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HISTOPATHOLOGY AND HISTOCHEMISTRY OF THE INSECTS TREATED WITH CHEMOSTERILANTS-III NUCLEIC ACIDS, PHOSPHOLIPIDS AND PHOSPHATASES IN THE TESTES OF CHEMOSTERILISED POECILOCERUS PICTUS.[1]

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A B S T R A C T

Histochemical localization of nucleic acids phospholipids in the testes of apholate and tepa treated and control P. pictus are discussed. The testes of the treated insects show a gradual decrease in the nucleic acids and phospholipids. Acid and alkaline phosphatases are found to be absent in the testes of even untreated insects.

Histochemistry of the chemosterilized insects has not yet received the due attention of the researchers. Very little information is available on this aspect. A few workers have contributed towards the studies on nucleic acids in sterilized insects but phosphatases, phospholipids, etc., appear to be completely neglected. Mention may be made of Kilgore and Painter (1964, 1965) and Painter and Kilgore (1967), Mitlin (1964), Borkovec (1966), Ochoa and Hirschberg (1967) and Saxena & Vikram Aditya (1969). In the present investigation histochemical localization of nucleic acids, phospholipids and phosphatases is made in P.pictus treated with alkylating agents (apholate and tepa).

MATERIAL AND METHOD

Required concentrations of apholate and tepa were obtained by diluting them with either distilled or tap or glass distilled water or acetone. These were administered to Poecilocerus pictus either by oral feeding or contact or injection.

The insects after decapitation were dissected under physiological saline solution *(.7%* saline solution to every 100 c.c. of which 2 c.c. of 10% CaCl has been added; Baker, 1944), and the tissues were fixed immediately in proper fixatives.

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Histochemical reactions for nucleic acids, lipids and acid and alkaline phosphatases were performed. The various fixatives used, and methods applied are given in Table I.

The extractive techniques employed for the confirmation of a particular compound are as follows:

(a) Ribonuclease extraction for the removal of RNA as described by Bonhag (1955).

(b) 10% Perchloric acid at 4°C for 18 hours for removal of RNA (Erickson et al. 1949) as described by Pearse (1960).

(c) 5% Perchloric acid at 60°C for removal of both the nucleic acids as described by Pearse (1960).

(d) Pyridine extraction for phospholipids followed by staining with Acid Haematin as described by Davenport (1960).

The acid and alkaline phosphatases were confirmed by incubating the slides into their respective substrate. The controls were not incubated with the substrate.

The DNA by Feulgen reaction was confirmed by the control slides which were not hydrolysed in HCL.

Controls for all the treatments were run.

R E S U L T S

Nucleic acids, lipids and phosphatases in P. pictus (Control):

Nucleic acids: All the stages in spermatogenesis show a positive reaction for DNA in their chromatin, intense positive reaction in the mono some and faint in the rest of the cromatin in spermatids (Fig. 1). The negative reaction is shown by the cytoplasm of all the stages of spermatogenesis, cyst wall, outer follicular covering except its nuclei which are positive, the tail of the sperm (head being positive) and the debris in the zone of transformation.

The basophilic staining with toluidine blue is shown by the chromatin and the cytoplasm. The reaction is postive in the spermatozoa, the cytoplasmic debris and the outer follicular covering and slightly more intense in the spermatocytes. A pretreatment with RNase and cold perchloric acid removed the reaction showing the presence of RNA, while it is completely removed by pretreatment with hot perchloric acid (Fig 2,3).

The chromatin in methyl green toluidine blue orange G preparations is green exhibiting the presence of DNA while the cytoplasm is blue because of the RNA. The sperm head is green while the tail is blue. The cytoplasmic debris and the sperm tail are stained blue whereas the sperm head takes

T A B L E I

TECHNIQUES FOLLOWED FOR HISTOCHEMICAL REACTIONS

S.No.	Compound	Test applied	Fixative	Embedding material	Reference
1	Nucleic acids (i)	Feulgen reaction with and without hydrolysis	Carnoy	Paraffin	After Feulgen and Rossenbeck (1929) modified
	(ii)	Methyl green Pyronin Y	"	"	After Kurnick (1955)
	(iii)	Methyl green Toluidine blue	"	"	" Korson (1951)
	(iv)	Toluidine blue	"	"	" Bonhag (1955)
2	Lipids (i)	Sudan Black B in 70% ethanol. MacManus's (Followed by post chromation)		"	" MacManus (1946)
	(ii)	Acid Haematin (followed by (post chromation)	Ca-formol.	Gelatin	" Baker (1946)
	(iii)	Acid Haematin. (After Pyridin extraction)	Weak Boinn.	Paraffin	" Baker (1946)
	(iv)	Controlled chromation	5 gm $HgCl_2$, 5 gm chrome alum 2.5 gm $K_2Cr_2O_7$, 5 cc formalin in 100 cc distilled H_2O	"	" Elftman (1957)
3	Acid Phosphatase	Calcium Cobalt Nitrate (with and without sub-strata)	Cold acetone or 80% ethanol	"	" Gomori (1952)
4	Alkaline phosphatase	Lead Nitrate (with and without substrata)	Cold acetone or	"	" Gomori (1952)

the green colour. The extraction with cold perchloric acid and RNase removes all the basophilia which is due to RNA staining blue, and the treatment with hot perchloric acid, results in the removal of both the nucleic acids.

In the apical cell very little cytoplasmic basophiilia is exhibited: a negative reaction around the nucleus and faintly positive a little away from it which is removed by RNase pretreatment. The nucleus of the apical cell shows basophilic reaction due to DNA in form of small blobs (Fig. 4).

The various reactions performed and the localisation of the nucleic acids are tabulated (Table II).

Lipids: For phospholipids the nucleus and outside of the nuclear membrane in all the stages of spermatogenesis are negative and positive respectively. Cytoplasm of apical cell is positive (Fig. 5). A small positive body present just outside the nuclear membrane in spermatids appears to be the mitochondrial nebenkern (Fig. 6.). The head of the sperm shows negative reaction whereas the tail is faintly positive (Fig. 7). The cyst walls and the outer follicular coverning give positive reaction (Fig. 8).

For gross lipids Sudan Black B in 70% ethanol reaction is more intense, covering more area but the localisation is similar.

Acid and alkaline phosphatases: For both the phosphatases the reaction appears to be in negative as the same type of reaction comes in the preparations which are incubated in the respective substrates or in distilled water.

Nucleic acids and lipids in treated P. pictus.

Similar histochemical changes are recorded on treating the grasshoppers either with apholate or tepa.

24 hours after treatment: No change was observed in nucleic acids and lipids in any stage of spermatogenesis in the grasshoppers after 24 hours of treatment.

3 days after treatment:

Nucleic acids: An increase in intensity for DNA is recorded in the pycnotic cells with both Feulgen reaction and toluidine blue. No basophilia with toluidine blue is shown by the surrounding cytoplasm in such cells but the normal reaction was observed wherever the cells show fragmentation of chromatin material.

Lipids: An increase in the intensity of Sudanophilia showing phospholipids around the pycnotic nucleus is recorded. The nucleoplasm remains negative. The cystwall appears to be somewhat positive.

TABLE II

NUCLEIC ACIDS IN TESTES OF CONTROL P. PICTUS

S.No.		Spermatogonia		Spermatocytes		Spermatides		Spermatozoa		Cyst wall	Debris	Follicular wall
		Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Head	Tail			
1	Feulgen reaction	+	-	+	-	±	-	+	-	-	-	-
2	MTO	G	B	G	B	G	B	G	B	B	B	B
	(a) Cold perchloric acid	G	-	G	B	G	-	G	-	-	-	-
	(b) RNase pretreatment	G	-	G	B	G	-	G	-	-	-	-
3	Toluidine blue	+	+	+	+	±	±	+	+	+	+	+
	(a) Cold perchloric acid	+	-	+	-	±	-	+	=	-	-	-
	(b) RNase pretreat-treatment	+	-	+	-	±	-	+	-	-	-	-
	(c) Hot perchloric acid pretreatment	-	-	-	-	-	-	±	-	-	-	-

Abbreviations used:
+ = Positive
± = Faintly positive
G = Green
G = Faintly green
B = Blue
- = Negative
MTO = Methylgreen toluidine blue organge G

5 days after treatments:

Nucleic acid: Only those cells which show fragmentation and clumping of chromatin material exhibit an increase in cytoplasmic basophilia which is removed by RNase treatment (Fig. 9, 10). An increased intensity for DNA in the pycnotic cells is shown. In the spermatocytes the condensed mitochondrial mass shows a positive reaction for DNA and a strong reaction with toluidine blue (Fig 11). It is resistant to RNase.

Lipids: An increased intensity around the nucleus of the damaged cells continues to the extent that a positive reaction in the whole cell is exhibited by some pycnotic cells (Fig. 20). A few of the hypertrophied cells are completely negative. Similar effects are shown by several spermatogonia and spermatocytes.

15 days after treatment:

Nucleic acid: In damaged cells the cytoplasmic basophilia with toluidine blue show a marked reduction (Fig. 12). Similarly a reduction in the reaction for DNA is exhibited by spermatogonia and spermatocytes which are damaged (Fig. 13).

Lipids: Disintegrated cells show a general decrease in reaction. Giant cells show negative reaction except at one region around the single nucleus (Fig. 21).

21 days after treatment:

Nucleic acids: Chromatin material is found absent in some cells and the cytoplasm shows reduced basophilia. Many basophilic droplets are seen in the zone of transformation (Fig. 14). The Feulgen reaction in general is diminished. Some of the giant cells exhibit vacuolisation and an increased reaction which in general is diminished (Fig. 15).

Lipids: In all the stages of spermatogenesis the debris show an increased reaction for lipids which in some of the hypertrophied spermatids and the giant cells is only on one side of the cell (Fig. 22). A large number of degenerating bodies which give different intensities with Sudan Black B are present in the zone of transformation. The tails of developing spermatozoa show intense positive reaction.

28 days after treatment:

Nucleic acids: Only debris showing a positive reaction to Feulgen reagent and toluidine blue (Fig. 16) are left in the tubule along with pycnotic or abnormal metamorphosing spermatids. They are RNase resistant (Fig. 17) which indicates that DNA is thrown out in the tubule.

Lipids: The tubule is full of intensely lipid positive debris (Fig. 23). Many of the giant cells are negative except a few showing the same type of reaction as described above. The phospholipids appear completely absent in disintegrated cells.

35 days after treatment:

Nucleic acid: Most of the debris loose basophilia (Fig. 18), while in some the toluidine blue staining is removed by RNase pretreatment showing the presence of RNA. Some exhibited the resistance to RNase pretreatment (Fig. 19) showing the presence of DNA.

Lipids: For lipids similar observations as after 28 hours of treatment were recorded. The difference was only of intensity.

DISCUSSION

In the present investigation histochemical localization of nucleic acids, lipids and phosphatases in the testes of P.pictus treated with apholate and tepa and in the normal insects are compared. The presence of nucleic acids in the stages of spermatogenesis has been reported by several authors and is established. DNA has been localised in chromatin and monosome nuclei of outer follicular covering and head of the sperm and found absent in cytoplasm, cyst wall, outer follicular covering excluding its nuclei, the tail of the sperm and the debris in the zone of transformation.

The presence of RNA is shown in the cytoplasm, sperm tail, follicular covering and debris, by the extractive techniques. The Feulgen reaction is specific for DNA but the extractive techniques differentiate between DNA and RNA.

The nucleus of the apical cell shows the presence of DNA.

In treated insects an initial increase of DNA in pycnotic nucleus is recorded after 3 hours of treatment. This increase does not appear to be due to an increase in DNA content but may be because of the clumping of chromatin material. The increased intensity for DNA persists as evident by histochemical preparations 15 days after treatment. The reaction for DNA continues to be reduced, although even after 28 and 35 days after treatment some debris show its presence. RNA meets a similar fate. It appears to undergo depolimerization. The metabolism in the nucleic acids in the testicular cells of P. pictus greatly appears to be disturbed as is evident by negative as well as positive reaction for both the nucleic acids in the debris. These observations are in accordance to Mitlin (1964), Borkovec (1966) and Ochoa and Hirschberg (1967) who suggested that the alkylating chemosterilants affect the nucleic acids including their synthesis. Inhibition of DNA synthesis in the sterilized housefly eggs has already been reported by Kilgore and Painter (1964) and Painter and Kilgore (1967). In view of the role of nucleic acids as a genetic controlling factor, the morphological abnormalities on the progeny may be attributed to their disturbances caused by alkylating agents.

In general the lipids are energy reserves and are found to occur in several forms which include phospholipids. The specific localisation significance of lipids in the testes of P. pictus has not been reported. In the present work the localisation of phospholipids is established besides others by controlled chromatin method after Elftman as this technique

PLATE I

(CONTROL TESTIS)

Explanation of figures

Fig. 1 - Showing spermatids and spermatozoa.
Carnoy feulgen x 400

Fig. 2 - Showing spermatocytes
Carnoy toluidine blue x 400

Fig. 3 - Showing spermatocytes.
Carnoy, Toluidine Blue, RNase pretreated x 400

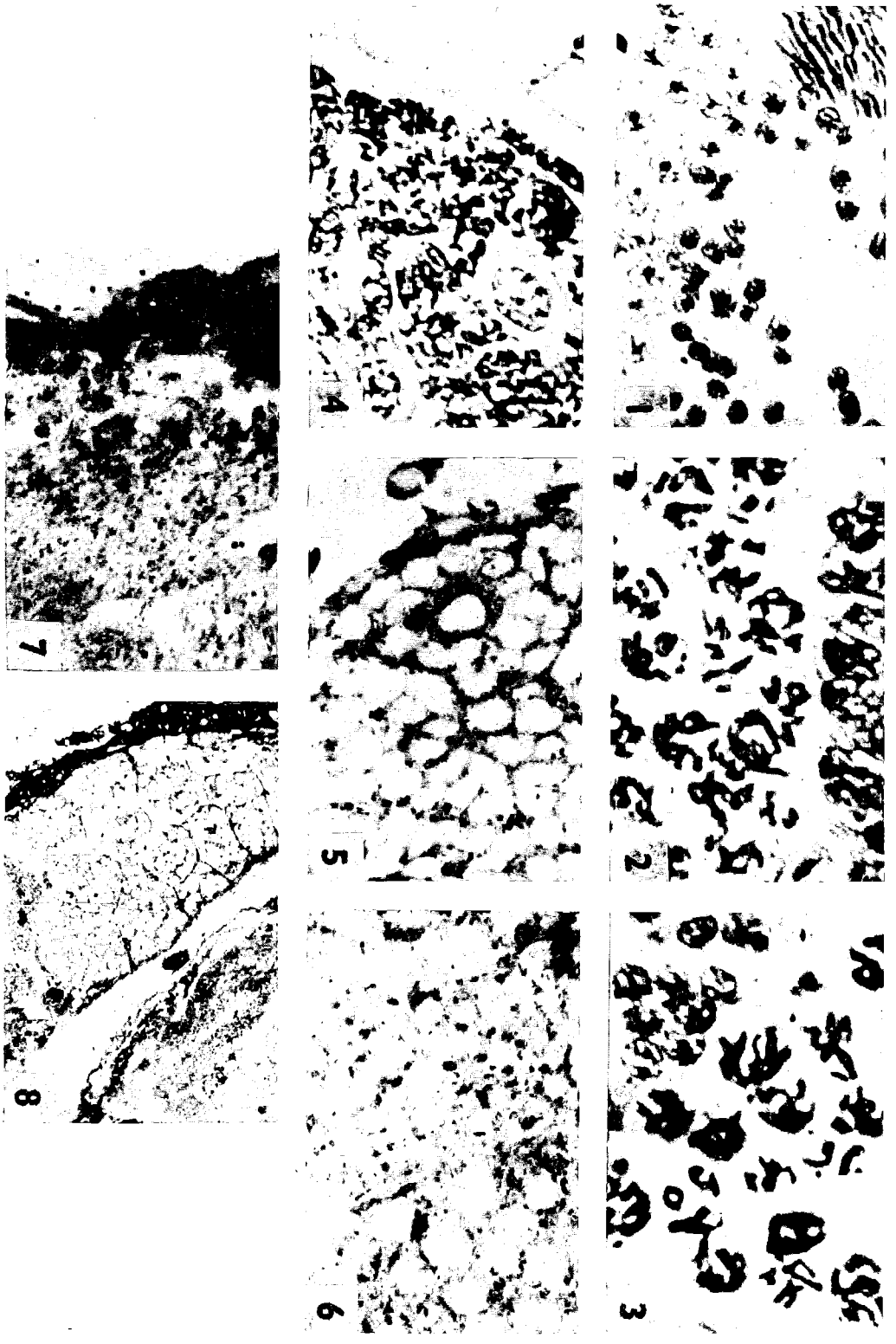
Fig. 4 - Showing apical cell and spermatogonia.
Carnoy, Methyl green toluidine blue orange G. x 400

Fig. 5 - Showing apical cell and surrounding cells.
Elftman's control chromation, Sudan Black B x 450

Fig. 6 - Showing spermatids and spermatocytes.
Elftman's control chromation, Sudan Black B x 450

Fig. 7 - Showing spermatozoa.
Elftman's control chromation, Sudan Black B x 450

Fig. 8 - Note cyst wall and outer follicular coverings.
Elftman's control chromation, Sudan Black B x 100



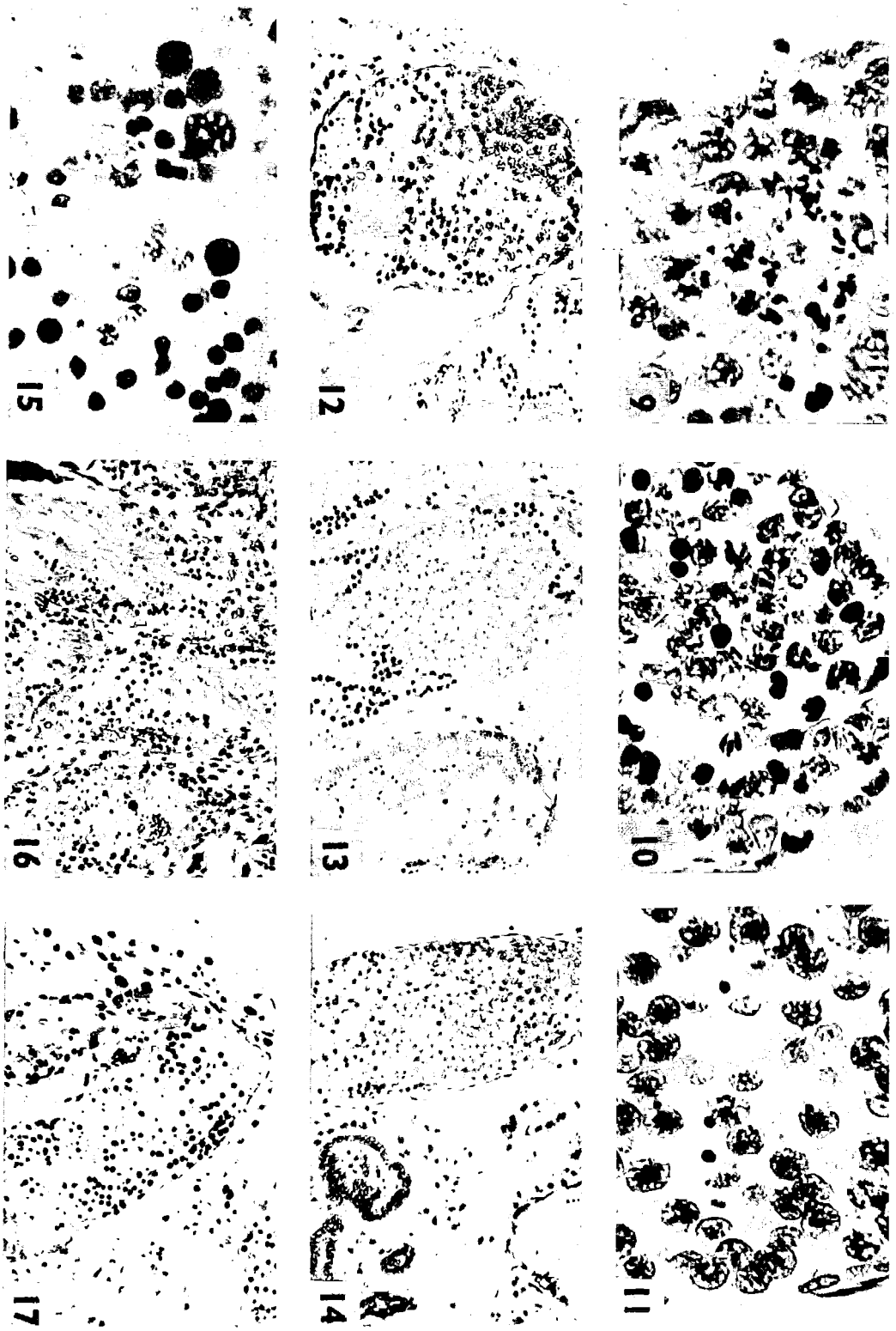

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PLATE II -

(TREATED TESTIS. SHOWING NUCLEIC ACIDS)

Fig. 9 - 5 days after injecting 0.1 mg apholate/insect. Showing increased reaction in cytoplasm and clumped chromatin (Anaphases). Carnoy. Toluidine blue x 400.

Fig.10 - 5 days after injecting 0.1 mg apholate/insect. Showing increased reaction in pycnotic cells. Carnoy. Toluidine blue. RNase treated x 400.

Fig.11 - 5 days after injecting 0.1 mg apholate/insect. Showing intensely positive mitochondrial mass. Carnoy. Toluidine blue. RNase treated x 400.

Fig.12 - 15 days after injecting 0.1 mg apholate/insect. Showing decreased cytoplasmic basophilia in the damaged cells. Carnoy. Toluidine blue x 400.

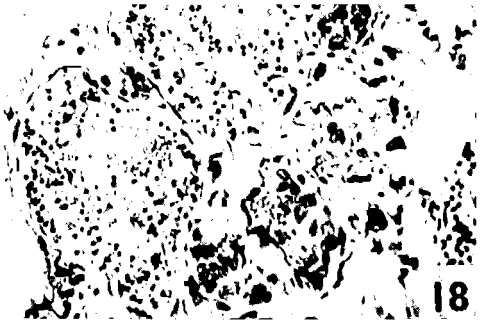
Fig.13 - 15 days after injecting 0.1 mg apholate/insect showing decreased reaction for DNA. Carnoy. Feulgen x 100.

Fig.14 - 21 days after injecting 0.1 mg apholate/insect showing reduced cytoplasmic basophilia and basophilic droplets in the zone of transformation. Carnoy. Toluidine blue x 100.

Fig.15 - 21 days after injecting 0.1 mg apholate/insect showing increased reaction for DNA in giant cells. Carnoy. Feulgen x 400.

Fig.16 - 28 days after injecting 0.1 mg apholate/insect showing Toluidine positive debris. Carnoy. Toluidine blue x 100.

Fig.17 - 28 days after injecting. 0.1 mg apholate/insect showing RNase resistant condition. Carnoy. Toluidine blue RNase treated x 100.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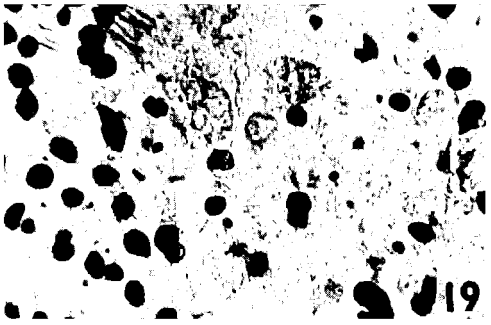
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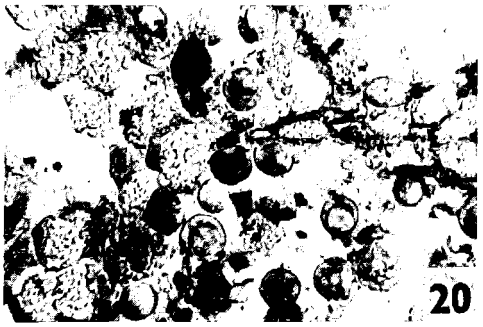
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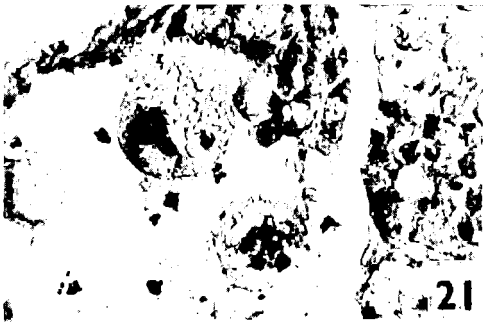
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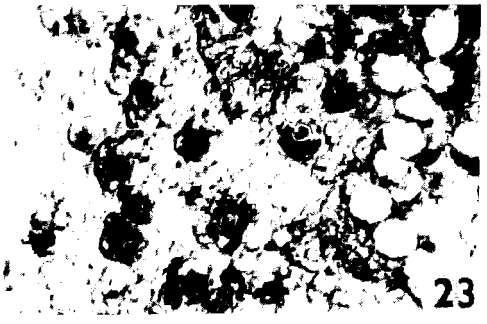
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PLATE III

(TREATED TESTIS. SHOWING NUCLEIC ACIDS)

Fig.18 - 35 days after injecting 0.1 mg apholate/insect showing unaffected connective tissue cells. Carnoy. Toluidine blue x 100.

Fig.19 - -5 days after injecting 0.1 mg apholate/insect showing presence of DNA in debris, hence resistance to RNase pretreatment. Carnoy Toluidine blue RNase treated x 450.

(TREATED TESTIS SHOWING LIPIDS)

Fig. 20 - 5 days after injecting 0.1 mg apholate-insect shwoing intensely positive pycnotic cells. Elftman's control chromation. Sudan Black B x 400.

Fig. 21 - 15 days after injecting 0.1 mg apholate/insect showing intensely positive reaction near the single nucleus of the giant cell. Elftman's control chromation. Sudan Black B x 400.

Fig. 22 - 21 days after injecting 0.1 mg apholate-insect, showing increased reaction for lipids in debris on one side only in hypertrophied spermatids and giant cells. Elftman's control chromation. Sudan Black B x 400.

Fig. 23 - 35 days after injecting 0.1 mg apholate/insect showing intensely positive debris. Elftman's control chromation. Sudan Black B x 400.

has several advantages over other methods; the main being the elimination of certain artifacts reported by Singh (1964-66).

The increase in the phospholipids around the pycnotic nucleus in different stages of spermatogenesis 3 days after the treatment which persisted up to 7 days after treatment is not fully understood. Such an increase has also been reported by Saxena and Srivastava (1969) in the midgut of insecticide (Pyrethrum) treated P. americana. Their suggestion based on Gilmour's (1965) observation that following sublethal doses of insecticide (BHC) inosital (a phospholipid in insect) counteracts the toxic effects leading to the accumulation of abnormal amounts of cholesterol may come up as a probable explanation but needs further investigations. 15 days after treatment and after it, there is a general decrease in the reaction for phospholipids in disintegerating cells. The giant cells, as well, show a negative reaction. However, during this period an increased intensity is exhibited by the debris but it seems to be heading for sudanophobia. The observations thus show that the effect of apholate and tepa lies in reducing the phospholipids which is expected to involve some reproductive physiological disorders probably due to failure of energy reserves.

Acid and alkaline phosphatases could not be localized even in the testes of untreated P. pictus, showing that either the phosphatases are absent in testes or are in too low concentrations to be detected qualitatively by the techniques employed.

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