IMMUNOLOGICAL STUDIES OF INSECT METAMORPHOSIS

II. The Role of a Sex-Limited Blood Protein in Egg Formation by the cecropia Silkworm

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The blood proteins of the *cecropia* silkworm undergo a complex sequence of changes which are synchronized with the morphological alterations comprising the insect's metamorphosis (15). This relationship indicates that the blood proteins are participants in the biochemical processes which bring about the caterpillar's transformation into the adult moth. In the previous study of this series, six blood proteins were investigated by means of immunological techniques. Each was found to undergo changes in concentration that differed in both magnitude and timing from those of the other five. In order to characterize the cellular mechanisms responsible for these changes, an experimental analysis of the functions of the individual proteins in the metamorphosing insect has been undertaken. The first to be studied in this manner is antigen 7, a female blood protein which occurs only in traces in the blood of males.

Methods

1. Preparation of Antigen Solutions.—The antigen solutions were derived from the *cecropia* silkworm (*Platysamia cecropia*), and included the blood of animals at various stages of metamorphosis, saline extracts of whole adult moths, and the centrifuged yolk of unfertilized eggs. Melanin formation, which normally occurs upon exposure of these solutions to air, was prevented by the addition of a neutralized cyanide solution or a few crystals of phenylthiourea (twice recrystallized from the Eastman product). The collection of blood samples and the preparation of saline extracts of whole animals were performed according to procedures previously described (15).

Eggs were obtained from the ovaries of newly emerged female moths. In *cecropia* as in other Lepidoptera each mature ovary consists of four thin walled tubes (ovarioles) enclosing a linear sequence of up to thirty unfertilized eggs. The ovaries were dissected from anesthetized insects, rinsed several times with saline, and stored at −20°C. When subsequently thawed and crushed in a mortar along with a few crystals of phenylthiourea, the eggs yielded a fluid yolk containing large amounts of particulate

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matter. The latter was removed by centrifugation at 10,000 *g*. A clear yellow liquid was then pipetted from between the sediment and a superficial lipid layer.

Several antisera were obtained from rabbits that had been injected with the distilled water–soluble fraction of blood or yolk. This fraction was obtained as follows:—

A sample of blood or yolk was diluted with two times its volume of neutralized cyanide solution (0.015 M KCN and 0.135 M NaCl, the pH being adjusted to neutrality by the addition of 0.15 M KH_2PO_4). The sample was then dialyzed against at least six changes of distilled water. A copious precipitate of water-insoluble globulins was removed by centrifugation, and the supernatant finally dialyzed against 0.15 M NaCl in preparation for injection into rabbits.

2. Preparation of Antisera.—Each rabbit was presensitized by a single intravenous injection of one of the antigen solutions under consideration. 3 weeks later, a series of four subcutaneous injections was begun in which 2 ml. samples of antigen solution were administered to the rabbit on alternate days. On the 6th or 7th day after the conclusion of the injection series, the blood was collected by cardiac puncture.

"Adult antiserum *d*" was a pool of antisera obtained from three rabbits that had been immunized with a saline extract of adult moths. Certain reactions of this antiserum have already been described (14, 15). "Blood antiserum *a*" was a pool of antisera obtained from three rabbits that had been injected with the water-soluble proteins of female pupal blood. "Egg antisera *a* and *b*" were each obtained from a rabbit after immunization with the water-soluble proteins of centrifuged yolk.

3. Antigen-Antibody Reactions.—

(*a*) *Antiserum-Agar Tests.*—*Cecropia* blood and tissue extracts contain a large number of different antigens, and, when injected into rabbits, elicit the production of antibodies which precipitate a number of different antigens. In order to resolve such complex antigen-antibody reactions into their component reactions, the antiserum-agar technique of Oudin was utilized (7, 10, 12).

By this technique the precipitation of antigen 7 was observed in small bore glass tubes, each of which contained a sample of blood or yolk layered over an appropriate antiserum which had been solidified with agar. The band of antigen-antibody precipitate attributable to the precipitation of antigen 7 in the antiserum-agar layer was identified with the aid of the mutual dilution and absorption tests discussed below. As has been described for other antigen-antibody systems (7, 11), band 7 advanced through the antiserum-agar at such a rate that

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

in which h is the distance from the interface to the sharp leading edge of the zone of precipitation, t is the time elapsed since the antigen solution was layered over the agar, and k is a coefficient characteristic of the band under specified experimental conditions. The k value was used with the aid of previously described procedures (14, 15) as an index to the relative concentration of antigen 7 in the various yolk and blood samples. The reliability of this interpretation of the k value in studies of *cecropia* blood antigens has already been considered in detail. The present investigation provides further confirmation of this reliability.

(*b*) *Quantitative Precipitin Tests.*—An antiserum specific for antigen 7 was developed by absorbing blood antiserum *a* with pupal male blood. As will be shown sub-

sequently, the supernatant from this absorption retained antibodies capable of precipitating only antigen 7 from female blood or yolk. The absorbed antiserum was therefore suitable for determinations of the relative concentration of antigen 7 by the quantitative precipitin technique.

Absorption was accomplished by mixing the antiserum with an equal volume of a 1:10 dilution of pupal male blood. The mixture, after storage at 3°C. for 48 hours, was centrifuged at 10,000 *g*.

In the quantitative precipitin tests, 1.0 ml. of the absorbed antiserum was mixed in a conical centrifuge tube with 1.0 ml. of a desired dilution of blood or centrifuged yolk. In order to prevent melanin formation, all dilutions were performed with the neutralized cyanide solution described above. The mixture of antiserum and antigen solution was maintained at room temperature for 1 hour and then at 3°C. for 48 hours. The precipitate was then sedimented by centrifugation at 2,000 *g* for 30 minutes. The supernatant was preserved for later study, while the precipitate was washed twice in 3.0 ml. of 0.15 M NaCl at 3°C., and then dissolved in 5.0 ml. of 0.25 N acetic acid. The optical density of this solution at 277 mμ was determined with a Beckman spectrophotometer. By this procedure, the amount of precipitate was estimated by the ultraviolet light absorption of its constituent aromatic amino acids (3).

A standard curve was obtained by reacting the antiserum with a series of dilutions of a sample of female pupal blood. From the resulting data, the optical density was plotted as a function of the volume of female blood that had been added to the antiserum. The relative concentrations of antigen 7 in other samples of blood or yolk were estimated with the aid of the standard curve. Two dilutions of each sample of blood or yolk were used in each determination.

4. Electrophoresis.—In their preparation for electrophoretic analysis, blood samples were first diluted with up to two times their volume of neutralized cyanide solution. After 1 day of dialysis against the cyanide solution, the sample was dialyzed for 3 days against three changes of an appropriate phosphate buffer (ionic strength, 0.1). It was then centrifuged at 2,000 *g* and transferred to a 2 ml. cell for analysis with the Perkin-Elmer electrophoresis instrument.

5. Surgical Procedures.—Surgical procedures were utilized in experiments designed to elucidate the function of antigen 7. All surgery was carried out *via* an incision at the tip of the abdomen of pupae anesthetized with carbon dioxide. At the conclusion of each such procedure, several crystals of phenylthiourea and, in certain cases, streptomycin sulfate and potassium penicillin G were implanted at the site of the incision. The opening was then covered with a plastic coverslip and sealed with melted paraffin (18). Animals surviving the surgical procedures underwent complete adult development at 25°C. according to the same time-table which obtains for normal insects at that temperature.

(*a*) *Ovariectomy.*—The tip of the abdomen of previously chilled, diapausing, female *cecropia* pupae was cut away just behind the eighth abdominal segment. As much blood as possible was drained into a tube containing a few crystals of phenylthiourea. The gut and most of the fat-body were maneuvered into a ventral position, thereby exposing the imaginal discs of the ovaries on each side of the heart at the level of the fifth abdominal segment. Each ovary was grasped with forceps and, along with a small amount of adjacent fat-body, was cut free, and removed. The blood was then poured

back into the pupa and insect Ringer's solution (18) added to complete the filling of the body cavity. 4 to 6 weeks later, at the completion of adult development, the blood was drained from these insects just prior to their emergence from the pupal cuticle; at this same time the absence of ovarian tissue was confirmed by dissection.

(*b*) *Implantation of Ovarian Imaginal Discs into Male Pupae.*—Ovarian imaginal discs were excised from previously chilled diapausing female pupae and washed free of blood with insect Ringer's solution. Undamaged discs were implanted into the body cavity of male pupae. Blood and eggs were collected from the latter after the completion of adult development.

(*c*) *Intraspecific Blood Transfusions.*—Male and female pupae of *Telea polyphemus*, a saturniid species closely related to *Plastysamia cecropia*, were emptied of blood through an opening at the tip of the abdomen, and refilled with freshly drawn blood from female *cecropia* pupae. The blood and eggs were collected from these animals after the completion of adult development.

RESULTS

1. Demonstration of a Sex-Limited Antigen in Pupal Female Blood.—When pupal blood was layered over agar containing the serum of *un*immunized rabbits, no precipitation developed. However, when layered over agar containing a 1:3 dilution of adult antiserum *d*, the blood caused the appearance of a series of bands of precipitate which varied in pattern according to the sex of the pupa which had donated the blood. Male blood produced approximately six weak bands of precipitate and a single extremely dense band. Female blood produced a similar number of weak bands, but differed from male blood in giving rise to two dense bands of precipitate. The sexual difference observed in these reactions suggested that pupal female blood contains an antigen which is either absent or in extremely low concentration in the blood of males.

The validity of this interpretation was confirmed by absorption tests. A sample of adult antiserum *d* was absorbed with male blood by the addition of an equal volume of a 1:10 dilution of pupal male blood. The supernatant from the reaction mixture was solidified with agar in glass tubes so that the dilution of the original antiserum was 1:3. Male pupal blood, when layered over this antiserum-agar, produced no bands of precipitate, a finding which indicates that the previous absorption had eliminated all antibodies detectably reacting with male blood. Female blood, by contrast, produced a single dense band of precipitate. We designate this band of precipitate as band 7 and the corresponding blood antigen as antigen 7.

As observed by Oudin (10), an antigen may be present in the overlying solution in such low concentration that, owing to its inability to diffuse into the agar layer against a high concentration of antibodies, no band of precipitate appears. Under such circumstances, the band of precipitate can be made to appear if the antibody concentration in the agar layer is sufficiently reduced. Therefore the ability of male blood to produce band 7 was tested with a 1:50 dilution of the male-absorbed antiserum in the agar layer.

Undiluted female pupal blood, when layered over this antiserum-agar, produced a single weak band of precipitate which advanced rapidly through the antiserum-agar, while a 1:1000 dilution of the same blood produced a ring of precipitate which was unable to penetrate the antigen-agar interface. Blood samples from each of fifteen diapausing male pupae were added to tubes of this antiserum-agar. Six of the fifteen undiluted blood samples produced a ring of precipitate at the interface, while eight samples produced a band of precipitate that penetrated the agar extremely slowly. The fifteenth sample of male blood produced no visible antigen-antibody precipitate, although it presumably would have done so if the sensitivity of the test had been greater. The precipitation produced by the male blood samples was identified as band 7 by the fact that mixtures of these samples with equal parts of pupal female blood which is known to produce band 7 produced only one band of precipitate rather than two bands, as would otherwise be expected. The blood of male pupae thus contains antigen 7, but at approximately one thousandth its concentration in the blood of female pupae.

Antigen 7, like all the other blood antigens we have studied, is evidently a protein, since it is non-dialyzable, labile at 75°C., and precipitable by 75 per cent saturated ammonium sulfate. Since pupal female blood retained the capacity to produce band 7 after dialysis against either 50 per cent saturated ammonium sulfate or distilled water, antigen 7 shows the solubility characteristics of an albumin.

A sexual difference in the blood proteins of *cecropia* pupae can also be seen by electrophoresis. Electrophoretic patterns of pupal female blood at pH 7.7 show one major peak not encountered in pupal male blood at this pH (Fig. 1). This peak has an intermediate position between two large peaks which characterize both male and female patterns. Antiserum-agar tests of electrophoretic fractions indicate that, at pH 7.7, antigen 7 is associated with the female characteristic electrophoretic component, and probably to some extent with the more slowly moving major component. The association of antigen 7 with more than one electrophoretic component is probably attributable to protein-protein interactions of the sort which have been observed previously in complex protein solutions (6). Such interactions, by affecting the mobility of one or more protein, could also account for the fact that the fast component, while appearing as a single peak in the female pattern at pH 7.7, can be resolved into two peaks in the male pattern.

It is worth noting that electrophoretic patterns of *cecropia* pupal blood are exceedingly complex. Indeed, the two largest peaks visible at pH 7.7 (Fig. 1) can be subdivided into additional components by varying the pH of the buffer medium. The multiplicity of proteins encountered in electrophoretic analyses is therefore in good agreement with the immunological evidence that *cecropia* pupal blood is comparable in complexity to mammalian blood serum (15).

A sexual difference in the protein nitrogen content of *cecropia* pupal blood

was noted by Chefurka (2), from whose data it can be calculated that the blood of female pupae contains 7.6 per cent protein, and the blood of male pupae only 5.7 per cent. It is possible that this difference is entirely attributable to antigen 7.

A sex-limited blood protein homologous to *cecropia*'s antigen 7 is widely distributed among saturniid moths, and probably among other Lepidoptera. This finding will be considered in detail at a later time.

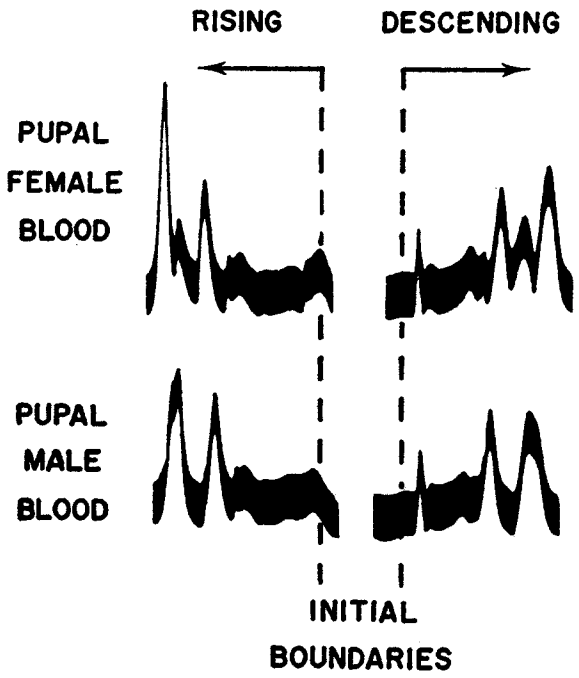

FIG. 1. Electrophoretic patterns of the blood of male and female *cecropia* pupae (pH 7.7; ionic strength 0.1; time 98 minutes for the male sample and 105 minutes for the female sample; dilution 1:3 before dialysis).

2. Demonstration of Antigen 7 in Eggs.—Since antigen 7 occurs in the blood of female pupae at approximately a thousand times its concentration in the blood of male pupae, it seemed reasonable to presume that this protein functions in some physiological process peculiar to the female. For this reason the eggs which the female insect produces during the pupal-adult transformation were tested for the presence of antigen 7.

When a 1:10 dilution of the clear liquid fraction of centrifuged yolk was mixed with an equal volume of adult antiserum *d*, a copious antigen-antibody precipitate appeared. The supernatant from this reaction mixture was solidified

with agar in glass tubes and overlayered, respectively, with samples of pupal female blood, pupal male blood, and centrifuged yolk. No bands of precipitate appeared in any of these antiserum-agar tubes. Thus it appeared that the yolk had precipitated all of the antibodies capable of combining with the blood proteins, a finding which suggests a close relationhsip between the soluble proteins of the yolk and those of the blood. This relationship will be considered in detail at a later time; for present purposes attention is directed to antigen 7.

If the yolk can inactivate the antibodies responsible for the production of band 7 in antiserum-agar tests, the yolk should itself be able to produce band 7 in antiserum-agar tests. This proved to be the case. When layered over solidified adult antiserum *d* that had previously been absorbed with one-tenth its volume of pupal male blood, centrifuged yolk produced a single heavy band of precipitate. When the antiserum had been previously absorbed with pupal female blood, the heavy band failed to appear. The band produced by the reaction between yolk and male-absorbed antiserum can thus be identified as band 7.

These results demonstrate that the yolk contains a substance which is immunologically similar to antigen 7 of pupal female blood. In order to ascertain whether antigen 7 of the blood is actually *identical* with the related substance of the yolk, we sought to determine whether the two antigens were immunologically distinguishable. To this end, antisera were prepared against the distilled water-soluble proteins of yolk and of female blood, respectively. The reaction of each of these antisera with yolk and with female blood will be considered in turn.

(*a*) Blood antiserum *a*, which had previously been absorbed with an equal volume of a 1:10 dilution of pupal male blood, produced a single dense band of precipitate when reacted in antiserum-agar with pupal female blood. With the aid of mutual dilution tests, this band of precipitate was identified as band 7.

Adult antiserum *d* and blood antiserum *a*, absorbed in each case with male blood, were combined in a graded series of mixtures, solidified with agar in glass tubes so that the dilution of total antiserum was 1:3, and overlayered with pupal female blood. Only a single band of precipitate was visible in any one tube; the *k* value of this band underwent a gradual transition in the intermediate mixtures from that of band 7 in adult antiserum *d* to that of the band produced by blood antiserum *a* (Table I). If the two antisera precipitated different antigens, two bands of precipitate should have been visible in one or more of the intermediate tubes and, one would not have expected a gradual transition in the *k* value of the single band through the intermediate tubes (15). Consequently, both antisera must have precipitated the same antigen from female blood and we can conclude that blood antiserum *a* contains antibodies against antigen 7.

Blood antiserum *a*, after previous absorption with centrifuged yolk, produced no visible bands of precipitate when overlayered in antiserum-agar with pupal female blood. This result indicates that absorption with yolk removes antibodies capable of

combining with antigen 7. If any residual antibodies capable of combining with antigen 7 remained in the antiserum after yolk-absorption, their concentration must necessarily have been lower than that required to produce a visible band of precipitate. With the aid of serial dilutions of blood antiserum *a*, it was found that, in order to render band 7 invisible, the yolk must have precipitated more than 98 per cent of the antibodies which could precipitate the blood's antigen 7. Thus, one can conclude in this case that all of the antibodies produced by rabbits against the blood's antigen 7 are precipitable by yolk. The fact that this reaction invariably appeared as a single band of precipitate in antiserum-agar suggests that a single antigen is responsible for all of the yolk's immunological similarity to antigen 7.

TABLE I

The k Values of Band 7 as Produced in Antiserum-Agar Reactions between Pupal Female Blood and a Graded Series of Mixtures of Adult Antiserum d with Each of Three Other Antisera

Composition of antiserum-agar:	Antiserum mutually diluted with adult antiserum *d*		
Adult antiserum *d* / Other antiserum	Blood antiserum *a*	Yolk antiserum *a*	Yolk antiserum *b*
	$cm.\ hr.^{-1/2}$	$cm.\ hr.^{-1/2}$	$cm.\ hr.^{-1/2}$
$\frac{0}{4}$	0.086	0.062	0.065
$\frac{1}{3}$	0.082	0.065	0.068
$\frac{2}{2}$	0.078	0.066	0.071
$\frac{3}{1}$	0.079	0.072	0.073
$\frac{4}{0}$	0.077	0.077	0.077

(*b*) Yolk antiserum *a*, after previous absorption with one-tenth its volume of pupal male blood, reacted in antiserum-agar with pupal female blood to produce a single dense band of precipitate. With the aid of mutual dilution tests, this band was identified as band 7 (Table I).

Yolk antiserum *a* that had been absorbed with pupal male blood produced two bands of precipitate when reacted in antiserum-agar with centrifuged yolk: one band could be identified by its high density as band 7; the other was a weaker band which advanced more rapidly through the antiserum-agar. This antiserum thus reacts with at least two antigens which are present in yolk but which are undetectable in pupal male blood.

After absorption with pupal female blood, yolk antiserum *a* reacted with yolk to produce a single band of precipitate whose *k* value and density were identical to those of the weak band produced by male-absorbed antiserum. This result suggests that, after absorption with female blood, the antiserum, while unable to produce band

7 when overlayered by yolk, retained the ability to precipitate a second yolk antigen: an antigen undetectable in either male or female blood. However, an alternative interpretation must be considered. It is possible that the weak band of precipitate produced in this reaction was due to a small amount of residual antibodies against the yolk's counterpart of antigen 7. If this were the case, it would be necessary to conclude that antigen 7 of the blood and that of the yolk are immunologically different molecules. Such a possibility was excluded, however, by the results of the following experiment.

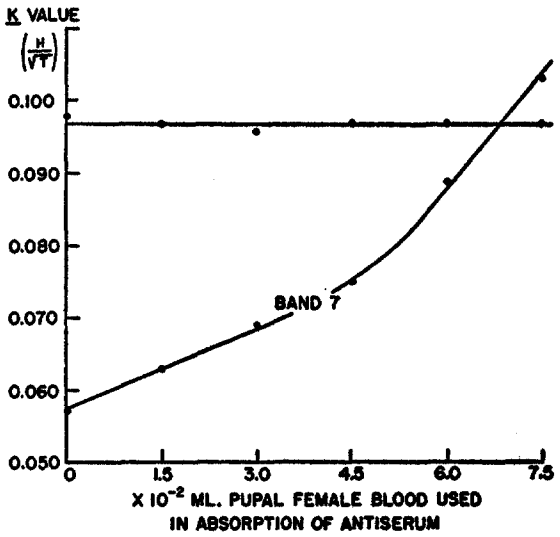

FIG. 2. The effects of prior absorption with graded volumes of pupal female blood on the k values of two bands of precipitate produced in antiserum-agar by yolk antiserum a. The antiserum was initially absorbed with one-tenth its volume of pupal male blood; it was then absorbed with graded volumes of female blood, solidified with agar and overlayered by centrifuged yolk.

Aliquots of male-absorbed antiserum were further absorbed with a graded series of volumes of pupal female blood. The supernatants from these reaction mixtures were solidified with agar so that the antiserum dilutions were 1:3, and overlayered by aliquots of a sample of centrifuged yolk. The k values of band 7 and of the weaker band produced in this reaction were plotted as functions of the volume of pupal female blood used for the preliminary absorption (Fig. 2).

As the male-absorbed antiserum was further absorbed with increasing volumes of pupal female blood, the density of band 7 in this test steadily decreased; simultaneously its k value, as recorded in Fig. 2, steadily increased. These changes can be attributed to a progressive reduction in the concentration of antibodies producing band 7 (11). By contrast, the faster and weaker band of precipitate remained constant with respect to both k value and density, indicating that the corresponding antibodies had not been significantly precipitated by female blood. Therefore, it can be

concluded that the yolk antigen producing this particular weak band of precipitate is undetectable in both male and female pupal blood.

A critical question for present purposes is whether these two antigens, the yolk antigen which is undetectable in the blood and the yolk's counterpart of antigen 7, are independent constituents of the yolk or whether they are part of the same molecule. Since the corresponding bands of precipitate can differ greatly in k value when simultaneously produced in antiserum-agar (Fig. 2), these two antigens appear to separate readily on diffusing through an antiserum-agar column. This evidence indicates that the two antigens are, in effect, independent constituents of the yolk. One can thus conclude that the single weak band of precipitate produced in the reaction between yolk and yolk antiserum *a* that had previously been absorbed with female blood was unrelated to band 7 and that, consequently, band 7 was not visible in this reaction.

Antiserum-agar tests with serial dilutions of yolk antiserum *a* indicated that, in order to render band 7 invisible, female blood must have combined with more than 98 per cent of the antibodies which can precipitate the yolk's counterpart of antigen 7. The fact that, under a variety of conditions of antiserum and blood dilutions, this reaction appeared in antiserum-agar as a single band of precipitate indicates that antigen 7 alone was responsible for this absorption.

(*c*) Yolk antiserum *b* proved to be qualitatively similar in all its reactions to yolk antiserum *a*.

In summary, we can state that rabbit antibodies against antigen 7 of the blood were completely precipitated by a single yolk antigen. Conversely, antibodies against the yolk's counterpart of antigen 7 were completely precipitated by antigen 7 of the blood. Antiserum-agar tests thus indicate that centrifuged yolk contains an antigen which is indistinguishable from antigen 7 of pupal female blood. This conclusion is confirmed by both the immunological and physiological evidence discussed in the following sections.

3. Quantitative Studies of Antigen 7 in the Blood and Yolk.—The demonstration of antigen 7 in the yolk led to a search for a mechanism which could account for the occurrence of this protein in both the blood and the eggs. Do the ovaries synthesize antigen 7 and secrete it into the blood and the yolk? Or do the ovaries acquire antigen 7 from the blood and deposit it in the yolk during the period of egg formation? In order to distinguish among these and other possibilities, a study was made of the changes occurring in the concentration of antigen 7 during the course of metamorphosis.

The relative concentrations of antigen 7 in the blood of animals at various stages of metamorphosis and in the yolk of mature unfertilized eggs were determined by the antiserum-agar and quantitative precipitin techniques. In the antiserum-agar tests, the index of the relative concentration of antigen 7 in a particular sample of blood or yolk was the k value of band 7 produced in antiserum-agar. The antiserum-agar contained a 1:3 dilution of adult antiserum *d*, and band 7 was identified by its characteristic density.

The blood of *cecropia* males was unable to produce band 7 at any stage of metamorphosis. The only exceptions to this rule were two out of seven samples of adult male blood which produced a ring of precipitate at the antigen-agar interface. A similar ring was produced by adult male blood in antiserum which had been rendered specific for antigen 7 by absorption with a small amount of pupal male blood. Thus, antigen 7, which is undetectable in pupal male blood by the methods used here, must increase somewhat in concentration during the pupal-adult transformation of the male.

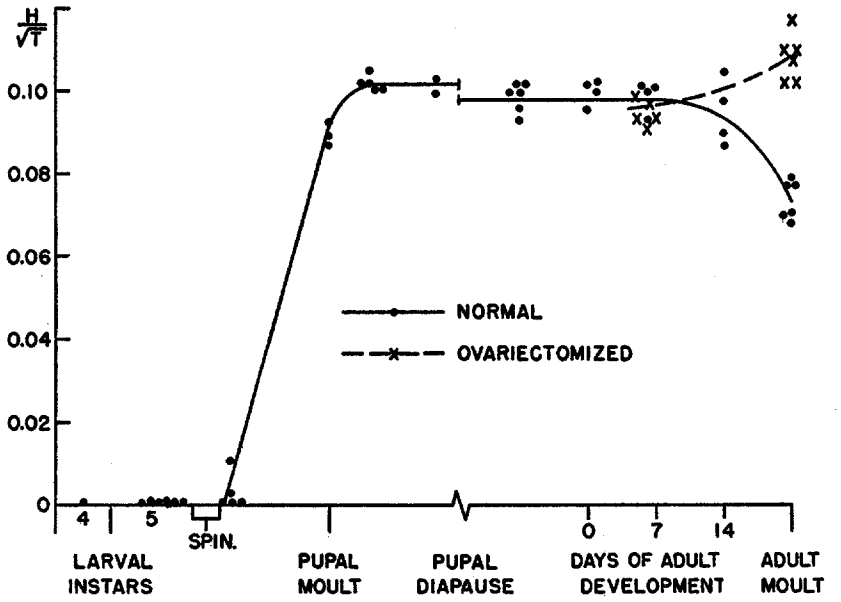

FIG. 3. The k value of band 7 as produced in antiserum-agar by the blood of *cecropia* females at the various stages of metamorphosis. The antiserum-agar contained a 1:3 dilution of adult antiserum *d*.

The blood of female larvae was unable to produce band 7 until the animal had completed the spinning of its cocoon. Before this stage of metamorphosis antigen 7 must either be absent from female blood or present in extremely low concentration.

The blood of females which had completed pupation produced band 7 with a high k value (Fig. 3). Throughout the pupal diapause and the first half of adult development, the blood continued to produce band 7 with this high k value. During the final half of adult development, however, the k value decreased significantly, a fact which suggests that the concentration of antigen 7 undergoes a corresponding decrease in the blood at that time. The magnitude of this decrease can be estimated by the fact that band 7, when produced by

adult female blood, had a k value equal to that of the band produced by a 1:3 dilution of pupal female blood.

That such a decrease in antigen 7 actually occurs was confirmed by quantitative precipitin tests. Absorption of blood antiserum *a* with one-tenth its volume of pupal male blood yielded an antiserum which, according to the following tests, contained antibodies only against antigen 7 in female blood

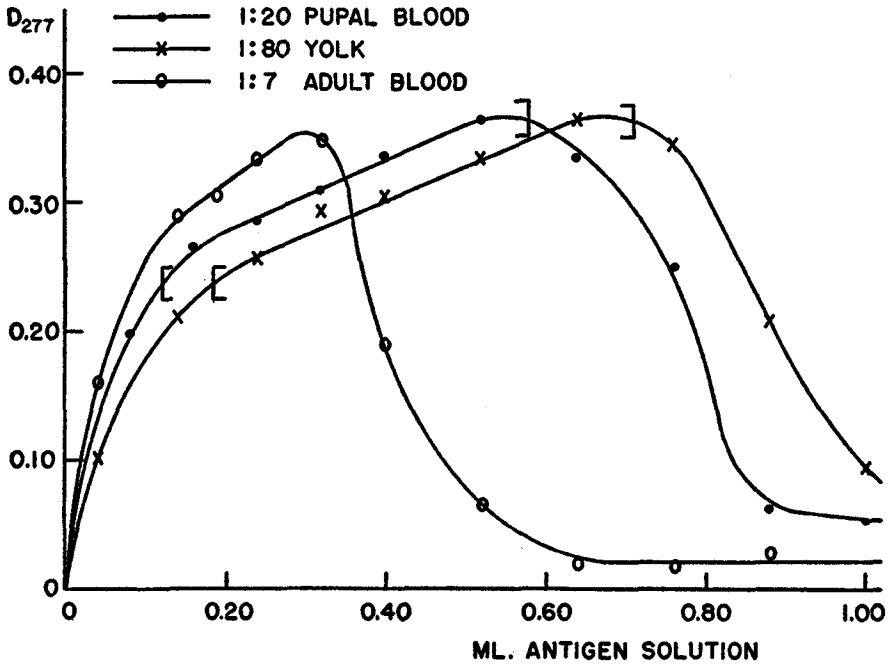

FIG. 4. The precipitin reactions of three *cecropia* antigen solutions with blood antiserum *a* which had been absorbed with pupal male blood. The amount of antigen-antibody precipitate was measured by its capacity to absorb light at 277 mμ when dissolved in dilute acetic acid. Brackets indicate the equivalence zone.

and yolk. The reactions of the absorbed antiserum with pupal female blood, with adult female blood, and with centrifuged yolk, respectively, appear to be quantitatively identical when allowance is made for differences in concentration of the antigen in the three samples (Fig. 4). Moreover, excess antigen and antibody were never simultaneously present in the supernatants from these reactions. Indeed, there appeared to be an exceptionally broad equivalence zone in which neither antigen nor antibody excess was detectable (Fig. 4). Finally, only a single band of precipitate was visible when this antiserum was reacted in antiserum-agar with female blood or with yolk; this was true in a

large number of tests in which the agar contained antiserum in dilutions ranging from 1:3 to 1:150. From these several lines of evidence, it was concluded that blood antiserum *a*, after absorption with pupal male blood, reacted specifically with antigen 7 and therefore was suitable for measuring concentrations of antigen 7 by means of the quantitative precipitin technique.

As recorded in Table II, the quantitative data obtained by the antiserum-agar technique were confirmed by the precipitin tests. Here again, antigen 7 was found to persist at high concentration during the first part of adult development, and then to fall to a much lower concentration.

TABLE II

The Relative Concentrations of Antigen 7 in Female Blood and Yolk as Determined by the Quantitative Precipitin and Antiserum-Agar Techniques

Antigen solution	Relative concentration of antigen 7 as determined by*	
	Quantitative precipitin tests	Antiserum-agar tests
Blood samples derived from:	Conc. ± S.D.	Conc. ± S.D.
Diapausing pupae	1.00 ± 0.16	1.00 ± 0.15
Females on 6th day of adult development	1.01 ± 0.17	1.01 ± 0.18
Adult moths	0.19 ± 0.09	0.36 ± 0.06
Ovariectomized females on 6th day of adult development	—	0.81 ± 0.14
Ovariectomized adult moths	2.2 ± 0.4	1.9 ± 0.3
Yolk	4.5 ± 0.6	>3.0

* Adjusted to set the pupal values at unity.

In the clear liquid fraction of centrifuged yolk the concentration of antigen 7 is approximately four times higher than that in pupal female blood (Table II). Since the eggs of *cecropia* are formed during the second half of adult development, it is of special interest that the decrease in the blood's concentration of antigen 7 also occurs at this particular time. This correlation suggests that the decrease of antigen 7 in the blood is due to the accumulation of the antigen in the yolk.

The quantitative data described here are summarized in Fig. 5.

4. Surgical Experiments.—

(*a*) *Ovariectomy.*—If a causal relationship exists between the elaboration of yolk and the simultaneous depletion of the blood's antigen 7, removal of the ovaries prior to this time should prevent such a depletion. This prediction was confirmed in experiments in which a series of pupae were ovariectomized and then allowed to undergo adult development.

As recorded in Table II, the concentration of antigen 7 in the blood of ovariectomized animals actually increased during adult development. This result

was obtained with both the antiserum-agar and quantitative precipitin techniques. In the blood of ovariectomized adult females, the concentration of antigen 7 was five to ten times higher than that of the blood of normal adult females (Fig. 5).

An influence of ovariectomy on the composition of adult blood was also observed electrophoretically (Fig. 6). An electrophoretic component with the

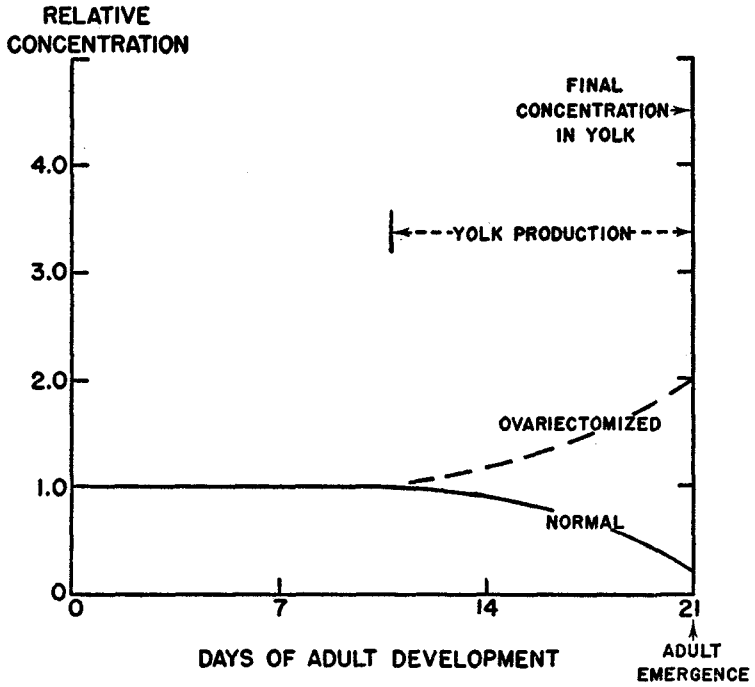

FIG. 5. The relative concentrations of antigen 7 in the blood during the adult development of normal and ovariectomized *cecropia* females, and in the yolk of eggs produced by normal females.

mobility of the female characteristic component of pupal blood was present in much larger amount in the blood of ovariectomized adult females than in the blood of normal adult females. The electrophoretic patterns further reveal that ovariectomy affects a single component preferentially, a finding which suggests that we are dealing here with a mechanism specific for antigen 7.

(*b*) *Implantation of Ovaries into Males.*—The increase in concentration of antigen 7 in the blood of ovariectomized animals during the pupal-adult transformation indicates that some tissue other than the ovaries is capable of synthesizing antigen 7. However, there remained the possibility that the ovaries also are able to synthesize antigen 7. In order to test this possibility, the imagi-

nal discs of from two to four pupal ovaries were implanted into each of a series of normal diapausing male pupae. Six recipients remained healthy and completed adult development several months later. The ovaries underwent considerable development within the male hosts. The eggs were less numerous and somewhat smaller than those produced within females, but possessed a typical chorion and a yellow, particulate yolk. This finding is reminiscent of the results obtained by Kopeć (5) who cross-transplanted the imaginal discs

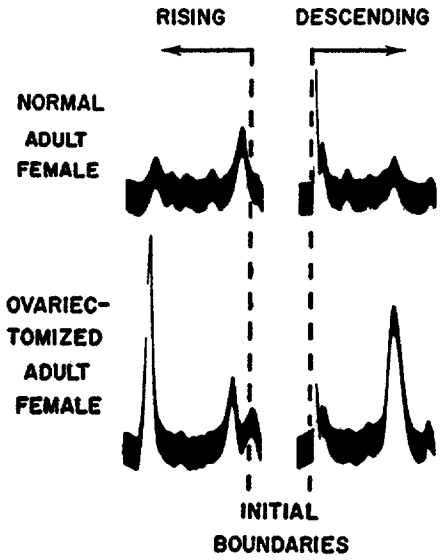

FIG. 6. Electrophoretic patterns of the blood of normal and ovariectomized *cecropia* female moths (pH 7.7; ionic strength 0.1; time 95 minutes; dilution 2:3 before dialysis).

of the gonads between male and female caterpillars of *Lymantria dispar* and related species.

The yolk obtained from the eggs of the ovarian transplants as well as the blood of their adult male hosts was tested in antiserum-agar for the presence of antigen 7. For this purpose, the antiserum-agar contained a 1:3 dilution of adult antiserum *d* which had previously been absorbed with pupal male blood. Antigen 7 was undetectable in either the blood or yolk of five of the six males. In the sixth male, a small amount of antigen 7 appeared to be present in both the yolk and blood.

Thus, in five of the six male hosts, the ovaries, even though established well enough to produce eggs, were unable to synthesize antigen 7 in detectable amounts. The sixth male host, in which detectable amounts of antigen 7 were encountered, was presumably one of the few adult males whose blood, even

under normal circumstances, contains a concentration of antigen 7 high enough to be detected by the methods employed in these tests. The fact that the ovaries were able to produce eggs in the male suggests that their failure to synthesize antigen 7 was due to an inherent inability to do so rather than to an environmental deficiency.

(*c*) *Cross-Specific Blood Transfusions.*—Conclusive evidence that the ovarian antigen 7 is derived from the blood was obtained in experiments in which female pupae of the silkworm *Telea polyphemus* were transfused with blood from *cecropia* female pupae.

The blood and eggs of *polyphemus* female pupae contain an antigen which corresponds to *cecropia*'s antigen 7. Adult antiserum *d*, after absorption with the blood of *cecropia* male pupae, reacted in antiserum-agar with the blood of

TABLE III

The k Value of cecropia's Band 7 Produced by the Blood and Yolk of Telea polyphemus Females after Transfusion in the Pupal Stage with cecropia Female Blood

Animal	*k* value of band 7 produced by		
	Pupal blood	Adult blood	Yolk
	$cm.\ hr.^{-1/2}$	$cm.\ hr.^{-1/2}$	$cm.\ hr.^{-1/2}$
1	0.106	0.040	0.106
2	0.107	0.026	0.107
3	0.110	0.044	0.112
4	0.108	0.029	0.098
5	0.108	0.076	0.104

polyphemus female pupae and with centrifuged yolk to produce a single band of precipitate. The blood of *polyphemus* male pupae was unable to produce this band of precipitate. Consequently this reaction can be attributed to the precipitation of a *polyphemus* antigen which corresponds both immunologically and physiologically to *cecropia*'s antigen 7.

One can, however, distinguish *polyphemus* antigen 7 from that of *cecropia*. Adult antiserum *d*, after absorption with the blood of *polyphemus* female pupae, retained its capacity to produce band 7 when overlayered by the blood of *cecropia* female pupae. The band 7 produced by *polyphemus*-absorbed antiserum had a lower density and a higher rate of advance than that produced by unabsorbed antiserum, a fact which indicates that *polyphemus* blood was able to precipitate some, but not all, of the antibodies against *cecropia* antigen 7. Because of the immunological characteristics of these antigens, the fate of *cecropia* antigen 7 could be followed after its transfusion into *polyphemus* pupae. Needless to say, this procedure was feasible because of the apparent inability of insects to produce antibodies in response to injections of foreign proteins (1).

Of ten *polyphemus* female pupae transfused with the blood of *cecropia* fe-

male pupae, five developed into healthy moths. The blood of the transfused pupae and the blood and egg-yolk of the subsequently produced moths were reacted in antiserum-agar with a 1:3 dilution of adult antiserum *d* that had previously been absorbed with the blood of *polyphemus* female pupae. Band 7 was identified by its characteristic density.

In every case, both the blood and the yolk produced *cecropia*'s band 7. Indeed, the yolk obtained from each of the five *polyphemus* female moths produced *cecropia*'s band 7 with a k value which was considerably higher than that produced by the blood sample drawn from the same moth (Table III). This result provides conclusive evidence that antigen 7 of the yolk is derived from the blood. Moreover, if the k value is taken in this case as an index of antigen concentration, *cecropia* antigen 7 was present in *polyphemus* yolk in a concentration considerably higher than that of the adult blood from which the antigen had been withdrawn.

DISCUSSION

The experimental results provide a consistent body of evidence that antigen 7 is secreted into the blood by some tissue other than the ovaries, and that during the period of egg formation the ovaries remove the antigen from the blood and deposit it in the yolk. Thus, the blood's concentration of antigen 7, while normally decreasing during the period of egg formation, is caused to increase when, by ovariectomy, the insect is prevented from forming eggs. The ovaries themselves do not synthesize antigen 7 because they are unable to incorporate the antigen in the yolk when placed in an environment lacking this protein. Finally, the demonstrated ability of *polyphemus* ovaries to incorporate *cecropia*'s antigen 7 from transfused *cecropia* blood affords conclusive evidence that the antigen is taken up by the ovaries from the blood.

In 1926, Umeya (16) reported evidence which indicated that the yolk pigments of *Bombyx mori* eggs are derived from the blood. The yolk produced by ovaries which had been transplanted between females of the colorless and yellow-blooded races of silkworm invariably assumed the color of the host's blood. In the light of later evidence that the blood pigments of Lepidoptera are protein-bound (2, 4), Umeya's findings suggest that the proteins of the eggs are also at least in part obtained from the blood. Additional evidence for such a proposition was provided by Wigglesworth (17) who observed that, in the bug *Rhodnius*, dietary hemoglobin which finds its way into the blood as cathemoglobin enters the eggs without further modification. Reasoning from this observation, Wigglesworth proposed that the normal egg proteins might also be derived from the blood. This suggestion proves definitely to be the case in the *cecropia* silkworm with regard to antigen 7.

Conceivably, the transfer of proteins from the blood may account for some of the immunological similarities that have frequently been detected between

the egg and blood proteins of vertebrates (8). It seems not unlikely that analogous mechanisms are frequently utilized in the management of proteins by cells.

It is noteworthy that the concentration of antigen 7 in the undiluted yolk is four times greater than the maximum concentration attained in the blood during metamorphosis. Indeed, during the period of egg formation, the concentration of antigen 7 in the blood drops to approximately one-twentieth its final concentration in the yolk. It thus appears that the ovaries, or their contained eggs, are endowed with the capacity to concentrate antigen 7.

The available evidence indicates that dehydration of the fully formed yolk can scarcely account for this concentration; the fact that mature lepidopterous eggs contain 48 to 66 per cent water (9) renders this hypothesis untenable. Furthermore, dehydration should be detectable in terms of an appreciable shrinkage of the yolk during the final stages of egg formation, while, in fact, the yolk's volume appears to increase progressively from the initiation of yolk deposition until the production of the chorion.

The apparent transfer of antigen 7 against a concentration gradient argues that an active transport mechanism is here involved. However, the presence of antigen 7 in higher concentration in the yolk than in the blood is not, in itself, adequate evidence for the occurrence of active transport, for the fluid matrix of the yolk may differ profoundly from the blood with regard to its properties as a solvent for antigen 7. Indeed, as Rosenberg (13) points out, thermodynamic considerations lead to the prediction that a protein, by virtue of properties associated with its large size, can be extraordinarily sensitive even to minor differences between solvents. It is thus conceivable that, during the period of yolk production, antigen 7 is in diffusion equilibrium between blood and yolk, even though its distribution between the two is conspicuously one-sided. Further insight into the mechanism for concentrating antigen 7 in the yolk must therefore await more extensive physical and chemical investigation of the system.

Since only a trace of antigen 7 is present in the blood of *cecropia* male pupae, the large amounts of this protein in pupal female blood is presumably an expression of the female's potentialities for egg formation. *Cecropia* can feed neither as a pupa nor as a moth, and the eggs must therefore be produced entirely from stored materials. Since the adult moth lives only a few days and must oviposit immediately after its emergence, it would be a matter of economy and efficiency for the stored materials to take the form of definitive constituents of the future eggs. The production of antigen 7 during the larval-pupal transformation may therefore be considered a precocious step in the production of yolk. It is a biochemical step which precedes by 8 to 10 months the first microscopically visible production of yolk.

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SUMMARY

1. In the pupal stage of the *cecropia* silkworm, antigen 7, a protein with the solubility characteristics of an albumin, is present in female blood in approximately a thousand times higher concentration than in the blood of males. Antigen 7 is undetectable in the blood of larvae of either sex. It first appears in the blood after the larva has spun its cocoon, and is present throughout all subsequent stages of metamorphosis. Late in the pupal-adult transformation, when the eggs are produced, the concentration of antigen 7 in female blood decreases significantly.

2. An antigen which could not be distinguished from antigen 7 immunologically is present in solution in the yolk of unfertilized eggs.

3. In females which, by ovariectomy, were prevented from forming eggs, the concentration of antigen 7 in the blood increased during the usual period of egg formation rather than undergoing the normal decrease. Ovaries transferred to the hemocoel of males produced eggs but were unable to incorporate antigen 7 in the yolk unless a detectable amount of the protein was present in the blood. The ovaries of *polyphemus* females which had been transfused with *cecropia* blood incorporated *cecropia* antigen 7 into the eggs they produced. These lines of evidence indicate that antigen 7 is secreted into the blood by some tissue other than the ovaries, and that it is subsequently drawn from the blood and deposited in the yolk.

4. The concentration of antigen 7 in the clear, liquid fraction of the yolk is four times higher than the maximum concentration attained in the blood during metamorphosis, and twenty times higher than that of the blood at the conclusion of egg formation. The protein thus appears to be transferred from blood to yolk against a concentration gradient.

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