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DIGESTIVE ENZYMES OF AN INSECT.*

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INTRODUCTION.

Digestion is a phase of Insect Physiology on which surprisingly little research has been done, considering the economic importance of the food of insects and the fact that our most important control measures involve the action of digestive juices on poisons taken into the digestive tract.

The cockroach (*Blatta orientalis* Linn.) was chosen for this study as it was available in quantities, and represented a generalized type of insect. Methods found of value in its study can be readily used upon such specialized types as the wood-, meat-, starch-, and sugar-feeders.

Our little knowledge of this problem is due to the investigations of F. Plateau (1), carried on about 1875. He came to the following conclusions:

1. The saliva of the cockroach changes starch to glucose, and is either neutral or alkaline. The reduction of copper in Barreswil's solution, a reagent similar to Fehling's solution, was taken as an indication of the presence of glucose.

2. The fore gut does not secrete digestive fluids. The contents are neutral, alkaline, or acid, depending on the food eaten.

3. The gastric cœca secrete a fluid which digests proteins. In his paper, "Les phenomenes de la digestion chez la Blatte americaine," he reports the fluid alkaline. Miall and Denny (2) cite him as having found it acid.

4. In the mid gut proteins are digested and fats emulsified(1). Miall and Denny (2) report Plateau in 1886 as having believed that no digestion takes place there.

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In the first series of tests in the present work, tissue suspensions were used, while in the second series the canal contents were tested directly. Macrochemical enzyme tests were used in the first case, and microchemical methods were developed for use in the second series.

I wish to acknowledge my great indebtedness to Drs. J. F. Lyman, C. H. Kennedy and R. C. Osburn, Ohio State University, for criticisms and suggestions.

Macrochemical Tissue-Suspension Method.

Tissue suspensions of the various organs were prepared and the presence of the enzymes tested for. An equal number of checks and tests were run for each experiment. The checks were made up in exactly the same manner as the tests, except that the tissue suspensions were first boiled to destroy the enzymes.

Preparation of the Tissue-Suspensions.

Insects, freshly killed with chloroform, were dissected under distilled water. The digestive tracts were removed, split open, and the contents thoroughly washed out, using distilled water. Each tract was then divided into the following four parts: fore gut, gastric cœca, mid gut, and hind gut. The salivary glands were also removed, and each of the tissues placed in a separate flask containing 30 cc. of pure glycerin.

After these tissues had been secured from 100 roaches, the glycerin-whole tissue suspensions were poured into mortars and the tissues reduced to a pulp with a pestle. The resulting suspensions were then replaced in the flasks and were ready for use. They will keep indefinitely.

AMYLASE.

This enzyme hydrolyzes starch, forming maltose.

Davis' Potato Starch Substrate (6): Add 10g potato starch to 250 cc. water. Boil with constant stirring till opalescent. Transfer to a 2 liter flask containing 500 cc. boiling water. Boil under reflux condenser for two hours. Cool, and make up to 1 liter. Preserve with 2% toluene.

Procedure: To 5 cc. of the above solution, add 1 cc. tissue suspension and .5 cc. toluene.

Iodine Test: Test every two minutes with iodine solution for the presence of starch. Record time taken to reach achromic point.

Fehling's test: After 48 hours, the above solution was tested for the presence of reducing sugars with Fehling's solution.

Results: Salivary gland.....heavy precipitate.
Other tissues and checks.....no precipitate.

Conclusion: Amylase is present only in the salivary gland.

MALTASE.

This enzyme hydrolyzes maltose, forming glucose.

Procedure: 35 cc. of 1% maltose solution plus 1 cc. tissue suspension plus 2 cc. toluene. Incubate 24 hours at room temperature.

Barfoed's test for monosaccharides:

Salivary gland	no precipitate.
Fore gut	slight "
Gastric cœca	heavy "
Mid gut	heavy "
Hind gut	no "
5 checks	no "

Saccharimeter Readings (Bates' type instrument):

Tissue	18 hours	22 hours
Salivary glands	+6.1	+6.1
Check	+6.1	+6.1
Fore gut	+5.5	+5.4
Check	+6.1	+6.1
Gastric cœca	+4.5	+4.1
Check	+6.1	+6.1
Mid gut	+5.5	+5.0
Check	+6.1	+6.1
Hind gut	+6.2	+6.2
Check	+6.2	+6.2

Conclusion: Maltase is present in the foregut, gastric cœca, and mid gut.

INVERTASE.

This enzyme hydrolyzes sucrose, forming fructose and glucose.

Procedure: 5 cc. of 1% sucrose solution + 1 cc. tissue suspension + .5 cc. toluene. Incubate 18–24 hours at room temperature.

Fehling's test for reducing sugars:

Salivary gland	no precipitate
Foregut	slight "
Gastric cœca	heavy "
Mid gut	heavy "
Hind gut	no "
5 checks	no "

Conclusion: Invertase occurs in the fore gut, gastric cœca, and mid gut.

Results: Salivary gland, 20 minutes. Other tissues and checks showed no digestion in 48 hours.

LACTASE.

This enzyme hydrolyzes lactose, forming glucose and galactose.

Procedure: 5 cc. of 1% lactose solution + 2 cc. tissue suspension + 2 cc. toluene. Incubate 24 hours at room temperature.

Saccharimeter Readings (Bates' Type Instrument):

Salivary gland	+3.00
Check	+3.00
Fore gut	+3.00
Check	+3.00
Gastric cœca	+3.00
Check	+3.00
Mid gut	+3.00
Check	+3.00
Hind gut	+3.00
Check	+3.00

Conclusion: Lactase is not present.

LIPASE.

This enzyme splits fats into fatty acids and glycerol.

Bloor-Raper (6, 9) *Neutral Olive Oil Emulsion:*

To 4 g. of casein, on a water bath, add water till a thin paste is formed Add two drops of phenolphthalein and enough N/1 NaOH to give a permanent pink tinge. Stir in 8 cc. olive oil till the globules disappear. Cool, dilute to a 4% emulsion.

Procedure: 5 cc. emulsion+2 cc. tissue suspension +.5 cc. toluene. Incubate 18 hours at room temperature. Titrate the freed acid with N/20 NaOH. Phenolphthalein indicator.

Tissue	Cc. N/20 Acid present
Salivary gland	.3cc.
Check	.3cc.
Fore gut	.9cc.
Check	.3cc.
Gastric cœca	1.3cc.
Check	.4cc.
Mid gut	1.0cc.
Check	.3cc.
Hind gut	.4cc.
Check	.4cc.

This experiment was repeated with the same results, using a 2% Acacia-Olive Oil emulsion (5) with thymol as a preservative.

Conclusion: Lipase is present in the fore gut, gastric cœca, and mid gut.

PEPSIN.

This enzyme, in an acid solution, breaks complex proteins down into peptones. Fibrin, at the suggestion of Dr. Lyman, was dyed in a 1% aqueous solution of amaranth red for 15 minutes. All surplus stain was then removed by thoroughly washing with water whose acidity was adjusted to pH=3 with HCL. This gave a red fibrin whose color would not come out in an acid solution.

Even the slightest digestion of the fibrin is then made apparent by a noticeable red color in the digested fluid.

Procedure: Adjust the tissue suspensions to an acidity whose pH=2 with HCL. 2 cc. tissue suspensions +colored fibrin+.5 cc. toluene.

Incubate 96 hours at room temperature.

No digestion was apparent.

Conclusion: Pepsin is not present in this insect.

TRYPSIN.

This enzyme, in an alkaline solution, breaks complex proteins down into peptones.

Procedure: The tissue suspensions were adjusted to an acidity whose pH=7.4 with $Na_2 CO_3$.

2 cc. tissue suspension +fibrin +.5 cc. toluene.

Gastric cœca.....digestion complete in 24 hours.
Mid gut.....digestion complete in 48 hours.
Other tissues and checks.....no digestion in 96 hours.

Conclusion: Trypsin is present in the gastric cœca and mid gut.

EREPSIN.

This enzyme splits peptones into amino-acids.

Salaskin's Phosphotungstic Acid Method (4, 3): To 25 cc. of a 1% peptone solution, whose acidity was adjusted to pH=7.6, add 2 cc. enzyme solution and 2 cc. toluene. Incubate at room temperature for 48 hours. Add phosphotungstic acid till no further precipitation occurs. Filter, and determine the nitrogen content of the precipitate by the Kjeldahl method. The presence of erepsin is indicated by a decrease in the N-content of the precipitate from that in the checks.

Tissue	g. Peptone-N(Precipitate)	Digested Peptone N.
Salivary gland	.01036g	0.0000g
Check	.01036g	0.0000g
Fore gut	.01050g	0.0000g
Check	.01050g	0.0000g
Gastric cœca	.00196g	0.0854g
Check	.01050g	0.0000g
Mid gut	.00826g	0.0124g
Check	.01050g	0.0000g
Hind gut	.01050g	0.0000g
Check	.01050g	0.0000g

Conclusion: Erepsin is present in the gastric cœca and mid gut.

SUMMARY OF MACROCHEMICAL TISSUE SUSPENSION METHOD.

Enzyme	Where Found
Amylase	Salivary gland.
Maltase	Fore gut, gastric cœca, mid gut.
Invertase	Fore gut, gastric cœca, mid gut.
Lactase	Absent.
Lipase	Fore gut, gastric cœca, mid gut.
Pepsin	Absent.
Trypsin	Gastric cœca, mid gut.
Erepsin	Gastric cœca, mid gut.

Microchemical Method of Enzyme Analysis.

The tissue suspension method used above is open to the criticism that it merely demonstrates the presence of enzymes in the tissues. It does not prove that these enzymes are secreted into the digestive canal nor that they function in the portion of the canal where they are secreted. Therefore, to check the above results, the contents of the various parts of the canal were collected and tested for the presence of those enzymes which had been found by the tissue suspension method.

In this investigation tests were sought which would be of use with smaller amounts of material, since the macrochemical tissue suspension method used above can find only limited application due to the large numbers of insects required for analysis.

By the following microchemical methods, the complete enzyme analysis of the canal contents can be carried out, with definite results, using the material obtained from two roaches. These tests can also be used with little or no change in tissue suspension analyses, although they were not so used in this investigation.

General Procedure.

The alimentary canals were obtained from freshly killed roaches, divided into the fore, mid and hind guts, and the contents removed. These materials were then diluted with equal volumes of distilled water, and the acidity regulated as desired. They were then ready for use.

The incubations were carried on at room temperature in micro-tubes, about 1¼ inches long, made from ¼-inch glass tubing.

The checks were made up with boiled enzyme solutions.

To each check and test, in the micro-tubes, was added enough toluene to cover the surface of the liquid. This prevents the action of microorganisms.

AMYLASE.

Starch-Iodine Test: To a dilute starch paste, add sufficient I-KI solution to give a dark blue color. Place in micro tubes, add a small amount of the enzyme solution, and cover with toluene. Incubate 48 hours. When the starch is digested, the solution will be colorless. Then, to the colorless tubes add several drops of I-KI solution. If no blue color develops, digestion is complete. This last check should be made to make sure that the loss of color was not due to the absorption of iodine by fats in the enzyme solution.

Fluckiger test for reducing sugars (12:) Incubate starch paste+enzyme solution+toluene for 48 hours. Add one drop of the resulting solution to a drop of Fluckiger's reagent on a slide. Add a cover glass and heat gently. A red precipitate of copper indicates the presence of reducing sugars.

Fluckiger's reagent: Mix a drop of 20% NaOH with an equal amount of powdered copper tartrate on a slide till the copper is dissolved. The reagent is then ready for use.

Results: Amylase was found in the fore gut, mid gut and hind gut.

MALTASE.

Procedure: 5% Maltose solution+enzyme solution+toluene. Incubate 72 hours in micro tubes.

Phenylhydrazine-Osazone Test (10): Mix one drop of Phenylhydrazine-HCL (1-10 glycerin) and one drop of Sodium acetate (1-10 in glycerin) on a slide. Add one drop of the incubated solution. Add the cover glass.

Heat on the water bath for 15 minutes. The glucose osazone will appear within an hour on cooling. Maltose osazones appear 3–4 days later.

Results: Maltase was present in the fore and mid guts.

INVERTASE.

Procedure: 5% sucrose solution+enzyme solution+toluene. Incubate 48–72 hours.

Phenylhydrazine-Osazone test: (10): This test is given above. Heat 15 minutes on the boiling water bath. Fructose osazones appear immediately; Glucose osazones within an hour on cooling.

Fluckiger test for reducing sugars: Fructose reduces the copper instantly in the cold. Glucose reduces it on warming.

Methyl-phenylhydrazine-Osazone test: Mix 1 drop of Methylphenylhydrazine acetate (1-10) in glycerin and 1 drop of sodium acetate (1-10 in 50% glycerin). Add sugar solution. Gives osazone with fructose on heating gently for 7–8 minutes.

Results: Invertase was present in the fore gut, mid gut and hind gut.

LACTASE.

Procedure: 5% Lactose solution+enzyme solution+toluene. Incubate in micro tubes 48–72 hours.

Phenyl-hydrazine-Osazone test (12): This test is described above. Glucose osazones are taken as an indication of the presence of lactase.

Results: No lactase was present.

LIPASE.

B. T. B.-Tristearin test: Wash commercial tristearin repeatedly with 70% alcohol to rid of free fatty acids. Melt, and add sufficient KOH to give it an acidity whose pH = 7.2. Pour into the micro tubes to a depth of ½ inch. Allow to harden.

Add several drops of Brom Thymol Blue to the enzyme solution and sufficient $Na_2 CO_3$ to give it a blue color (pH = 7.2).

Add the blue enzyme solution to the tristearate in the micro tubes, cover with toluene, and incubate 48–96 hours.

As the fat is digested a green to yellow zone forms at the junction of the fat and liquid, and eventually the entire solution becomes greenish yellow to yellow due to the liberation of fatty acids.

B. T. B.-Emulsion test: Add several drops of Brom Thymol Blue to the emulsions prepared as directed under the tissue suspension method for lipase. Add sufficient NaOH to give a blue color. Prepare the enzyme solution as in the above test and add it to the blue emulsion in micro tubes. Incubate 48–96 hours.

The change from blue to greenish yellow or yellow indicates the presence of lipase.

Results: Lipase was present in the fore gut and mid gut.

PEPSIN.

Colored Fibrin method: Color fibrin with Amaranth red as directed under the tissue suspension method for Pepsin. Place a small piece of the red fibrin in the bottom of a micro tube. Add the enzyme solution whose acidity is adjusted to pH = 2 with HCL. Cover with toluene. Incubate 48–96 hours. Digestion is indicated by a strong red color in the solution.

Results: Pepsin was not present.

TRYPSIN.

Colored Fibrin Method: Stain fibrin 15–30 minutes in Anilin Blue. Wash with alkaline water (pH = 7.6) till all surplus stain is removed. Place a small piece of the blue fibrin in the bottom of the micro tubes and add the enzyme solution whose acidity is adjusted to pH = 7.6 with $Na_2 CO_3$. Cover with toluene. Incubate 48–96 hours. Digestion is indicated by a strong blue color in the solution.

Results: Trypsin was present in the fore gut, mid gut, and hind gut.

EREPSIN.

Modified Sorensen Method: Solution A: Add several drops of Brom Thymol Blue to a 4% peptone solution, and then sufficient KOH to give a blue color (pH = 7.6).

Solution B: Add several drops of Brom Thymol Blue to 40% Formalin and then sufficient 2% KOH to give a blue color. Add additional 2% KOH a drop at a time till when 1 cc. of this solution is mixed with 2 cc. of solution A, the resulting color remains blue.

Procedure: To solution A in the micro tubes, add the enzymes solution and toluene. Incubate 48–96 hours.

Then add a volume of solution B equal to one-half the volume of A in the micro tubes.

Digestion by erepsin with the liberation of amino acids is indicated by a greenish yellow to yellow color on the addition of solution B.

Results: Erepsin is present in the fore gut, mid gut, and hind gut.

Summary of Microchemical Analysis.

Enzyme	Found in contents of:
Amylase	Fore gut, mid gut, hind gut.
Maltase	Fore gut, mid gut.
Invertase	Fore gut, mid gut, hind gut.
Lactase	Absent.
Lipase	Fore gut, mid gut.
Pepsin	Absent.
Trypsin	Fore gut, mid gut, hind gut.
Erepsin	Fore gut, mid gut, hind gut.

General Conclusions.

In the tissue suspension experiments, no maltase could be detected in the salivary glands. Plateau states that the saliva can change starch to glucose, in which case maltase would necessarily be present. However, he took the positive Barreswil's test as an indication of the presence of glucose. This test is given by any reducing sugar, since Barreswil's and Fehling's solutions are practically identical. It was, without doubt, maltose and not glucose which gave the positive test.

The gastric secretion must be alkaline or at least only slightly acid since trypsin is the only enzyme present splitting complex proteins.

From the tissue tests, it appears that three enzymes, namely, maltase, invertase, and lipase are present in the cells of the fore gut. Plateau states that no enzymes are secreted by the fore gut, but that the digestive fluid from the gastric cœca rises into that portion of the gut and that digestion takes place there. It is also generally accepted, from morphological studies, that the fore gut does not secrete digestive juices. In these experiments, the contents of the fore gut were washed out thoroughly with distilled water. Any enzymes found there then, must be in the cells or the chitinous lining of the tract.

These enzymes therefore, must either have been formed by the cells of the fore gut, or absorbed by them from the digestive cavity. This cannot be settled by the evidence at hand. However, in the latter case, it would be expected that all the digestive enzymes found by the microchemical analysis in the fore gut, should also be found in the tissue preparation. This of course, is not necessarily so. Regardless of the origin of the

enzymes, we at least know that they are present in the fore gut and that most of the digestion takes place there.

Plateau's contention that the secretion from the gastric cœca rises into the fore gut is borne out by the fact that enzymes were found in the fore gut contents which could have been secreted only by the gastric cœca or the mid gut. As the mid gut secretes only small amounts, the gastric cœca would appear as the logical source of these enzymes.

The salivary glands and the gastric cœca are the most important sources of the digestive enzymes. The mid gut produces the same enzymes as the gastric cœca, but less abundantly. Its main function appears to be absorption rather than secretion.

The hind gut does not secrete digestive enzymes. Its main function appears to be the elimination of waste. Enzymes may be found in the contents of that portion nearest the mid gut, but are not demonstrable near the rectum. From this, it would appear that the enzymes are either reabsorbed or destroyed in the hind gut.

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