CONTROL OF A SEX-LIMITED HAEMOLYMPH PROTEIN BY CORPORA ALLATA DURING OVARIAN DEVELOPMENT IN PERIPLANETA AMERICANA (L.)

K. K. THOMAS [1] AND J. L. NATION

Zoology Department, University of Florida, Gainesville, Florida

There is considerable evidence to suggest that in a wide variety of insects corpora allata are involved in yolk formation, egg maturation and functional activity of accessory sex glands. The control of yolk formation and egg maturation by corpora allata was first demonstrated by Wigglesworth (1936) in *Rhodnius prolixus* and subsequently has been confirmed in other insects (see Johansson, 1958). In some insects, such as *Calliphora erythrocephala* (Thomsen, 1952) and *Schistocerca gregaria* (Highnam, 1962), it appears that egg maturation and yolk deposition are largely under the hormonal control of the median neurosecretory cells of the brain. In certain insects there is evidence to suggest that corpora allata are involved in various phases of protein metabolism and that the effects on egg maturation and yolk deposition could be due to their influence on protein metabolism (Gilbert and Schneiderman, 1961; Vanderberg, 1963).

The present study is an attempt to test the relationship between corpora allata and protein synthesis during ovarian development in *Periplaneta americana,* and to correlate any changes in protein metabolism with yolk deposition and egg maturation. Haemolymph was chosen as a likely medium to reflect changes in protein metabolism.

Materials and Methods

Periplaneta americana adults were taken from a stock colony maintained in the laboratory on dog biscuits and water. The stock colony was started from a larger colony which had been maintained for more than ten years by the Department of Entomology, University of Florida. Newly molted adult females were separated daily and kept separately for surgical procedures. Allatectomy was performed in all cases on females no later than 48 hours after imaginal molt. The allatectomized females and sham-operated controls of the same age were kept with males, for Griffith and Tauber (1942) reported that mating influences egg maturation in *Periplaneta* during the first pre-oviposition period.

Allatectomy

Surgical procedure for removal of corpora allata was that described by Bodenstein (1953). Corpora allata were removed with a pair of finely tipped forceps by severing the allatal nerves without damaging the corpora cardiaca. The pulled-

[1] Present address: Department of Biological Sciences, Northwestern University, Evanston, Illinois.

out corpora allata were later examined microscopically to insure complete removal. After the operation a few crystals of streptomycin sulfate were added to the wound and the flap of cuticle pulled back into place and sealed with molten paraffin. Mortality was 15%. The corpora allata of sham-operated controls of the same age as experimental animals were exposed but not removed. All operated animals were able to move about and feed in the normal way.

Ovariectomy

Ovaries were removed from 7-day-old adults. A roach was anaesthetized with ether and the adbomen placed ventral side down into a groove in a wax plate and firmly held in position with a strip of "plasti-Tak." A small transverse incision was made on the intersegmental membrane between the sixth and seventh abdominal segments. Through the slit the ovaries were removed with a pair of forceps. In controls the same procedure was followed except that ovaries were not actually removed. The wound was sealed with molten paraffin, and unless damage to the gut occurred, the insects made a quick recovery after the operation and fed in a normal manner.

Collection of haemolymph

Haemolymph for electrophoresis and protein determinations was collected by the centrifugation technique described by Siakotas (1960) with the modification that the alimentary canal was not removed from the animals, but mouth and anus were sealed with molten paraffin. Haemolymph was used immediately after collection or stored at −18° C. for future use.

Electrophoretic procedures

Electrophoretic separation of haemolymph proteins was carried out on cellulose acetate strips in a Shandon Universal model electrophoresis tank with a Beckman Duostat power supply. The buffer system, freshly prepared each week, was that of Brackenbridge (1960). Cellulose acetate strips, 2.5 × 12 cm. (obtained from Consolidated Laboratories Inc., Chicago Heights, Illinois), were processed as suggested by Brackenbridge (1960) and placed in position in the electrophoresis tank. Three hundred milliliters of buffer solution were poured into each of the electrode wells. Whatman 3 MM wicks, saturated with the buffer, connected the buffer solution in the electrode wells with the strips. With a calibrated micropipette 2.0 μl. of the centrifuged haemolymph were applied to the center of the strip in the tank. A constant current of 0.4 milliamperes per cm. width of strip was used in all cases. An initial potential of 200 volts fell to 150 volts at the end of a two-hour run, at which time the strips were taken out and dried in an oven at 100° C. for 15 minutes.

Strips were stained with 0.002% Nigrosin in 2% acetic acid for 15–20 minutes, rinsed in water, dried at room temperature and stored in the dark until scanning. Strips were stained for glycoproteins using periodic acid-Schiff reagent as described by Bodman (1957). Stained strips were cleared in paraffin oil and scanned in a model 52-C densitometer (Photovolt Corporation, New York).

Quantitative determination of total haemolymph proteins

Total haemolymph protein concentration was measured by the colorimetric method of Lowry *et al.* (1951). Haemolymph protein determinations were made simultaneously on both allatectomized and sham-operated females of the same age. After sample color development, optical density was measured at 700 mμ against a water blank and a crystalline bovine serum albumen standard.

Radioisotope experiments

A sample of 14 allatectomized and 14 sham-operated females was used for these experiments 2–4 days after operation. Each female received 5 μc. of tritium-labelled amino acids from a mixture of equal parts of glycine, leucine, and histidine (specific activity 2.5 mc./m*M*) in 0.1 ml. of physiological saline. Injection was accomplished through the femoral membrane of the metathoracic legs following anesthetization with CO_2. At intervals of 1.5, 3, 6, 12, 24 and 48 hours after injection, haemolymph was removed and protein precipitated with 95% ethanol to remove all radioactive amino acids not incorporated into the haemolymph proteins. The precipitate was dried, weighed, and samples converted to a uniform condition for counting by a modification of the Schoniger combustion technique reported by Buyske *et al.* (1961) and Oliverio *et al.* (1962). The samples were ignited in a Thomas-Ogg safety oxygen flask igniter (Arthur Thomas Co., Philadelphia, Pennsylvania), whereby tritium was converted to water. A 15.0-ml. sample of toluene-phosphor solution containing 20% ethanol was added to the flask and a 10.0-ml. sample withdrawn for counting in a transistorized Tricarb Liquid Scintillation Counter (Packard Instrument Co.). The result of triplicate 10-minute counts of each sample is presented as specific activity (counts/minute/mg. protein).

Results

1. *Effects of allatectomy on ovarian development*

In all roaches allatectomized within 48 hours after imaginal molt, the ovary remained small and the oocytes neither grew nor deposited yolk. The sham-operated controls laid eggs 15–20 days after the operation. Allatectomy performed on females later than 48 hours after the imaginal molt did not completely prevent egg maturation.

2. *Effects of allatectomy on the electrophoretic pattern of haemolymph proteins*

A typical electrophoretic pattern of haemolymph proteins of allatectomized and sham-operated females at intervals after the operation is shown in Figure 1. The relative amounts of the different fractions of haemolymph proteins, obtained by scanning the strips of Figure 1, are shown in Figure 2. Of the five electrophoretically separable haemolymph protein fractions (arbitrarily numbered 1 through 5), 1–4 migrate toward the anode while 5 migrates toward the cathode. Quantitative analyses of the protein fractions after staining with light green S.F. in potassium biphthalate buffer and subsequently eluting the stains according to the method of Brackenbridge (1960) revealed that in unoperated females, fraction 4 makes up

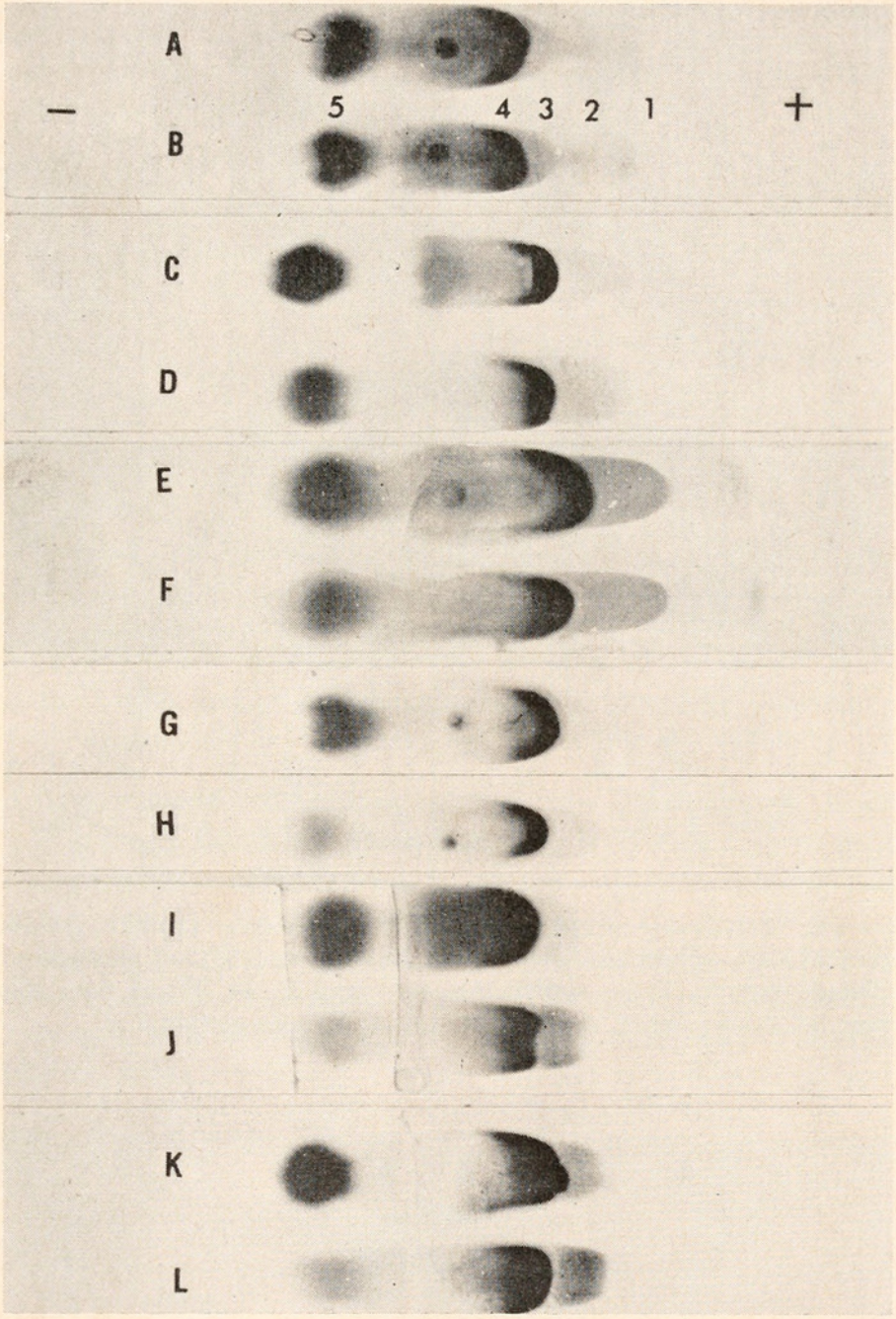

FIGURE 1. Electropherograms of haemolymph proteins of sham-allatectomized (A, C, E, G, I, and K) and allatectomized (B, D, F, H, J, and L) female roaches at respective intervals of 3, 7, 14, 17, 21, and 28 days after the operation. Separated fractions arbitrarily numbered 1–5.

45–51% of the haemolymph proteins, while fraction 5 constitutes 39–45% of the total proteins.

The general results in Figures 1 and 2 have been verified in several electropherograms of allatectomized and sham-operated roaches at each interval after operation. Fraction 5 decreases in concentration after allatectomy, in contrast to a relatively constant concentration in this fraction from the sham-operated females. No significant changes were evident in fraction 4. The other fractions were too small to detect any differences.

The decrease in concentration of protein in fraction 5 observed in the allatectomized females prompted studies to observe the nature of this fraction in the

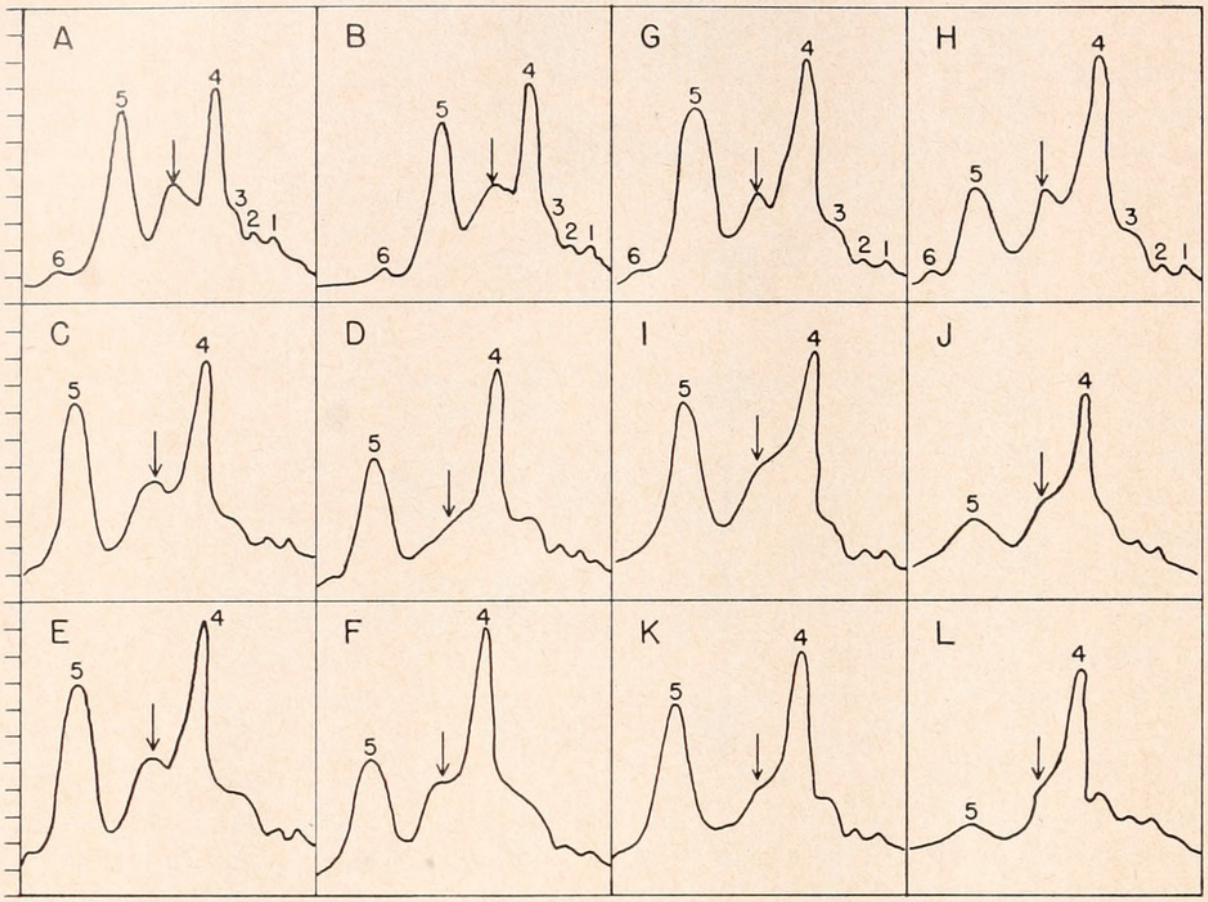

FIGURE 2. The relative concentrations of haemolymph protein fractions 1–5 from electropherograms of sham-allatectomized (A, C, E, G, I, and K) and allatectomized (B, D, F, H, J, and L) female roaches at respective intervals of 3, 7, 14, 17, 21, and 28 days after the operation. Arrow indicates point of application of haemolymph sample.

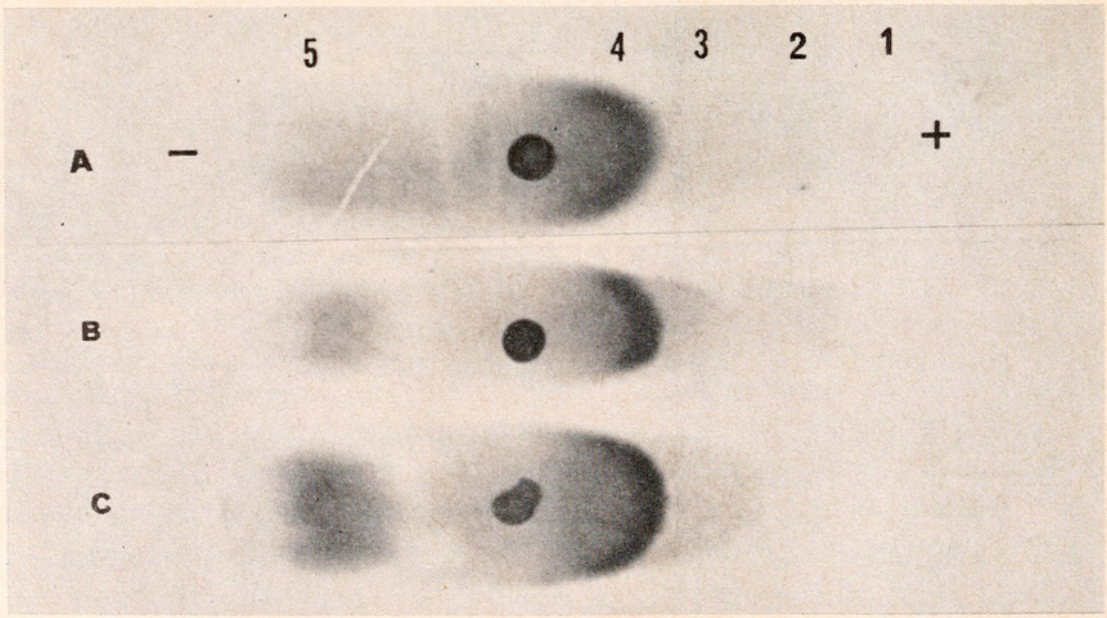

FIGURE 3. Electropherograms of haemolymph proteins from a sham-allatectomized male (A), allatectomized female (B), and sham-allatectomized female (C) roach 14 days after the operation.

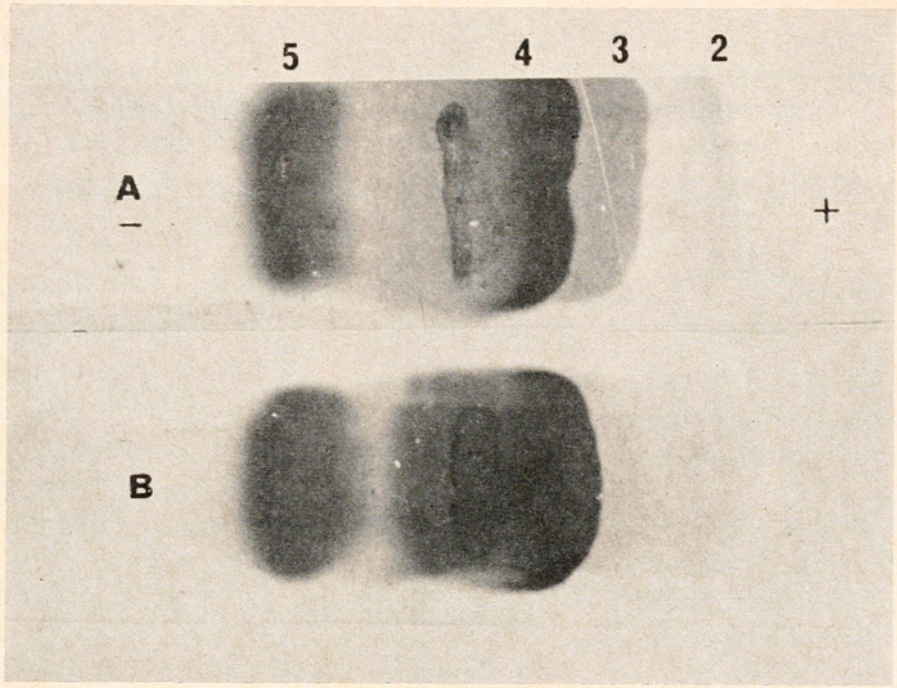

FIGURE 4. Electropherograms of haemolymph proteins from sham-ovariectomized (A) and ovariectomized (B) roaches 7 days after the operation.

male. A typical electrophoretic pattern of the male haemolymph proteins is shown in Figure 3. Protein of fraction 5 from the male is low in concentration compared to that from the female of the same age. This suggests that fraction 5 may be sex-linked, being present in greater concentration in the female than in the male. To test this further, electrophoretic studies were made on haemolymph of ovariectomized females, and a typical electrophoretic pattern obtained 7 days after ovariectomy, along with that of a sham-ovariectomized female, is shown in Figure 4. Protein in fraction 5 has increased in concentration in the ovariectomized roach in excess of that in the sham-operated control. Fractions 4 and 5 in ovariectomized roaches showed additional changes in composition during aging. After the initial

TABLE I

Comparison of haemolymph protein concentrations of allatectomized and sham-operated control females of the American cockroach, Periplaneta americana

Days after operation*	Allatectomized		Sham-operated control	
	Number of females	Haemolymph protein concentration (g./100 ml.) $\bar{X} \pm SD$	Number of females	Haemolymph protein concentration (g./100 ml.) $\bar{X} \pm SD$
7**	5	1.47 ± 0.07	5	1.86 ± 0.13
14	5	1.41 ± 0.16	5	2.03 ± 0.25
21	5	1.29 ± 0.08	5	1.75 ± 0.09
28	5	1.25 ± 0.13	6	1.85 ± 0.14
35	5	1.09 ± 0.14	6	1.71 ± 0.10

* All insects operated upon 8–48 hours after imaginal molt.

** Five newly molted females had an average concentration of 0.85 ± 0.02 g./100 ml. haemolymph.

TABLE II

Comparison of haemolymph protein concentration of ovariectomized and sham-operated females of the American cockroach, Periplaneta americana

Ovariectomized		Sham-operated	
Number of females	Haemolymph protein concentration (g./100 ml.) $\bar{X} \pm SD$	Number of females	Haemolymph protein concentration (g./100 ml.) $\bar{X} \pm SD$
4	2.59 ± 0.18	5	1.82 ± 0.13

All insects operated upon 7 days after imaginal molt.
All protein determinations were made 14 days after operation.

increase in stainable material following ovariectomy, fraction 5 began to decrease after about 14 days in stainable material, and after 30 days very little protein was left. In the meantime fraction 4 began to increase in material which stained as glycoprotein.

3. *Effects of allatectomy and ovariectomy on total haemolymph protein concentration*

The total haemolymph protein concentration of allatectomized and sham-operated females is shown in Table I. Total haemolymph protein concentration of allatectomized females decreases far below that of sham-operated controls.

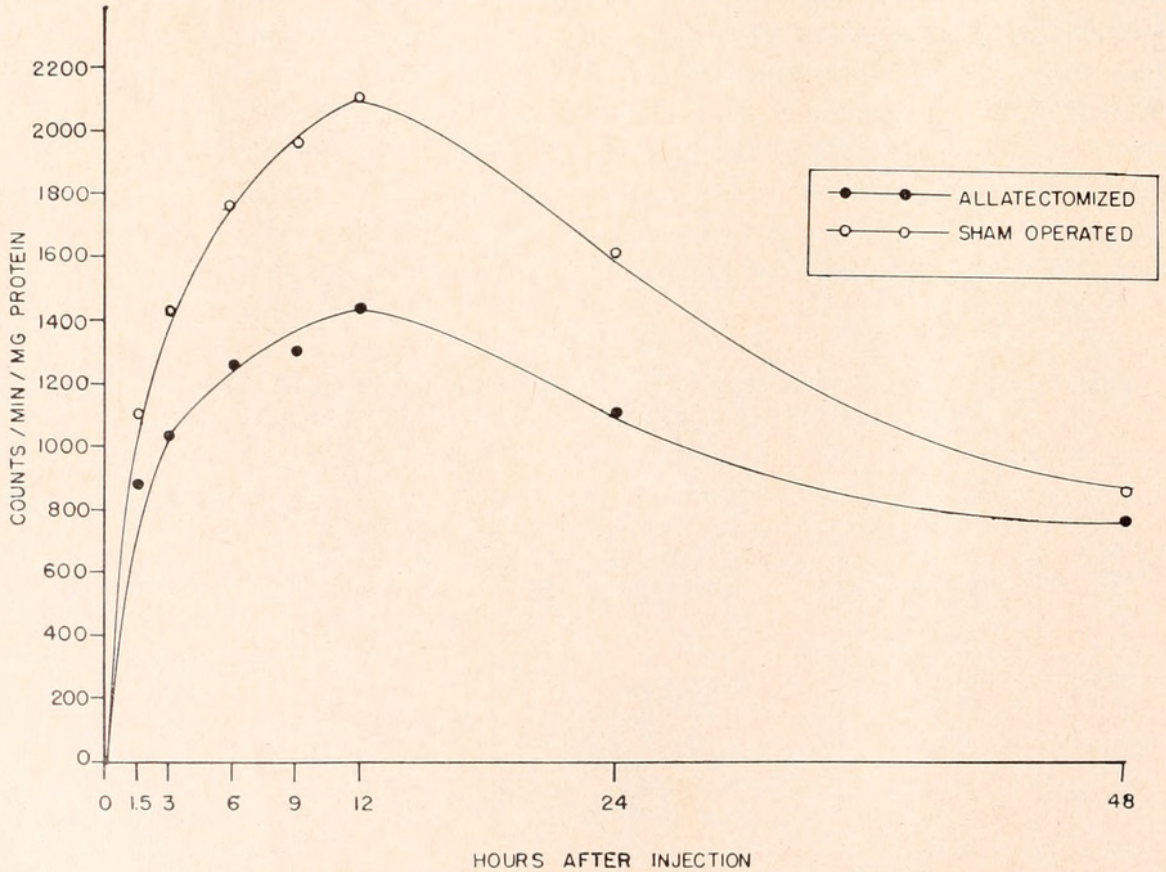

FIGURE 5. Comparison of incorporation of tritium-labelled glycine, leucine, and histidine into the haemolymph proteins of allatectomized and sham-allatectomized female roaches (5 μc./roach). Points are averages of two experiments.

Haemolymph protein concentrations of 14 days post-ovariectomized females and of sham-ovariectomized females of the same age are shown in Table II. It is clear that an increase in blood protein concentration follows ovariectomy.

4. *Effects of allatectomy on incorporation of H^3-labelled amino acids into haemolymph proteins*

Figure 5 shows that allatectomy inhibits incorporation of H^3-labelled amino acids into haemolymph proteins. The maximum rate of incorporation occurred 12 hours after injection of the amino acids, at which time the controls exhibited a rate which was $1\frac{1}{2}$ times that of allatectomized females. We have no evidence that labelled proteins were synthesized in the haemolymph; they may have been synthesized elsewhere and then released into the haemolymph.

Discussion

The interactions of the various hormones involved in insect reproduction are not fully understood. The role of corpora allata in egg maturation and in the functional activity of the accessory sex glands has been well established in all the major orders except Lepidoptera (see Johansson, 1958). Among roaches several species have been studied and the influence of corpora allata on egg maturation confirmed. The present study with *Periplaneta* supports the findings of Scharrer (1946) in *Leucophaea,* Engelmann (1959) in *Diploptera,* and Roth and Stay (1959) in *Blatella,* that allatectomy does interfere with the normal development of the ovaries.

Though endocrine control of egg maturation has been well documented, the nature of the hormonal control is still a matter of dispute. Pfeiffer (1945) has shown in *Melanoplus* that the corpora allata are the source of a metabolic hormone which controls metabolism of fat and mobilization of some non-fatty materials and facilitates the production of materials necessary for yolk deposition. The concept of a metabolic hormone was further strengthened by L'Hélias (1953), who postulated that the hormone from the corpora allata favors synthesis of proteins. Wigglesworth (1954) maintains that deficiency of yolk deposition in the oocytes of *Rhodnius* may be due to decreased protein synthesis. Recently Vanderberg (1963) presented evidence to show that corpora allata are involved in synthesis of proteins and RNA. Highnam *et al.* (1963) argue that oocyte growth in *Schistocerca* is facilitated by an allatum-produced gonadotrophic hormone exerting its influence, not by general metabolic effects, but by mediating the uptake of proteins from the haemolymph by the oocytes. Furthermore, Highnam *et al.* (1963) found that haemolymph protein levels in allatectomized *Schistocerca* adults exceeded the levels found in sham-operated controls.

The failure of the haemolymph protein concentration in allatectomized *Periplaneta* females to reach the level characteristic of sham-operated control females, and the depressed protein synthesis from labeled amino acids in allatectomized females, are interpreted by us to support the data of Pfeiffer (1945), L'Hélias (1953), and Wigglesworth (1954) that allatectomy does interfere with at least certain aspects of protein synthesis and metabolism.

The gradual decline in concentration of protein fraction 5 (Figs. 1 and 2)

following allatectomy further suggests that ovarian development in *Periplaneta* is related to a protein or proteins whose synthesis is somehow under control of the corpora allata. Fraction 5 may be sex-limited, for in all males tested fraction 5 stained only weakly, giving much the appearance of allatectomized females after 14 days. Stephen and Steinhauer (1957) and Siakotas (1960) demonstrated by electrophoretic techniques not directly comparable with the technique we have used that sex-limited or sex-related differences in concentrations of protein in separated bands do exist in *Periplaneta* haemolymph. Telfer (1954) not only demonstrated the existence of a sex-limited protein in haemolymph of *Platysamia cecropia* females, but by immunological means showed that this same protein enters the eggs during maturation.

Although we have no data to indicate that fraction 5 protein enters the egg during maturation, ovariectomy does allow an increase in stainable material in fraction 5 for a few days following the operation (Fig. 4), which is certainly suggestive that fraction 5 protein may be removed from the haemolymph by the ovaries. After 14 days post-ovariectomy, however, there is clear evidence that the protein compositions of both fractions 4 and 5 are being altered, with fraction 5 decreasing in stainable material and fraction 4 increasing in stainable material which stains as glycoprotein. These changes have not been observed in control females. We interpret the 14 days post-ovariectomy changes as a failure of the maintenance of normal allatum activity in the absence of the ovaries. Since *Periplaneta* frequently lives beyond the reproductive period, it would be interesting to know if similar changes occur during senescence.

Recently Englemann (1965) has observed a correlation between the haemolymph protein concentration and changes in activity of innervated and isolated corpora allata of *Leucophaea*. He thinks that nutrition acts directly on the corpora allata and not through the mediation of the central nervous system. Protein metabolism in turn is also influenced by the corpora allata. He attributes the hypertrophy of the corpora allata after ovariectomy to a persistently high protein level in the haemolymph. Our results support the observations of Englemann (1965).

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Summary

1. The removal of corpora allata from females of the roach, *Periplaneta americana,* soon after the imaginal molt prevented growth and yolk formation in the oocytes.

2. No immediate effect on ovarian development was noticed when mature adult females were allatectomized.

3. The haemolymph protein concentration of allatectomized females failed to reach the level of the sham-operated female roaches of the same age.

4. Electrophoresis of haemolymph proteins on cellulose acetate strips revealed a gradual decrease and final disappearance of one of the main protein fractions in the allatectomized females.

5. The haemolymph protein concentration of ovariectomized roaches rose to a level far in excess of that of sham-ovariectomized roaches of the same age. A significant increase in protein fraction 5 has been observed in the ovariectomized roaches compared with that of sham-ovariectomized roaches.

6. In male roaches fraction 5 remained at low concentration at all times, suggesting that protein fraction 5 is sex-limited in character.

7. Allatectomized roaches exhibited a slower rate of incorporation of tritium-labelled amino acids into haemolymph proteins than the sham-operated roaches.

8. It is concluded that the effects of corpora allata on egg maturation and yolk deposition may be due to their influence on metabolic processes, particularly the synthesis of a sex-limited haemolymph protein which presumably enters the growing oocytes.

LITERATURE CITED

Bodenstein, D., 1953. Studies on the humoral mechanisms in growth and metamorphosis of the cockroach, *Periplaneta americana*. II. The function of the prothoracic gland and the corpus cardiacum. *J. Exp. Zool.*, **123**: 413–433.

Bodman, J., 1957. The separation of serum glycoproteins by continuous electrophoresis. Laboratory Practice, Part 1, Sept., p. 517.

Brackenbridge, C. J., 1960. Optimal fractionation conditions for the quantitative analysis of human serum protein fractions by cellulose acetate electrophoresis. *Anal. Chem.*, **32**: 1357–1359.

Buyske, D. A., R. Kelly, J. Florin, S. Gordon and E. Peets, 1961. Determination of tritium and C^{14} in biological samples by rapid combustion techniques. *Atom Light*, **20**: 1–7.

Engelmann, F., 1959. The control of reproduction in *Diploptera punctata* (*Blattaria*). *Biol. Bull.*, **116**: 406–419.

Engelmann, F., 1965. The mode of regulation of the corpus allatum in adult insects. *Archives d'Anat., microscopique et de Morphol. expér. Paris*, **54**: 387–404.

Gilbert, L. I., and H. A. Schneiderman, 1961. Some biochemical aspects of insect metamorphosis. *Amer. Zool.*, **1**: 11–51.

Griffith, J. T., and O. E. Tauber, 1942. Fecundity and parthenogenesis of the American cockroach, *Periplaneta americana*. *Physiol. Zoöl.*, **15**: 196–209.

Highnam, K. C., 1962. Neurosecretory control of ovarian development in the desert locust. *Mem. Soc. Endocrin., No. 12*: 379–390.

Highnam, K. C., O. Lusis and L. Hill, 1963. The role of the corpora allata during oocyte growth in the desert locust, *Schistocerca gregaria* Forsk. *J. Ins. Physiol.*, **9**: 587–596.

Johansson, A. S., 1958. Relation of nutrition to endocrine-reproductive functions in the milkweed bug *Oncopeltus fasciatus* (Dallas) (Heteroptera: Lygaeidae). *Nyt. Mag. Zool.*, **7**: 1–132.

L'Hélias, C., 1953. Rôle des corpora allata dans le metabolisme des glucides, de l'azote et des lipides chez le phasme *Dixippus morosus*. *C. R. Acad. Sci. Paris*, **236**: 2164–2166.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

Oliverio, V. T., C. Denham and J. D. Davidson, 1962. Oxygen flask combustion in determination of C^{14} and H^3 in biological materials. *Anal. Biochem.*, **4**: 188–189.

Pfeiffer, I. W., 1945. Effect of the corpora allata on the metabolism of adult female grasshoppers. *J. Exp. Zool.*, **99**: 183–233.

Roth, L. M., and B. Stay, 1959. Control of oocyte development in cockroaches. *Science*, **130**: 271–272.

Scharrer, B., 1946. The relationship between corpora allata and reproductive organs in adult *Leucophaea maderae* (Orthoptera). *Endocrinology*, **38**: 46–55.

Siakotas, A. N., 1960. The conjugated plasma proteins of the American cockroach. I. The normal state. *J. Gen. Physiol.*, **43**: 999–1013.

STEPHAN, W. P., AND A. L. STEINHAUER, 1957. Sexual and developmental differences in insect blood proteins. *Physiol. Zoöl.*, **30**: 114–120.

TELFER, W. H., 1954. Immunological studies of insect metamorphosis. II. The role of a sex-limited blood protein in egg formation by the cecropia silkworm. *J. Gen. Physiol.*, 37: 539–558.

THOMSEN, E., 1952. Functional significance of the neurosecretory brain cells and the corpus cardiacum in the female blow fly, *Calliphora erythrocephala* Meig. *J. Exp. Biol.*, **29**: 132–172.

VANDERBERG, J. P., 1963. The role of the gonadotropic hormone in the synthesis of protein and RNA in *Rhodnius prolixus* (Hemiptera). *Biol. Bull.*, **125**: 576–581.

WIGGLESWORTH, V. B., 1936. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). *Quart. J. Micr. Sci.*, **79**: 91–121.

WIGGLESWORTH, V. B., 1954. The Physiology of Insect Metamorphosis. Cambridge University Press, Cambridge, England, 152 pp.