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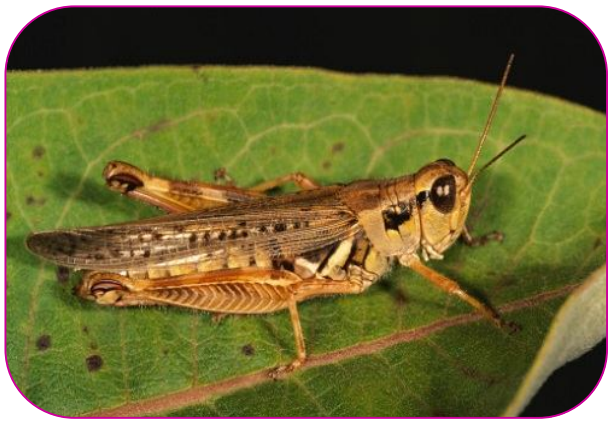
HISTOCHEMISTRY STUDIES OF THE DIGESTIVE SYSTEM OF TWO SPECIES OF GRASSHOPPERS (POECILOCERUSPICTUS,CHROTOGONUSTRACHYPTERUS)



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ABSTRACT

The grasshoppers which belong to the family "Acrididae" have been reported as one of the most serious insect pests of certain important cultivated crops of India and other parts of the world causing heavy damage. Two species of Grasshoppers was chosen for histochemical studies which plays important role in digestive system.Specific tests for Nucleic acids, connective tissue fibres, iron and calcium neutral and phospholipid and few enzymes were performed. We got significant results from the test like presence of RNA, collagen ,elastin, lipase, calcium etc.

KEYWORDS: *Grasshoppers, Digestive System, Iron, Calcium, RNA, Histochemical Studies.*

INTRODUCTION

Insects had over enjoyed a dominant place amongst animals on earth, in its all environment and continue in the same position even at present. The total number of species of insects in the world so far described, exceeds one and half million representing nearly 90 per cent of the animal kingdom. Insects are an ancient group of animals. They were one of the first to invade dry land in the dim past and were indeed the first to fly. They appeared fully 250 million years ago (Mani, 1973).

Economically, insects are very important. They give us clothing, supply food and medicine and numerous other useful products. They bring about the cross-pollination of flowers and thus assist in setting of seeds and growth of valuable fruits. They damage our crops, lay waste vast areas of cultivated fields spoil our food, sabotage our food industries and bring death and diseases with them. Hence, their influence on human life is so great that man has been compelled to study their mode of life, their feeding habits and various aspects of their physiology and histochemistry.Atwal (1965) has estimated a loss of about 500 Crores of rupees annually of the total production of food grains in India only because of insects.

The present problem relates to the histochemistry of the two species of Indian Grasshoppers (Acridids) feeding on different types of food plants. A thorough review of literature reveals that the orthopterous insects have been neglected except certain locusts and cockroaches. Due to this lacuna certain acridids were selected for the present work.

A perusal of the literature reveals that though a great deal of work has been done on various aspects of grasshopper's life but the studies on the histochemistryof the digestive system in relation to their varied food habits have suffered

almost complete neglect at the hands of the entomologists.

MATERIALS AND METHODS:

Adult specimens of Poeciloceruspictus were collected from St. John's College campus. This is a common type of grasshopper and generally feeds on leaves of Ak. This is abundantly found in the month of July and August. This is dark green in colour. The systematic position is as follows:-

Class	-	Insecta
Order	-	Orthoptera
Family	-	Acrididae
Genus	-	Poecilocerus
Species	-	Pictus

Adult specimens of Chrotogonoustrachypterus were collected from Prernasheel farm house of Aliganj town in Etah District. Here the crop of Jowar is cultivated on large scale and this grasshopper feeds on the leaves of Jowar plant. The maximum number of insects was collected in June and July at the time of Rainy season. They are reddish brown in colour. The systematic position is as follows:

Class	-	Insecta
Order	-	Orthoptera
Family	-	Acrididae
Genus	-	Chrotogonous
Species	-	trachypterus

Adult specimens of Atractmorphacrenulata were collected from the cultivated adjoining area of Sona village of Dhumri town of Etah District. They were found in abundance from March to June. They feed on leaves if Tobacco plants. They are yellowish green in colour. The systematic position is as follows:-

Class	-	Insecta
Order	-	Orthoptera
Family	-	Acrididae
Genus	-	Atractomorha
Species	-	crenulata

EXPERIMENTAL STUDIES

Histochemistry has been considered as a link between morphology and analytical chemistry. Its parameter was adopted in the present study. Specific tests for Nucleic acids, connective tissue fibres, iron and calcium neutral and phospholipid andfew enzymes were performed. Fixatives used and precautions taken are described in table No. 2

Localization of Nucelicacid :

A) Deoxyribonucleic acid – (Fuelgen&Rossen beck - 1924)

1. Brought sections to water and treated with schiff's reagent followed bysulphurus acid rinse.
2. Counter stained the cytoplasm

Result- sites of DNA were Magenta coloured.

B) Ribonuclic acid – (Methyl green pyronin.Y.Method), (Kurnick- 1955).

Preparation of stain

Made a 2% aqueous solution of pyronin Y. extracted with chloroform layer became colourless. Made a 2% aqueous solution of methyl green and extracted with chloroform until the chloroform layer was no longer violet coloured. For use, mixed 12.5 ml. Pyronin Y. solution & 7.5 ml. of methyl green with 30 ml. distilled water.

Method

1) Brought paraffin sections to water.
2) Stained for 6 min. in methyl green pyronin.
3) Blotted with filter paper.
4) Immersed in two changes of n-butyl alcohol 5 min. in each.
5) Immersed in xylene for 5 min.
6) Immersed in cedar oil for 5 min.
7) Mounted in DPX.

Result

RNA bright red, DNA blue, chromatin green.

Localization of Basic proteins:

A) Elastin: Elastin tissue is widely distributed in animal tissue and occurs as fine fibres sometimes as branching fibres or in the laminated sheets as in blood vessels.

Gomori(1950 b) aldehyde fuchsin stain for elastic fibres:

Fixation – 10% formalin gave a colourless back ground.
Section – Thin paraffin section.

Preparation

Basic fuchsin= 0.5gms.
70% Alcohol = 100ml.
Con. Hcl= 1ml.
Paraldehyde = 1ml.

Method:

Dissolved the dye in the alcohol and then added the acid and paraldehyde. Left at room temprature for 24-72 hours.Until the stain had the deep purple colour which indicated fitness for use.

The stain was stored in the refrigerator at 4°C and was replaced after 2-3 months.

Method:

1. Took sections to water.
2. Washed in tap water followed by 70% alcohol.
3. Stained in a jar of aldehyde fuchsin for 10 min.
4. Washed well in 95% alcohol.
5. Dehydrated, cleared and mounted.

Results:

Elastic fibres and some muco-polysaccharides and most cell granules – deep purple – violet. Rest structures according to counter stain.

(B) Collagen:

Collagen is derived from fibroblasts which is a cellular structure and occurs as wavy fibre either single or fused together in dense bundles. It is quite resistant to autolytic changes but swells in the acetic acid. It is bierfragment and the examinations of unstained sections with a polarizing microscopic and a powerful light source is often helpful.

Van Gieson's (1889) Stain:

Van Gieson's mixture of picric acid and acid fuchsin is the simplest for the different staining of collagen.

Fixation:

It gave good results after a wide range of fixative and mercuric chloride; is specially suitable.

Preparation:

Saturated aqueous picric acid – 100 ml, 1% acid fuchsin, in distilled water, 5-10 ml.

Method:

1. Took sections of water.
2. Stained nuclei with weigert's iron haematoxylin.
3. Washed well in tap water followed by distilled water.
4. Stained in Van Gieson's solution 2-5 minutes.
5. Rinsed in distilled water and did not wash in alkaline tap water.
6. Dehydrated in abs. alcohol, cleared in xylene and mounted in DPX.

Results:

Nuclei = Brown black to black
Collagen= Deep red
Muscles, cytoplasm = yellow.

(C) Fibrin (Mallory's Method)Mallory 1938:

Preparation of PTAH:

Haematoxylin or haemain = 0.1 gms.
P.T.A. (Phosphotungustic acid) = 2.0 gms
Dist. Water = 100ml.

Dissolved separately and mixed the solution which ripened after several months. When ripened keep for years for use.

Method:

1. Brought sections to water.
2. Washed in water.
3. Differentiated for 1 minute in acid permanganate.
4. Washed in water.
5. Rinsed in water and transferred to PTAH for 12-24 hours.
6. Dehydrated in absolute alcohol.
7. Cleared and mounted in DPX.

Results:

Fibrin - Dark blue
Nuclei - Light blue
Collagen - Rose red

Localization of Amio Acid:

A) Tyrosine (Simple Protein) Million's reaction (Bensley and Gersh Modification 1933).

Preparation of the Reagents:

Added 400 mL.Conc. HNO_3 (sp. Grav. 1.4) to 600 mL.distilled water and let it stand for 48 hours, dilutes 1:9 with distilled water and saturated with crystalline mercuric nitrate. Filtered and added to 400 ml. of the filtrate, 3 mL.of the original diluted nitric acid and 1.4 gms sodium nitrite.

Method:

Removed wax from sections with light petroleum. Rinsed in absolute acetone and allowed to dry in air. Covered sections with million's reagent and let it stand at room temperature until the maximum colour developed or placed in a covered petridish in 60° Cincubator.

The reaction proceeded slowly in the cold but was complete in 30-60 minutes in incubator. Rinsed in cold or warm 2% nitric acid (2mL. conc. acid sp. Gravity 1.42, 98ml. water) dehydrated rapidly in 70% and absolute alcohol. Cleared in xylene. Mounted in Canada Balsam.

Result:

Protein containing tyrosin stain-orange to rose red. The colour is stable for over 12 months.

B) Arginine:

Removed wax and covered with a thin film of 1% cellaidin (Optional). Brought sections to water. Removed from water and flicked off till neatly dry. Flooded withhypochloride solution for 15 minutes. (α-napthol) mixed 2 ml. of 1% NaOH, 2 drops of 1% α-napthol in 70% alcohol and 4 drops of 1% million drained and blotted. Immersed in a mixture of equal parts of dry pyridine and chloroform.Mounted in pyridine chloroform or dry pyridine.

Result:

Arginine containing proteins appeared in various shades of orange red.

Localization of Inorganic Metals:

A) Calcium: (Alizarin red method for calcium Dahl, 1952) Formalin fixed paraffin sections.

Preparation for staining solution:

Washed 2 gms. Nuclear fast red (G.T. Gaur Batch 3896) twice with 100 mL.distilled water. Took the residue (about 0.25 gms) with 100 mL distilled water.

Method:

Brought sections to water, stained in calcium red solution 1-10 minutes, washed in distilled water. Dehydrated in alcohol, cleared in xylene and mounted in DPX.

Result:

Calcium deposits red. Tissue various shaded of pink.

B) IRON: (Perl's method for ferric iron. After Lion and Bunting Pearse 1961)
Formalin fixed paraffin sections.Acid fixative and chromate were avoided.

Method:

Brought sections to distilled water, exposed sections to a fresh mixture of equal parts of 2% potassium or sodium ferrocyanide and 2% HCl for 30-60 minutes. Washed in distilled water. Counter stained nuclei with aqueous 1% neutral. red for 2 minutes. Washed dehydrated and cleared in xylene, mounted in DPX.

Result:

Ferric iron- Deep persion blue.Nuceli red.

Localization of Lipids:

A) Sudan Black – B Staining For Lipids in Paraffin Section(After Mc. Manus 1946)

Method:

1. Brought sections in 70% alcohol.
2. Stained for 30 minutes at room temperature in saturated sudan Black – B in 70% alcohol.
3. Removed excess dye by rinsing quickly in 70% alcohol.
4. Washed in running water.
5. Counter stained in 1% aqueous neutral red for 1 minute.
6. Washed in water mounted in glycerin jelly.

Results:

This stains fat globules red. A blue colour also was obtained by using a saturated solution of oil blue N in 60% isopropyl alcohol. Sections stained by this method could not be dehydrated.

Localization of Enzymes:

For the localization of enzymes (alkaline phosphatase, acid phosphatase and lipase) the alimentary canal from a freshly collected grasshopper was fixed in chilled acetone for 24 hours. After dehydration, by two changes of acetone at room temperature, it was cleared in benzene and embedded in paraffin. The paraffin sections were processed for enzymes according to Gomori revised method.

(A) Alkaline Phosphates:

Gomori revised method for alkaline phosphatase (1952) Substrate medium pH-9.4

Preparation:

Combined 25 ml. of 2% sodium glycerophosphate, 25 ml. of 2% sodium barbital, 50 ml. distilled water, 5 ml. of 2% calcium chloride, 2ml. of 2% magnesium sulphate and few drops of chloroform.

The sections were placed in distilled water after dewaxing. Then they were incubated for 1-16 hours at 37^0C in the substrate medium and rinsed with distilled water.

Method:

1. Brought sections to distilled water.
2. Incubated the section for 1-6 hours at 37°C in the substrate medium.
3. Rinsed in running water.
4. Treated with 2% cobalt nitrate solution for 5 minutes.
5. Treated with a dilute solution of yellow Ammonium Sulphide 1-2 minutes
6. Washed in water, counter stained in 1% eosin for 5 minutes.
7. Dehydrated in alcohol, cleared in Xylol and mounted in DPX.

Results:

Various structures were stained black or brownish-black in tissues possessing alkaline phosphatase activity.

(B) Acid Phosphate:

(Gomori Revised method for acid phosphatase 1952) substrate medium (pH 5.0)

Preparation:

Combined 30 ml. of 1M. acetate buffer (100 ml. 13.6% sodium acetate, $CH_3CooNa.3H_2O$ + 50 ml. of 6% acetic acid) 105ml. of 5% lead nitrate and 60ml. distilled water and added slowly while stirring 30 ml. of 2% sod. glycerophosphate. Shaked the mixture and let it stand for a few hours, stored in a refrigerator. Before use filtered a small amount and diluted it with 2-3 parts distilled water.

Method:

1. Brought sections to distilled water after dewaxing.
2. Incubated the sections for 1-24 hours at $37^{\circ}C$ in the substrate medium.
3. Rinsed in distilled water followed by 2% acetic acid and then in distilled water again.
4. Treated with a dilute solution of yellow ammonium sulphide for 1-2 minutes.
5. Washed and counter stained with 1% aqueous eosin for 5 minutes.
6. Dehydrated in alcohol, clear in Xylol and mounted in DPX.

Results:

The presence of acid phosphatase in the section was indicated by a black precipitate of lead sulphide.

(C) Lipase: (Gomori revised method for lipase 1945)

Substrate medium.

Stock Solution I :

Combined 150 ml. glycerol, 50 ml. of 10% calcium chloride, 50 ml. M/2 maleate buffer pH 7 to 7.4 (dissolve 5.8 gms maleic acid in 94 ml. of 4% sodium hydroxide and 6 ml. water) and distilled water to make 1000 ml.

Stock Solution II :

5% tween 40 or 60 of "Product 91" added methylate to 0.02% in each stock solution and stored in refrigerator. The solution may be used for many months. Before use, added 2 ml. of stock II to 50 ml. of stock solution I.

Method:

1. Brought the sections to distilled water after dewaxing.
2. Incubated the sections in the substrate medium for 6-12 hours at 37^0 and washed thoroughly in distilled water.
3. Immersed in 1% lead nitrate solution for 15 minutes.
4. Rinsed in repeated changes of distilled water.
5. Immersed in diluted yellow ammonium sulphide solution for 1-2 minutes.
6. Washed and counter stained with 1% aqueous eosin for 5 minutes.
7. Dehydrated in alcohol, cleared in gasoline and mounted in DPX.

Result:

Sites of lipase activity appeared golden brown

RESULTS & DISCUSSIONS

Chemical Peculiarities of the cellular components of the digestive tract of three different grasshoppers under study analysed qualitatively on a cytological scale-gave notable changes. These changes may be attributed to their feeding habits on different crops. Specific histochemical tests were employed to note various histochemical investigations. Estimation of DNA contents per cells provided rapid and precise tool to investigate the degree of proliferation. However qualitative changes in the state of Nucleic Acid are equally important. The observation includes the DNA contents inmidgut the DNA contents were almost similar among two grasshoppers. Same results were obtained in hindgut of all the two selected grasshoppers.

The presence of RNA was observed more in Poeciloceruspictus in comparison to Chrotogonustrachypterus. The presence of RNA was not observed in rectum region of digestive tract among two species of grasshoppers. RNA contents are more in foregut and midgut regions in Chrotogonustrachypterus.

In the history of entomology probably it is the first attempt ever made to examine the fate of Nucleic Acids among the digestive tract of three different grasshoppers feeding upon different crops. No statics is so far available. The observations may prove of great value in the application of diagnostic cytology.

The connective tissue fibres like elastin, collagen and fibrin were observed. It is summarized that the elastin fibres were noticed only foregut while midgut and hindgut showed a nil activity except ileum where elastin fibres were observed amongst the two grasshoppers. The elastin contents in foregut were more in Chrotogonustrachypterus in comparison to Poeciloceruspictus. Midgut and hindgut showed the same observation for all the three grasshoppers. For collagen fibres same observations were obtained in twograsshoppers. The collagen fibres were observed only in proventriculus, midgut and colon in all the two grasshoppers. Rest part showed poor and dull reaction while in crop and rectum collagen fibres were totally absent in all the three grasshoppers. The fibrin fibres were noticed in foregut and midgut in all the three grasshoppers, while hindgut did not give much response except ileum.

In addition to non-cellular (Basic) proteins amino acids were also examined histochemicaly like nucleic acids. Reports on amino acid also seems to be first of its kind. No comparison is to be made since other evidences are lacking. However, the presence of Tyrosine was observed in the foregut except crop where nil reaction was observed, while midgut and hindgut showed poor and dull reaction except rectum where tyrosine was totally absent in all the three types of grasshoppers.

The Agrinine was observed only in Pharynx, Proventriculus and midgut while oesophagus, crop, ileum showed poor and dull reaction. A nil activity was observed in colon and rectum in all the three grasshoppers. These findings are important from histochemical point of view.

Histochemical study of two essential trace elements viz. Calcium and Iron thus made could be helpful in establishing specific observations. Metal ions are involved in a number of different types of Biological oxidation. Iron, being a vital constituent of Porphyrine enzyme, it is essential for all living cells. It is also a constituent of non-porphyrin enzymes. Iron occurs only in traces in tissues. Protein (Haemoglobin) containing iron is associated with oxygen transport and storage.

In the present study of grasshoppers the iron was not traced in the digestive tract in all two selected grasshoppers. On the other hand calcium is involved in Physiological control and Trigger Mechanisms and plays a significant role in maintaining the structure and function of cell wall. In the present study calcium was observed in the foregut and midgut of Poeciloceruspictusand Chrotogonustrachypterus. The calcium contents were more in the foregut of Poeciloceruspictus in comparison to Chrotogonustrachypterus. While midgut showed more calcium in Chrotogonustrachypterus in comparison to Poeciloceruspictus.

Present study on lipids reflects the anticipated changes in the surface tension and mechanical properties of the tissues. The Topographical changes in the distribution of lipids thus observed justified the morphological alteration, more neutral lipids were observed in the oesophagus region of foregut in Chrotogonustrachypterus in comparison to Poeciloceruspictus in which oesophagus showed negative reaction. Same results were obtained in pharynx, midgut, colon and rectum regions in two grasshoppers.

For phospholipid, oesophagus, proventriculus, colon and rectum showed the same results for all the three grasshoppers. More phospholipid were observed in Chrotogonustrachypterus in pharynx region in comparison to Poeciloceruspictus, while crop region in Chrotogonustrachypterus showed less phospholipid in comparison to remaining two grasshoppers. In midgut region of Poeciloceruspictus more phospholipid was observed in comparison to other two grasshoppers. Ileum showed a negative reaction in Poeciloceruspictuswhile Chrotogonustrachypterus showed poor and dull reaction.

The phosphatase which hydorlyse wide variety of otho-phosphoric monoesters into alkaline and acid media respectively were localized in Poeciloceruspictus, Chrotogonustrachypterus.

Day (1949) studied the distribution of alkaline phosphatase histochemically in the different regions of the digestive tract in number of insect species and suggested the relation of the enzymes and mechanisms of transport across cell boundaries. According to Pearse (1961), an alkaline phosphate ion from one alcohol to another and a high activity indicates increased phosphate transfer rather than hydrolysis of phosphate esters.

In Poeciloceruspictus the alkaline phosphatase activity was strong in pharynx, oesophagus, crop and proventriculus, while midgut showed a vigorous activity. In ileum, colon and rectum a positive activity was also observed. In Chrotogonustrachypterus, the alkaline phosphatase activity was vigorous in crop, proventriculus and midgut while pharynx and oesophagus showed a strong activity. Ileum, colon and rectum were observed to be alkaline phosphatase positive. Proventriculus and midgut showed a strong activity while in crop a vigorous activity was found, but in rectum the activity was present only in traces.

The presence of alkaline phosphates in the different parts of the digestive tract of presently studied grasshoppers may suggested that there may be some transport of substances across cell membrane of that region. Present findings find support with Day (1949) who studied the distribution of alkaline phosphatase in insects. Horie (1955 and 1958) studied histochemically and physiologically the alimentary canal of Bombyxmori.

The relative difference in the distribution pattern of alkaline phosphatase in different regions of the presently studied grasshoppers may be accounted for low and high phosphate transfer in these regions. Srivastava (1966) and Pearse (1961) noticed that the high activity indicates increased phosphate transfer from one alcohol to the other. On the basis of the present study, it may further be suggested that the quantity of alkaline phosphate is directly proportional to the amount of transport across cell membrane.

Hiromu (1969) observed the cytochemical localization of acid phosphatase in the midgut of Bombyxmori, and suggested that the enzyme is concerned with active fluid transport during the absorption of digested food materials from the lumen. In Poeciloceruspictus the pharynx, oesophagus, proventriculus, midgut, ileum and colon showed the presence of acid phosphatase while the crop gave a strong activity. A negative activity of acid phosphatase was observed in the rectum. In Chrotogonustrachypterus, acid phosphatase activity was positive in the pharynx, oesophagus, crop, ileum and colon while proventriculus and midgut showed a strong activity. In rectum it was present only in traces. All the regions exhibited acid phosphatase activity in traces except in midgut where the activity in traces except in midgut where the activity was found strong. The difference noted in the distribution pattern of acid phosphatase in different grasshoppers may thus be attributed to relative importance in digestive physiology.

The present findings are supported by Gomori (1941), Drilhon and Busnel (1945) and Hiromouo (1969) of acid phosphatase presence in the digestive tract and assigned the similar reasons.

The source of lipase in the digestive tract of the presently studied grasshoppers is not clearly known. In Poeciloceruspictus a positive activity of lipase was observed in crop, proventriculus and midgut

region while lipase was observed in traces in pharynx, oesophagus, ileum, colon and rectum showed a negative lipase activity. In Chrotogonustrachypterus, the activity of lipase in all the regions was in traces except the colon and rectum, where the negative activity was noticed.

CONCLUSION

From the functional point of view the distribution pattern of acid phosphatase in the pharynx, oesophagus, crop, proventriculus, midgut, ileum, colon and rectum indicated that it is found in the tissues which play active role in the digestion and absorption of food. The presence of acid phosphatase further established its significance in metabolism and transphosphorylation process. The presence of lipase in the digestive tract of the presently studied grasshoppers may be attributed to the fact that there may be some hydrolysed fat in that region which breaks down in the presence of lipase. Present findings also find support with Eisner (1955) who studied the digestion and absorption of fats and noted the similar results. Gomori (1941 and 1949) also studied the presence of lipase and assigned the similar functions.

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