of proteins, it was necessary to test the possibility that some of these proteins have a non-specific, accelerating effect on the observed *k* values.

In order to explore this possibility, we required a sample of *Cecropia* blood which was deficient in at least one of the antigens under examination. That sample of blood was then used as the source of protein contaminants in experiments concerning the band of precipitate of the missing antigen. This condition was met in two cases.

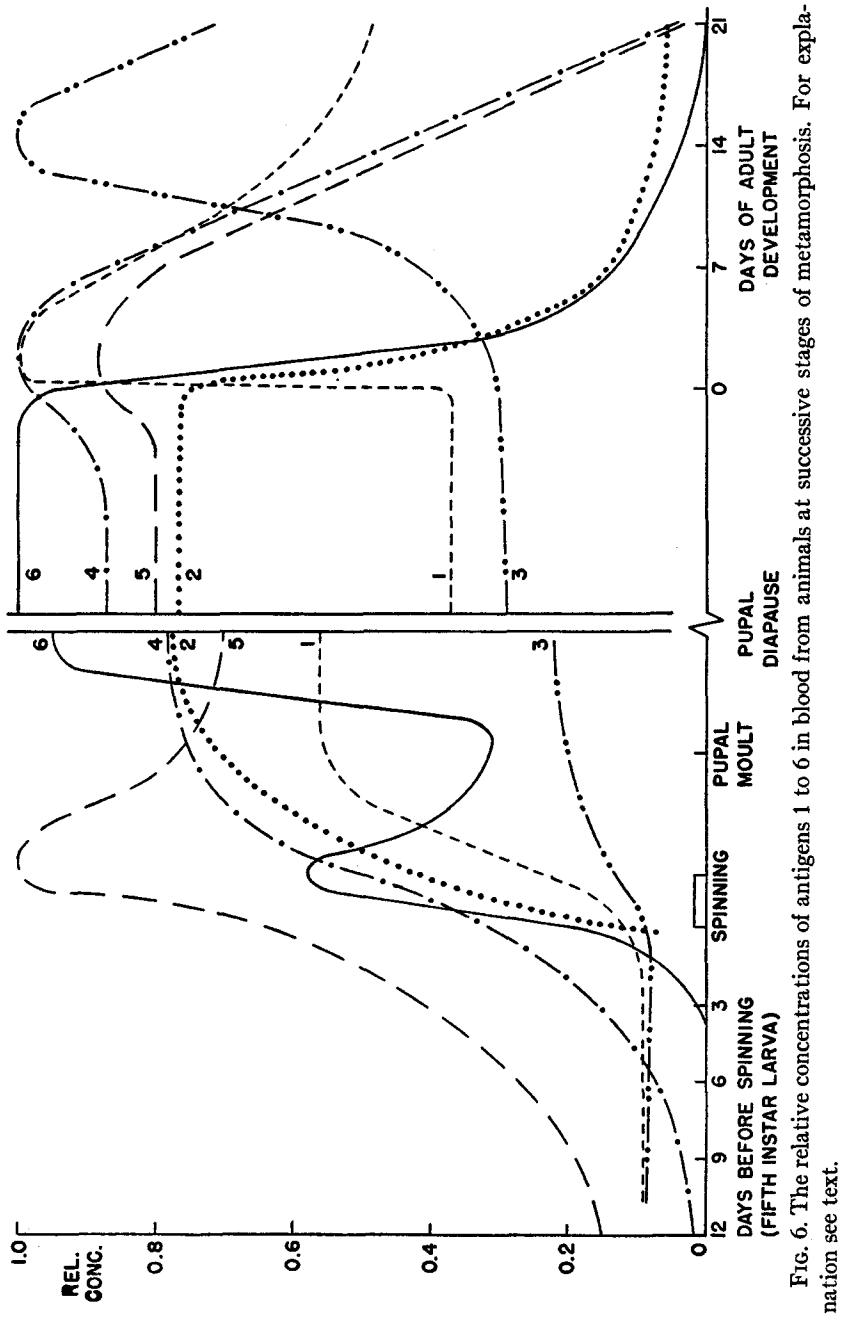


FIG. 6. The relative concentrations of antigens 1 to 6 in blood from animals at successive stages of metamorphosis. For explanation see text.

The blood of female pupae contains an antigen, to be described at a later time, which is absent from the blood of males. This fact has been demonstrated with adult antiserum *d* which reacts with the "female" antigen to form a dense band of precipitate that we designate as band 7. Consequently, it was possible to study the effect of the proteins of pupal male blood, which lacks this antigen, on the *k* value of band 7 as formed by female blood.

Six aliquots from a pool of pupal female blood were diluted to four times their initial volume with varying mixtures of 0.15 M NaCl and pupal male blood. Each sample was then reacted in an antiserum-agar tube with adult antiserum *d*. The composition of the antigen solutions and the *k* value of band 7 are presented in Table VI. It is evident that the proteins of pupal male blood had no effect on the *k* value of band 7.

TABLE VI

*The Effects of Mixtures of Saline and Pupal Male Blood on the k Value of Band 7 in the Reaction between Adult Antiserum d and Pupal Female Blood*

| Composition of antigen solution | | | *k* value of band 7 |
|---|---|---|---|
| Male blood | 0.15 M NaCl | Female blood | |
| *ml.* | *ml.* | *ml.* | *cm. hr.*$^{-1/2}$ |
| 0.30 | 0 | 0.10 | 0.071 |
| 0.24 | 0.06 | 0.10 | 0.071 |
| 0.18 | 0.12 | 0.10 | 0.071 |
| 0.12 | 0.18 | 0.10 | 0.073 |
| 0.06 | 0.24 | 0.10 | 0.073 |
| 0 | 0.30 | 0.10 | 0.073 |

A similar test was carried out on band 6, using adult blood which lacks antigen 6, as a source of protein contaminants. In this case, six aliquots of a sample of pupal blood containing antigen 6 were diluted to four times their initial volume with varying mixtures of 0.15 M NaCl and adult blood. The antigen mixtures were then reacted with larval antiserum *c*. The results recorded in Table VII indicate that adult blood is indistinguishable from 0.15 M NaCl in its effect on the *k* value of band 6. Therefore, there is no indication that either pupal or adult blood contains substances which non-specifically affect the *k* values of bands of precipitate in antiserum-agar.

Further evidence that the *k*-value changes during metamorphosis are not due to factors having over-all and non-specific effects on the bands of precipitate is seen in the lack of uniformity in the behavior of the individual bands, as recorded in Figs. 3 and 4.

(*c*) *The Diffusion Coefficient and Immunological Cross-Reactions.*—Little evidence has as yet been obtained with reference to two additional factors which could affect the *k* value of a band of precipitate. Constancy in the diffusion

coefficient of each antigen during metamorphosis has not been demonstrated, nor is there assurance that interaction between different bands of precipitate is entirely absent. The latter phenomenon could occur if two or more antigens had a partially common immunological specificity. Oudin (11) showed in his analysis of "complex precipitating systems" that, when the antigen layer contains two antigens which react to some extent with the same antibodies in an antiserum-agar tube, then both antigens influence the rate of advance of each of the two resulting bands of precipitate. However, it will not be possible to test whether such cross-reactions occur in the antigen and antibody solutions studied here until each of the antigens can be obtained free of all other antigens.

TABLE VII

*The Effects of Mixtures of Saline and Adult Blood on the k Value of Band 6 in the Reaction between Larval Antiserum c and Pupal Blood*

| Composition of antigen solution | | | $k$ value of band 6 |
|---|---|---|---|
| Adult blood | 0.15 M NaCl | Pupal blood | |
| *ml.* | *ml.* | *ml.* | *cm. hr.*$^{-1/2}$ |
| 0.30 | 0 | 0.10 | 0.090 |
| 0.24 | 0.06 | 0.10 | 0.090 |
| 0.18 | 0.12 | 0.10 | 0.089 |
| 0.12 | 0.18 | 0.10 | 0.090 |
| 0.06 | 0.24 | 0.10 | 0.090 |
| 0 | 0.30 | 0.10 | 0.089 |

Thus, of these possible sources of error in the use of the $k$ value as a criterion of antigen concentration, two have been largely ruled out, as far as applicability to the present study is concerned, while two others have not been readily accessible to direct experimental analysis. For this reason, we adopted an indirect approach to this problem by comparing the results obtained by the $k$ value criterion with those obtained by an alternative method. The latter consisted of a quantitative adaptation of the absorption technique.

(*d*) *Relative Antigen Concentration as Estimated by a Quantitative Adaptation of the Absorption Technique.*—In this method, relative antigen concentrations were determined by comparing the capacities of several samples of blood to precipitate the antibodies from an antiserum and thereby decrease that antiserum's ability to produce the corresponding bands of precipitate in subsequent antiserum-agar tests. Advantage was taken of the fact that the $k$ value of a band of precipitate increases with decreasing concentration of the corresponding antibody. Thus, when the concentration of a specific type of antibody is lowered by precipitation with its homologous antigen, the corresponding band of precipitate formed in antiserum-agar by the absorbed antiserum

has a higher $k$ value than that produced by unabsorbed antiserum. As increasing amounts of antigen are used in the preliminary absorption, the band of precipitate moves at a faster rate and also decreases in density. Finally, when the preliminary absorption has removed all of the specific antibody, the band fails to appear.

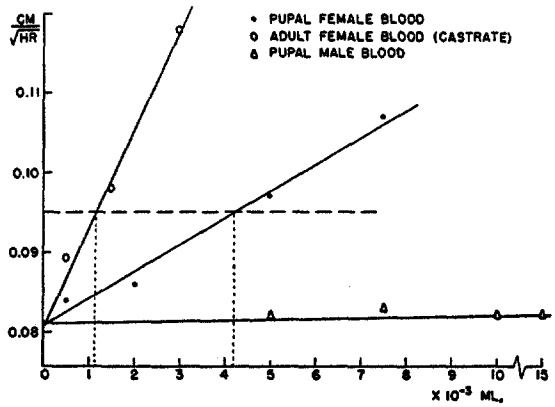


FIG. 7. The $k$ value of band 7 as produced in antiserum-agar reactions between pupal blood (diluted 1:3 with saline) and a standard concentration of adult antiserum $d$ that has been absorbed with various amounts of blood. The type of blood used for absorption is indicated on the figure.

The absorption tests were performed as described earlier, 0.4 ml. of a specific dilution of *Cecropia* blood being added to 0.3 ml. of antiserum. The supernatant from this reaction mixture was measured in volume and solidified with agar in glass tubes so that the final dilution of the antiserum was 1:3 and the final agar concentration was 0.25 per cent. The antiserum-agar was then overlayered with a standard sample of pupal blood and the $k$ value of the band of precipitate under consideration determined.

Fig. 7 shows that, when several different dilutions of a blood sample were used in the absorption of adult antiserum $d$, the $k$ value of band 7 behaved in subsequent antiserum-agar tests as predicted. As increasing amounts of blood were used in the preliminary absorption, the $k$ value of band 7 increased. The assumption that this increase in $k$ value is due to a decrease in the concentration of antibodies that can combine with antigen 7 is borne out by the fact that, as illustrated in Fig. 7, absorption with male blood which lacks antigen 7 did not affect the $k$ value of band 7 in subsequent antiserum-agar tests.

The broken line in Fig. 7 designates an arbitrarily selected $k$ value of 0.095. Under the conditions of this experiment, it also designates a particular concentration of the corresponding antibody in the antiserum-agar—a concentration which is lower than the antibody concentration of the unabsorbed anti-

serum. In order to reduce the antibody content of the antiserum to this extent, it is necessary to add a particular, although undetermined, quantity of antigen 7. The relative concentration of antigen 7 in a sample of blood can therefore be determined by measuring the volume of that blood sample required to cause the designated change in the concentration of the homologous antibody. According to the data in Fig. 7, for example, the concentration of antigen 7 in adult female blood, relative to its concentration in pupal female blood, is $4.2 \times 10^{-3}$ ml./$1.1 \times 10^{-3}$ ml., or 3.8:1.

TABLE VIII

*A Comparison of Relative Antigen Concentrations as Determined by the k Value Criterion and by the Absorption Technique*

| Antigen | Source of blood | Relative concentration* | |
|---|---|---|---|
| | | *k* value | Absorption |
| 6 | Chilled diapausing pupae | 1.00 | 1.00 |
| | 1st day of adult development | 0.95 | 0.91 |
| | 14th " " " " | 0.08 | 0.06 |
| | Adult | <0.08 | 0 |
| 7 | Chilled diapausing females | 1.00 | 1.00 |
| | Females on 14th day of adult development | 0.30 | 0.25 |
| | Chilled diapausing males | <0.10 | 0 |
| | Males on 14th day of adult development | <0.10 | 0 |
| | Late 5th instar larvae | <0.10 | 0 |
| | Castrated adult females | 2.50 | 3.80 |
| 3 | Chilled diapausing females | 1.00 | 1.00 |
| | Females on 14th day of adult development | 1.50 | 1.70 |
| | Chilled diapausing males | 0.95 | 0.90 |
| | Males on 14th day of adult development | 0.76 | 1.70 |
| | Late 5th instar larvae | 0.20 | 0.17 |
| | Castrated adult females | 1.90 | 3.90 |

* Chilled diapausing pupal values are arbitrarily designated as 1.0 and all other concentrations are expressed in relative terms.

The relative concentrations of antigens 3, 6, and 7 in several different samples of blood were estimated in this manner and compared with the values obtained on the same samples by the direct *k* value method described above in section *a*. The results of the two methods are presented in Table VIII. With a few exceptions, there is good agreement between the two sets of data. When high concentrations were being dealt with, the *k* value criterion yielded consistently lower values than did the absorption test. Otherwise, the only serious discrepancy was in the case of antigen 3 in the blood of males on the 14th day of development.

Antigen 3, the carotenoid protein, is peculiar among the antigens studied in

that it sometimes behaved in an anomalous manner. Thus, if a blood sample was exposed to the air and permitted to undergo melanin formation for 15 or 20 minutes, the $k$ value of band 3, and that of no other band, was always significantly low in subsequent antiserum-agar tests. Furthermore, heating the blood to 55°C. often caused an increase in the $k$ value of band 3, whereas the same treatment either failed to affect the $k$ values of the other bands or caused a decrease due, presumably, to heat precipitation of the antigens and a resulting decrease in their concentrations. These facts indicate that the $k$ value is not a consistently reliable index of the concentration of antigen 3. Yet it will be observed in Table VIII that, even for this antigen, good agreement was obtained in four out of six comparisons between the absorption test and the antiserum-agar test. The fact that two such dissimilar methods yield generally uniform results suggests that the data produced by both methods are probably in large measure reliable.

## DISCUSSION

The results presented here show that the blood antigens of the *Cecropia* silkworm undergo changes which are closely correlated with the morphogenetic events comprising metamorphosis. Though the qualitative changes are not extensive, Fig. 6 illustrates the fact that all the six antigens that were studied undergo pronounced quantitative changes during metamorphosis. This conclusion rests on the assumption that, in this investigation, the only variable that significantly affected the rate of advance of any of the bands of antigen-antibody precipitate through antiserum-agar was the concentration of the corresponding blood antigen. Although further study will probably lead to modifications of specific details in the description made possible by this assumption, we feel that the over-all picture presented here is substantially correct.

According to Fig. 6, the six antigens undergo a general increase in concentration during the last larval instar and a decrease in concentration during adult development. In view of the fact that these antigens have the properties of proteins, it is significant that both the timing and the order of magnitude of the over-all concentration changes agree with the results of protein nitrogen determinations on *Cecropia* blood (3).

There is considerable variation between individual antigens in the time and magnitude of their concentration changes, each antigen undergoing a distinctive pattern of change. Thus, the onset of the increase in concentration during the last larval instar, while precisely set for each antigen, varies from antigen to antigen over a period of 2 weeks. Similarly, the decrease in concentration during adult development is initiated on the 1st day of development by antigens 2 and 6, on about the 7th day by antigens 4 and 5, and, in the case of antigens 1 and 3, is preceded by a large increase in concentration.

These facts indicate that the quantitative changes in the blood antigens

result from physiological processes which produce changes in the concentration of individual blood antigens without necessarily affecting the concentrations of the other antigens. The degree of specificity required of these hypothesized physiological processes could scarcely result from non-specific mechanisms such as loss of water from the insect, for such a mechanism, operating by itself, would produce simultaneous changes in all the blood antigens. The observed diversity in the behavior of the antigens suggests that each antigen has an individually controlled synthetic mechanism, and that the growing tissues of the silkworm can selectively accumulate or utilize individual antigens. This aspect of the matter will form the substance of further communications.

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## SUMMARY

1. The cell-free blood of the *Cecropia* silkworm produces a maximum of nine bands of antigen-antibody precipitate when reacted in antiserum-agar tests with antisera prepared by injecting *Cecropia* extracts into rabbits. The blood antigens producing these bands of precipitate have the properties of proteins in that they are non-dialyzable, labile at 75°C., and salted out by 75 per cent saturated ammonium sulfate. One antigen was identified as a carotenoid protein.

2. Six bands of precipitate were selected for further study. Absorption tests revealed that the blood, at all stages of metamorphosis, is capable of precipitating the antibodies which produce five of these bands. This result indicates that five of the six antigens are present in the blood throughout metamorphosis. The sixth antigen is undetectable in blood from fourth instar larvae, appears in the blood late in the fifth instar, persists during the pupal stage, and disappears again during adult development.

3. When blood samples from various stages of metamorphosis were tested in antiserum-agar tubes, the rates of advance of the six bands of precipitate underwent systematic change in close correlation with the morphological stage of the silkworm's metamorphosis. These changes are interpreted in terms of concentration changes of the corresponding blood antigens. The validity of this interpretation was tested in several ways, with the conclusion that the interpretation was generally acceptable for the system under consideration.

4. All six antigens appear to increase in concentration during the last larval instar and to decrease in concentration during the period of adult development. However, each antigen has its own characteristic pattern of concentration change which differs from those of the other five. In order to explain this diversity, we conclude that the physiological mechanisms which regulate

the synthesis and utilization of the blood antigens control each antigen on an individual basis.

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