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EFFECT OF THE CHEMOSTERILANTS APHOLATE AND METEPA ON THE OVARIES OF THE RED COTTON BUG, *DYSDERCUS CINGULATUS* FABR. (INSECTA, HETEROPTERA, PYRRHOCORIDAE

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Studies on the histology and histochemistry of the ovaries in normal *Dysdercus cingulatus* and effects of the chemosterilants apholate and metepa on the ovaries of this animal are presented. Single injection of 10 μg metepa or 5 μg apholate per newly moulted adult female does not apparently interfere with oocyte growth or vitellogenesis. But the eggs laid after both these treatments do not hatch. Oocyte development is however inhibited after treatment of newly moulted adult females with 12.5 μg metepa or 7.5 μg apholate. Histological and histochemical studies of the ovary after this treatment indicate disintegration of germarium and follicular epithelium as well as reduction in size of oocytes and their resorption. Prefollicular tissue almost completely disappears and multiple oocytes are present in follicles after treatment with apholate. Histochemical studies reveal no yolk granules of protein, carbohydrate or lipid after treatment with both the sterilants. Treatment with apholate by contact method at 0.22 mg/ sq cm of surface of contact, inhibits oocyte growth after ptevitellogenesis, ultimately resulting in irreversible atrophy of the ovarian tubes.

INTRODUCTION

Chemosterilants affect fecundity and fertility of insects (LACHANCE *et al* 1968; CAMPION, 1972), but we know very little about the effects of the sterilants on female reproductive system. Studies on this aspect are limited mainly to flies and mosquitoes (MORGAN, 1967; MORGAN & LABRECQUE, 1962, 1964; RAI, 1964; LANDA & REZABOVA, 1965); our information in this regard extends very little to histological levels even in most of these studies. Detailed studies on the histopathological and histochemical effects of chemosterilants on hemipteran ovaries are fewer. Effects of 6-azauridine have been studied on the ovaries of *Pyrrhocoris apterus* (MASNER, 1971). Severe pathological effects on the ovaries by tepa have been reported in *Dysdercus cingulatus* on the basis of morphological studies SUKUMAR & NAIDU, 1973). The present investigations have been carried out to find out the action of the chemosterilants apholate and metepa on the ovaries of *Dysdercus cingulatus* at histological and histochemical level. Ovaries of the normal animal have also been studied.

MATERIALS AND METHODS

Rearing of animals

The red cotton bug *Dysdercus cingulatus* was reared in the laboratory on soaked cotton seeds kept on wire meshes in plastic basins of 30 cm diameter. The rearing basins were covered with clothing to prevent the animals from escaping. The seeds were changed daily. Mating started when they were two days old. The animals laid eggs when six or seven days old, among cotton seeds, in clutches. These eggs were removed to a Petri-dish while changing the cotton seeds in the morning. The eggs hatched by five days. The first instar nymphs were transferred to cotton seeds and were reared as above in basins. These animals have five nymphal stages and the freshly laid egg took about twenty five days to become adult, under the laboratory conditions.

Animals used

Newly moulted adults were collected from the stock colony. Females could be distinguished by

their larger size and the external genitalia. The newly moulted males and females were transferred to glass chimneys and reared on soaked cotton seeds; the mouth of the chimney was covered with clothing. Thus animals of known age were readily available for the study.

Histological and histochemical techniques

For the study of the normal ovary and vitellogenesis, females were sacrificed at one day interval upto six days post-emergence. The ovaries were dissected out and fixed in BOUIN'S fluid, CARNOY'S fluid, 10% formalin, or ZENKER'S fluid. For histological studies, ovaries fixed in BOUIN'S fluid or ZENKER'S fluid were processed in the routine manner. Paraffin sections were stained in HEIDENHAIN'S iron haematoxylin and eosin.

For histological studies, ovaries were embedded in either paraffin or gelatin. Proteins were demonstrated by MILLON'S reaction (after BAKER) or by mercury bromophenol blue method after BONHAG (PEARSE, 1968), using CARNOY–fixed or formalin–fixed material after embedding in paraffin or gelatin. For carbohydrates, Periodic acid Schiff technique (after MCMANUS) was employed using formalin–fixed gelatin sections or Carnoy–fixed paraffin sections. Lipids were stained using Sudan Black B or formalin–fixed gelatin sections or formalin-fixed paraffin sections. DNA was studied using Feulgen technique, on paraffin sections after fixation in CARNOY'S fluid or formalin fixation. Nucleic acids were also studied by methyl green pyronin Y method (CURNICK) employing formalin–fixed gelatin sections and Carnoy–fixed paraffin sections. Suitable controls were kept for all histochemical procedures.

Chemosterilants

Metepa and apholate were the chemosterilants employed. They were either injected into the haemocoel through pleural region after ether anesthesia by means of a microliter syringe, or the animals were treated by contact method. For injection the solutions were dissolved in distilled water at known concentration. After preliminary trials, 10 μg and 12.5 μg metepa and 5 μg and 7.5 μg apholate were chosen for detailed study. Controls received distilled water injection. For treatment by contact method, sets of PETRI-dishes of radius 5 cm and height 2.5 cm were taken and 50 mg apholate dissolved in 5cc acetone was applied uniformly to each set over all the surface of contact and allowed to dry. This gave a residue of 0.22 mg / sq cm of the surface of contact. Animals kept in acetone-treated dishes served as controls. Both experimental and control animals were kept in the PETRI-dishes for 4 hours. Only newly moulted animals were used for treatment.

Treated as well as control animals along with some normal males were reared on soaked cotton seeds in glass chimneys. Eggs laid by the animals were collected, counted and observed for hatchability studies. Treated insects were also dissected in insect Ringer 2, 3, 4, 5, 6, 12 and 19 days after treatment to note the progressive changes in the ovaries. Ovaries of control insects were dissected out 2, 3, 4, 5 and 6 days after treatment. Histology and histochemistry of the ovaries of these insects were studied as already desceribed.

OBSERVATIONS

Structure of the ovavries and vitellogenesis in the normal animal

The ovaries of *Dysdercus cingulatus* are telotrophic, each with seven ovarioles. The ovariole consists of terminal filament, germarium, vitellarium and pedicel (Fig. 1). In the germarium are the germinal cells at various stages of maturation, trophocytes, trophic core and prefollicular tissue. As each oocyte of the germarium enlarges and enters the vitellarium, prefollicular tissue which is at first multilayered, gets arranged around the oocyte in the form of follicular epithelium. This becomes ultimately one layer thick. During the previtellogenic stage which extends upto two days after emergence this differntiation of follicular epithelium takes place. The follicle cells which are at first small and mononucleate, subsequenly enlarge and become binucleate. Vitellogenesis begins on the third day and continues on the fourth and fifth days. Concomitant with the progress of vitellogenesis the abdomen swells to accommodate the enlarging oocytes.

The cytoplasm of the trophocytes, young oocytes, follicular epithelium as well as the trophic core and nutritive cords of the previtellogenic ovary are stainable rather homogeneously with techniques for proteins and RNA but no granules of these materials are visible now. The trophocytes apparently

contribute DNA, large quantities of RNA and proteins to the developing oocytes. Masses of DNA are visible in the trophocyte region, but the trophic core is not Feulgen positive. DNA appears to break down before reaching the trophic core. Small granules which are rich in both carbohydrates and proteins appear in the peripheral ooplasm of the basal oocytes on the third day. These enlarge in size and number forming the large yolk granules made up of protein-carbohydrate complex (Fig. 2). These subsequently fill the oocyte. When vitellogenesis is most active, spaces develop among follicle cells through which material can enter the oocytes from blood (Fig. 3). Smaller, lipid yolk granules which appear subsequently, are distributed amog the larger protein-corbohydrate yolk granules (Fig. 4). Vitellogenesis is complete by the end of the fifth day and chorion formation ensues subsequently. Completed, mature eggs are deposited on the sixth or seventh day.

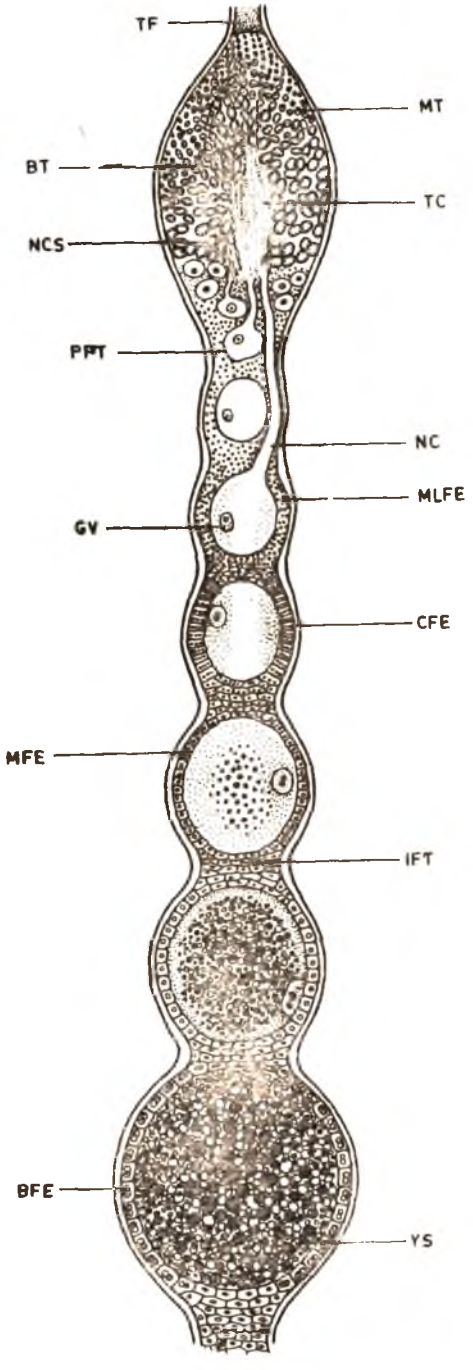

Fig. 1.

Effect of metepa

Animals treated with 10 μg metepa lay sterile eggs. This dosage does not interfere with the development of the ovary or with fecundity. Mating is normal in treated insects. Insects which receive 12.5 μg metepa appear normal on the day of the injection and on the next day. However, on the third day about 50% mortality is recorded among them. The remaining animals mate freely. Abdomen does not swell even after 12 days indicating absence of egg development. This is substantiated histologically. Infecundity is complete. Insects have been kept upto nineteen days to see whether the trend is reversed and eggs start developing again. They have been found to develop no eggs. Thus 12.5 μg metepa inhibits growth of the ovary and vitellogenesis completely.

Detailed studies on the histological changes in the ovaries after treatment with 12.5 μg metepa reveal that ovariole size as well as oocyte number are reduced in the treated insects. Visible histological changes in the

EXPLANATION OF FIGURES

Fig. 1. Diagram representing the longitudinal section of an ovariole of 3–4 day old *Dysdercus cingulatus*. BFE–binucleate follicular cell; BT–binucleate trophocyte; CFE–columnar follicle cell ; GV–germinal vesicle ; IFT–interfollicular tissue ; MFE–mononucleate follicle cell; MLFE–multilayered follicle epithelium ; MT–mononucleate trophocyte ; NC–nutritive cord ; NCS–nuclear clusters of trophocytes ; PFT–prefollicular tissue ; TC–trophic core ; TF–terminal filament; YS– yolk spheres.

Fig. 2. Section of a mature oocyte showing carbohydrate yolk granules (CARNOY'S fluid ; PAS technique).

Fig. 3. Section of a mature oocyte showing binucleate follicle cells (solid arrows). The thin connection between follicle cells (broken arrows) disappears except in certain regions.

Fig. 4. Section of a mature oocyte showing distribution of the lipid yolk spheres (dark) around the protein-carbohydrate yolk granules (unstained). (Formalin, Sudan Black B, cryostat sections).

Figs. 5 & 6. Section of the germarium of animals three days after injection of 12.5 μg metepa (Fig. 6) showing disintegration of the posterior region of the germarium (dark masses), and section of its control (Fig. 5) showing less intense trophocyte disintegration.

Fig. 7. Section of the germarium and part of the vitellarium of an animal seven days after injection of 12.5 μg of metepa showing the trophic tissue almost completely dis integrated (dark masses). Arrow shows hyperplastic follicle cells.

Fig. 8. Section of a part of the vitellarium of an ovariole four days after injection of 12.5 μg of metepa showing resorbing oocyte (RO) and the hyperplastic follicular epithelium (FE).

Fig. 9. Section of the ovariole of an animal nineteen days after injection of 12.5 μg metepa. Note the disintegrated content (G) at the basal region of the germarium. Vitellarium is almost empty.

Fig. 10. Section of the ovary of an animal two days after injection of 7.5 μg apholate. Note the disintegration of trophocytes (DT), absence of prefollicular tissue and poorly developed follicular epithelium.

Fig. 11. Section of the germarium of ovaries of an animal four days after injection of 7.5 μg apholate, showing dark staining granules in the oocytes and the disintegrated trophocytes.

Fig. 12. Section of the vitellarium of an animal four days after injection of 7.5 μg apholate, showing twin oocytes in a follicle (arrows) and poorly developed follicular epithelium.

Fig. 13. Section of the vitellarium showing resorption of the oocyte (dark masses) eight days after injection of 7.5 μg apholate.

Fig. 14. Section of the ovary of an animal nineteen days after apholate treatment by contact method, showing atrophied ovarian tube and invading follicle cells (arrows).

(All figures from 2–14, unless otherwise stated, are from preparations made from tissues fixed in BOUIN'S fluid and stained in HEIDENHAIN'S iron haematoxylin and eosin).


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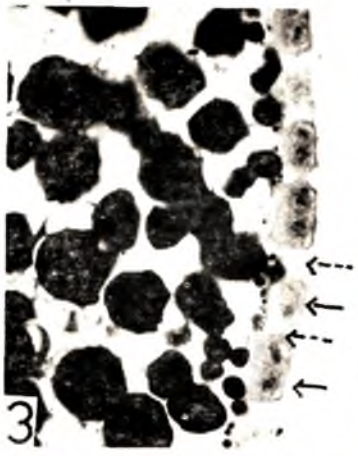
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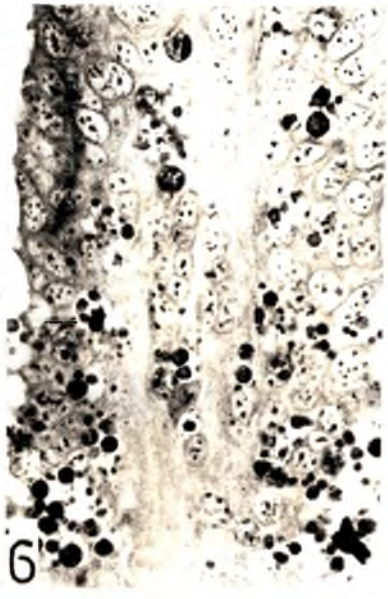
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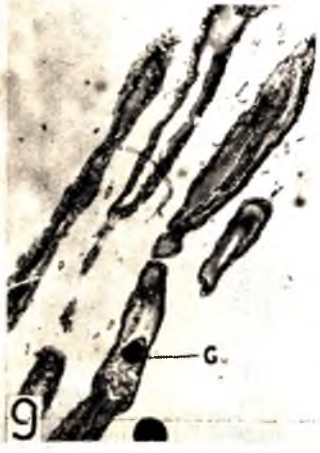

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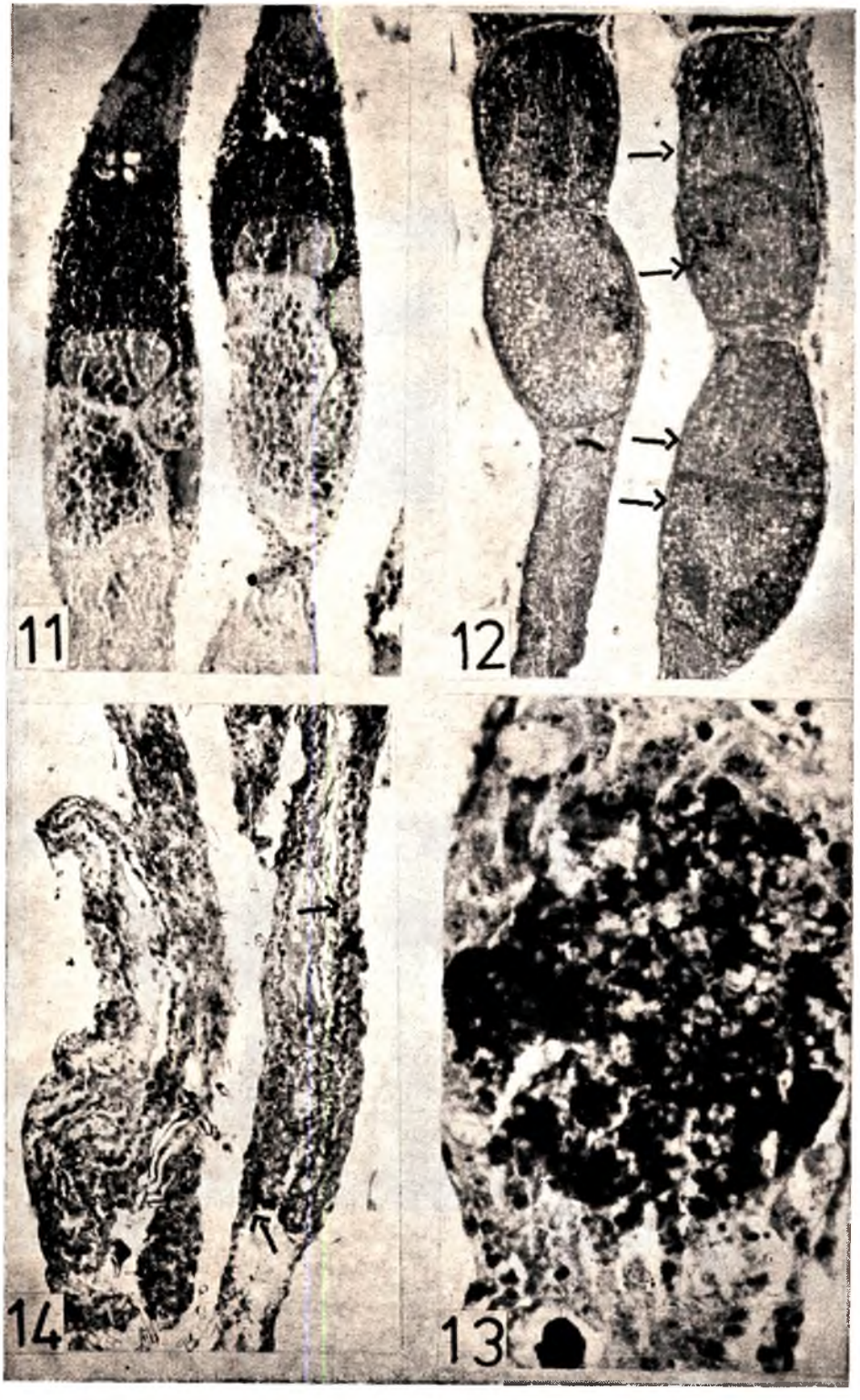
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ovary start two days after injection. Intense disintegration of the trophocytes starts from the posterior zone of the germarium and progresses forwards (Figs. 5&6). Ovariole at this stage measures 1.7 mm in length as compared to control ovariole which is 4 mm in length. There are now four to six oocytes in the vitellarium which are all at previtellogenic stage in the treated ovariole whereas in the corresponding controls the basal oocytes start deposition of yolk granules.

Disintegration of chromatin material of the trophocytes, prefollicular tissue and germ cells increases steadily, forming clumps. The cytoplasm also degenerates subsequently contributing to the clumping masses, which ultimately fill the entire germarium. These clumps unite to form large masses which develop among them vacuoles. Six to seven days after treatment the germarium is almost full of disintegrated tissue (Fig. 7).

Of the 4–6 oocytes in the vitellarium none show signs of any yolk deposition when examined on the fourth day. The treatment results in resorption of the oocytes even before yolk formation, and it starts most frequently from the anterior end of the vitellarium but occasionally from the posterior end also. Resorption starts on the second day or on the early third day after treatment. At first the follicular epithelium appears quite normal. Some light-staining patches appear in the homogeneous ooplasm which later develops vacuoles. Gradually vacuolation of the cytoplasm increases, resulting in masses of dark staining bodies. During this time the follicular epithelium shows considerable changes. In some ovaries, follicular epithelium appears multilayered and results in hyperplasia (Fig. 8). When compared to the follicle cells of the control which measure about 17 μ x 15μ, those of treated insects are very small measuring only 7μ x 3μ. They do not differentiate beyond this stage. Later they become irregular in shape, pycnotic and gradually appear to invade the oocytes. The shape of the oocyte becomes distorted and the oocytes grow smaller as a result of gradual resorption. By six or seven days after treatment almost all the oocytes are resorbed and their place is occupied by the follicle cells which now increase in size and fill the entire "ooplasm". The mass of follicle cells and the relics of the germarium get shrunk, ultimately leaving the ovariole as an almost empty tube (Fig. 9). No sign of development of a second batch of eggs is indicated even in animals kept under observation for nineteen days.

In the resorbing oocytes in animals six days after treatment, certain regions of the cytoplasm show abundant proteins and the vacuolated region contains very little of it. The clumped material of the germarium is lightly stainable with PAS. As vitellogenesis does not take place in the treated ovary the corresponding histochemical reaction is absent. Most of the clumps of degenerated material in the treated ovary consist of either DNA or RNA. Material taking up both methyl green and pyronin and hence containing both DNA and RNA are also present among them. The ovary of the control animal does not differ from that of the normal animal either histologically or histochemically.

Effect of apholate

Females treated with 5 μg of apholate show a mortality rate of about 25%. They deposit sterile eggs. Rather high mortality of about 50% is observed among insects treated with 7.5μg of apholate. These insects mate less frequently and swelling of the abdomen is not noted. Dissection of these insects reveals drastic influence on ovaries. Apholate-treated ovary presents a histological pattern of degeneration different from that of the metepa-treated ovary.

Changes in the ovary are noticeable even from the beginning of the second day (Fig. 10). There are fewer oocytes in the vitellarium and as a result the whole ovary is reduced in size. Disintegration of the germarium starts from the posterior region and proceeds anteriorly. Four days after treatment the whole germarium is filled with irregular clumps of chromatin material (Fig. 11). Almost complete elimination of perfollicular tissue is a striking feature of the ovary as early as two days after treatment (Fig. 10). As a result the follicular epithelium is almost reduced to a thin membrane without any distinct cells. More than one oocyte very often occur in a single follicle (Fig. 12). The linear arrangement of follicles in the vitellarium is also disturbed. Oocytes with apparently two nuclei are sometimes found in the germarium. Yolk deposition does not take place in these oocytes and these are never oviposited. They later disintegrate and are resorbed. Vacuoles appear in the homogeneous cytoplasm of oocytes three days after treatment along with granules which stain dark by iron haematoxylin. In oocytes four or five days after treatment, these dark staining granules increase in size, later forming dark masses filling the entire oocyte (Fig. 13). They differ from the yolk granules in that they first appear in the oocytes which are either in the germarium or are near it. They are also irregular in shape. These bodies later get resorbed and their place is occupied by the invading follicle cells.

Protein granules indicating resorption are observed in the cytoplasm and nuclei of oocytes, follicular epithelium and trophocytes of the treated ovary as early as one day after treatment, unlike the controls. In animals four days after treatment the degenerating material in the germarium and in the oocyte is rich in protein, but the controls contain no such granules. These however are not comparable to yolk granules. As vitellogenesis does not take place in the treated ovary, the protein-carbohydrate yolk granules and lipid yolk granules are entirely absent in them. The disintegrated mass of the trophocytes and what remains of the follicular epithelium and oocytes, contain DNA and RNA. These clumps fill the entire germarium as well as the vitellarium of the ovaries six days after treatment.

Effect of apholate treatment by contact method

Newly moulted females exposed to apholate residue fail to develop eggs. Oocyte growth proceeds upto the end of the previtellogenic period. Sometimes vitellogenesis starts but later those oocytes also get resorbed. The ovary nineteen days after treatment show atrophied ovarian tube, the interior of which is filled with the invading follicle cells (Fig. 14). The shrunken, disintegrated germarium consists only of the fragments of cytoplasm and disintegrated chromatin material. Control insects exposed to acetone treated surface are comparable to normal animals.

DISCUSSION

The structure of the ovaries and vitellogenesis in *Dysdercus cingulatus* are essentially comparable to that in *Dysdercus fasciatus* worked out in detail by BRUNT (1971). Yolk deposition in *Dysdercus cingulatus* starts on the third day after adult emergence, as already reported (JALAJA & PRABHU, 1971).

Apholate and metepa administered in various doses produce changes which range from an almost complete necrosis of the ovary to almost normal ovary resulting in the production of apparently normal but nonviable eggs. The severity and changes produced by the chemosterilants are dependent on dosage. The infertility of the apparently normal eggs produced as a result of

treatment of females with lower doses may be due to enzyme inhibition (MENDOZA & PETERS, 1968; TURNER & MAHESWARY, 1969), or due to induction of dominant lethal mutations as suggested by LACHANCE & CHRISTAL, (1963) in other insects.

The two chemosterilants apholate and metepa act differently on the ovarian tissue though the final result is the same. When higher doses are employed reduction in the number of oocytes as well as decrease in the size of the ovary are the common effects. The trophocytes of the germarium are the first to be affected by the sterilants. At the time of the treatment the vitellarium already contains three to four oocytes. Production of more oocytes is inhibited by the sterilants. This is due to disintegration of the posterior zone of the germarium where oocytes are differentiated. The disintegration of the germarium leads also to restriction of the supply of trophic material such as ribonucleoprotein and DNA necessary for the activation of young oocytes in the vitellarium, restricting their growth during the previtellogenic period. The observations on the house flies also indicate that damage to follicle cells and nurse cells of the egg chamber as well as destruction of the germarium are responsible for infecundity (LANDA & REZABOVA, 1965). Ovarian degeneration is also observed in *Drosophila* (CANTWELL & HENNEBERRY, 1963) after treatment with chemosterilants. In the telotrophic ovary of beetles disintegration of the upper part of the germarium and suppression of the division of trophocytes are noted following application of tepa (ONDRACEK & MATOLIN, 1971). Atrophy of prefollcular and follicular tissues and the occurrence of multiple oocytes within a single follicle without any linear arrangement of oocytes in the ovarioles are the striking characteristics of apholate treated ovary observed during the present study. In *Pyrrhocoris* this phenomenon has been described after treatment with 6-azauridine in which it has been suggested that the twin oocytes are formed as a result of disturbance of the division mechanism by the chemosterilant (MASNER, 1971). It may be due to oocyte fusion or due to more than one oocytes entering the same follicular chamber.

There is a possibility that yolk deposition does not start in the oocytes of the treated insects due to failure of follicle cells to differentiate properly. Synthesis of yolk in many insects does not start until follicular epithelium differentiates properly (ANDERSON, 1971). The sensitivity of the follicular epithelium to the sterilants observed during the present study is especially significant in the light of the findings that follicle cells play an important role in incorporating yolk into the oocytes (ANDERSON & TELFER, 1969). Chemosterilants also influence the neuroendocrine mechanism in insects (MASNER & MACHA, 1968; TAN, 1974; JALAJA & PRABHU, 1976). Median neurosecretory cells and corpus allatum are known to control vitellogenesis in this animal (JALAJA, 1974, 1975; JALAJA *et al.*, 1973). So the effects of the sterilants on the neuroendocrines might also play their role in affecting vitellogenesis in the treated ovary. Resorption in insects occurs after treatments with chemosterilants (SMITTLE *et al.*, 1966: BULYGINSKAYA *et al.*, 1967: CAMPION, 1968; KERNS & NAIR, 1972; SUKUMAR & NAIDU, 1973). In *Schistocerca* (KERNS & NAIR, 1972) the lower concentration of haemolymph proteins in the tepa-treated insects is one of the factors that contribute to the resorption of oocytes. However, in metepa treated cockroach *Periplaneta* haemolymph protein concentration does not fall, though there is a change in electrophoretic pattern of the blood proteins (PRABHU & NAYAR, 1972). It appears that a number of these factors might play their role in bringing about inhibition of vitellogenesis in *Dysdercus* after treatment with the chemosterilants.

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