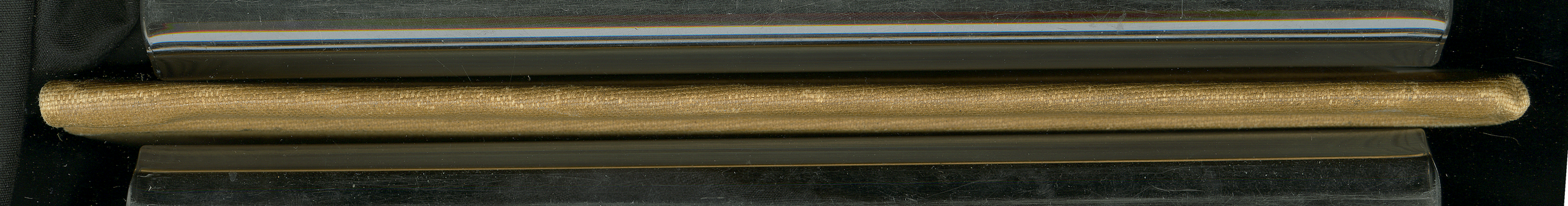

DIGESTIVE ENZYMES OF THE COELENTERATES HYDRA VIRIDIS AND HYDRA FUSCA

BY

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#2.

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Our knowledge concerning the digestive enzymes of the Coelenterates, especially the smaller and simpler ones, is very limited. A review of the literature of the subject shows that very little has been done toward a complete investigation of the digestive processes in the Coelenterates.

The present work had, as its basis, a piece of work done by Bodansky and Rose,(I),(which see for description and bibliography), on the digestive enzymes of the large Coelenterates, Stomolophus meleagris and Physalia arethusa. Tests were made by these men on the digestive tissues of these animals for proteolytic, lipolytic, diastatic, and rennitic enzymes, as well as for inulase, invertase, maltase, lactase, and raffinase. The following enzymes were found: Pepsin, trypsin, rennin, amylase, maltase, lipase, and invertase in negligible quantities. The methods of Dernby(3) were used in these investigations by Bodansky and Rose. These methods as well as a number of others were used in the following investigations.

A general review of the literature on the enzymes, found by Bodansky and Rose in Coelenterates, was first made, and the work was carried out with the purpose of throwing some light on the occurrence, or non-occurrence, of these in Hydra viridis and Hydra fusca. Most of the investigation was made with Hydra viridis only because of the scarcity of Hydra fusca during this present year.

The general method of the work has been that of testing the digestive power of tissue suspensions of the animals on

certain substances under certain definite conditions. No attempt was made in any of the experiments to determine the exact H-ion concentration of the digests. Special efforts were made, however, by the use of indicators, to make the H-ion concentration agree with the optimum H-ion concentration of the enzyme being sought. The indicator used in all cases was litmus. Litmus has a lilac color at true neutrality, that is, PH equals 7; at a PH value of 8, it is blue, and, at a PH value of 5, it is red.

MATERIALS USED

Tissue Suspension- Following the method of Bodansky and Rose, no attempt was made to isolate, or concentrate, the digestive enzymes from the tissues of the Hydras. The Hydras were picked out of aquaria and freed from mud and debris. They were then dried at room temperature; ground to a powder with crushed glass in a mortar, and preserved dry in a tightly stoppered bottle. When a tissue suspension was needed, some of the ground material was shaken with water; 0.2% HCl, Or N/IO NaOH for twenty-four to thirty-six hours in most cases. One drop of toluene was added for each cc. of the mixture to act as a preservative. The most potent digestive suspensions were found to be those that had stood for about twenty-four hours, having been made up with distilled water alone. The suspensions used were not all the same strength. The most satisfactory ones were those that contained at least seventy-five to one hundred Hydras per cc. of suspension.

Peptone Medium- Four gms. of Witte's Peptone were

dissolved in I00 cc. of water at 60 degrees C.; boiled for 30 minutes, and filtered. To 40 cc. of this 4% solution of peptone were added I cc. of 0.5 M potassium dihydrogen phosphate; 9 cc. of 0,5 M disodium hydrogen phosphate, and 0.2 gm. of thymol suspended in water to act as a preservative. Enough water was added to bring the volume up to I80 cc. Then N NaOH was added until the medium was just alkaline to litmus--PH equal to about 8. The phosphates were added as buffer salts. They prevent any great change in H-ion concentration of the medium during digestion.

Vanillan Solution- A 5% solution of vanillan in 95% alcohol.

Bromine Water- A saturated solution of bromine in water.

P-dimethylamidobenzaldehyde- A 2% solution of P-dimethylamidobenzaldehyde in 95% alcohol.

Colored Fibrin- Colored fibrin prepared by Gutzner's method (2) was used. Dried, commercial fibrin was swelled by soaking in an ammoniacal solution of carmine. This colored it a bright red. It was then rinsed in two changes of water; soaked for several hours in 0.2% HCl, and dried at about 40 degrees C.

Colored Gelatin- Colorless, commercial gelatin was dissolved in a warm ammoniacal solution of carmine; heated to boiling for fifteen to thirty minutes; dried on paper at room temperature; soaked for several hours in water, and washed with several changes of water.

Gelatin Medium- Thirty-five grams of colorless,

commercial gelatin were dissolved in 70 cc. of warm water, and ¼ gm. of thymol in water added to act as a preservative. This was diluted to 250 cc., and its acidity reduced by adding N/10 NaOH until it was just acid to litmus,(PH equal to about 5).

Mett Tubes- (2)- White of egg was drawn into a thin walled glass tube of about ½ mm. bore. The tube was immersed in water at 95 degrees C. for five minutes to coagulate the albumen. Then it was cut into pieces about four to five mms. long.

Water-Whenever water is mentioned in the preparation of reagents or media, distilled water is meant.

EXPREIMENTAL PROCEDURE

Tests were made for the following enzymes in Hydra viridis: pepsin, trypsin, trypsinogen, rennin, animal diastase or amylase, and lipase. Tests were made for trypsin, amylase, and lipase in Hydra fusca. The tests were usually run in pairs; one a digest and the other a blank to act as control. The optimum temperature for the above mentioned enzymes is 37-40 degrees C. Some of the tests were run at this temperature while others were run at room temperature,(about 23 degrees C.).

Pepsin- This is one of the main proteolytic enzymes found in the bodies of higher animals. It is the enzyme that starts the breaking down of proteins in the process of digestion. It works only in an acid medium. Its optimum H-ion concentration is about PH equal to 4.

The first tests for pepsin were made by using Mett Tubes(2). The Hydra suspension used was prepared by soaking

ground Hydra tissue in 0.2% HCl for twenty-four hours. Four pairs of test tubes were prepared as follows: to each ~~tube~~ of four tubes were added 2 cc. of the tissue suspension, while to each of the other four tubes were added 2 cc. of 0.2% HCl. The latter tubes were to act as controls. Two drops of toluene were added to each tube as a preservative. Two prepared Mett Tubes were placed in each test tube. The test tubes were tied in pairs--one digest and one control--and let stand at room temperature. The object of the experiment was to find out whether, or not, the tissue suspension would dissolve, or digest, the coagulated albumen in the Mett Tubes.

After twenty-four hours the Mett Tubes, from two sets of the test tubes, were examined. No signs of digestion, other than a slight swelling of the albumen, showed in the Mett Tubes from either the digests or controls. The remaining test tubes were placed in a water bath at 37-40 degrees C. for five hours more; then removed, and examined. No traces of digestion, other than the slight swelling previously mentioned, were detected in either the digests or controls. It was discovered later that the time interval was too short to show digestion in any of these tubes.

A series of tests were made with colored gelatin. Pieces of the gelatin were placed in prepared tissue suspension, and the solvent power of the suspension noted. Three pairs of tubes were prepared as follows: to each tube were added 6 cc. of 0.2% HCl; to each of three of the tubes I cc. of tissue suspension from Hydra viridis was added; to each of the remaining three tubes I cc. of water was added. These latter tubes were to act

as controls. Five drops of toluene were added to each tube as a preservative. The tubes were tied together in pairs, one digest tube and one control tube to a pair. A small piece of colored gelatin was suspended in each tube, care being taken to get the piece in a digest and its control as near the same ~~same~~ size as possible. Two sets of the tubes were warmed slightly and tilted back and forth occasionally. The other set was allowed to stand unshaken at room temperature. Solution took place in the tubes as follows:

Set #I (control-- complete solution, I9 minutes.
digest-- complete solution, II minutes.

Set #2 (control-- complete solution, 40 minutes.
digest-- complete solution, 36 minutes.

In the set that stood at room temperature, unshaken, the results were as follows:

Set #3 (control-- complete solution, 2 hrs. 25 minutes.
digest-- complete solution, I hr. 20 minutes.

It was here discovered that the HCl being used was made up with tap water that contained enough salts in solution to dissolve gelatin rather rapidly. The above tests with colored gelatin, therefore, are not dependable.

Another set of tubes was prepared in the same way as the above, using distilled water for diluting the HCl. At the end of thirty minutes, at room temperature, the gelatin in the digest tube showed signs of dissolving, while that in the control tube showed no indications of solution. After one hour, the gelatin in the digest tube was disintegrating perceptibly, while that in the control tube showed only a few bits broken off from the main mass.

Four pairs of tubes were prepared in the same manner as those above. Pieces of colored fibrin were placed in them instead of the colored gelatin previously used. After standing at room temperature for forty-eight hours, no appreciable difference was noted between the fibrin in the digest tubes and that in the controls.

Thus in none of the preceding experiments is the presence of pepsin in Hydra tissue suspension conclusively shown. It is the opinion of the author that the methods thus far used are not dependable. There is too much room for error in them.

The method of Dernby (3) and of Bodansky and Rose (I) was now tried for the detection of pepsin. This method consists of trying the liquefying power of tissue suspensions on gelatin medium. The digests were prepared as follows: fifteen cc. of the prepared gelatin mediumwere added to each of three Erlenmeyer flasks; to one of these flasks, 5 cc. of boiled tissue suspension were added, and enough water to bring the volume up to 30 cc. in the flask. To each of the other two flasks, 5 cc. of unboiled tissue suspension were added, and enough water to bring the volume in each flask up to 30 cc. The tissue suspensions had been made up in water instead of dilute HCl. The flasks were kept just warm enough to keep the gelatin liquid, 27-35 degrees C. Four cc. portions were removed from each flask at intervals; poured into small bottles, and immersed in an ice bath for fifteen minutes. The following comparative table was used to express the degree of liquefaction.

0- completely solid.

I- solid but small pieces may be torn off by strong shaking.

2- solid but the surface moves somewhat when the bottles are shaken.

3- soft.

4- half liquid.

5- almost liquid.

6- entirely liquid.

The following table shows the results obtained in the experiment.

Time	1½hrs.	7hrs.	22hrs.	44hrs.	72hrs.
#I control	0	0	0	0	0
#2 digest	0	0	0	0	0
#3 digest	0	0	0	0	0

The test does not give any evidence of pepsin in the tissue suspension. The tissue suspension used was some weaker than those used later in this work, and had been prepared for more than a week. Any pepsin in the suspension could easily have been destroyed, or become inactivated by standing in water for that length of time. The temperature of the digest was not high enough to insure the maximum activity of any pepsin present.

The experiment was twice repeated, using freshly prepared and, stronger, tissue suspension, and placing the flasks in an incubator at 37-40 degrees C. The following table gives the results of the two tests.

Time	7hrs.	24hrs.	48hrs.	72hrs.	96hrs.	I20hrs.	I44hrs.	I68
#I control	0	0	0	0	0	0	0	0
#2 digest	0	0	0	2	2+	3	3	4

#I control	0	0	0	0	0	0
#2 digest	0	0	I	I+	2	3

Under these improved conditions a pronounced liquefaction of the gelatin in the digest flasks took place while no liquefaction was noted in the controls.

Trypsin- This is one of the main proteolytic enzymes found in the digestive tracts of higher animals. It continues the work, begun by the pepsin, of breaking down proteins into assimmilable forms. It acts best in a slightly alkaline medium, (PH equal to 7.8-8.0).

One of the easiest methods for detecting trypsin is by testing for the products of tryptic digestion in a digest of some kind. Tryptophane always appears as one of the products of tryptic digestion. A number of tests were made in which peptone medium was used as a substrate. After certain intervals of time, the digests and controls were tested for the presence of tryptophane. The tissue suspensions used in the first experiments were two weeks old.

The procedure was as follows: to each of two test tubes 8 cc. of peptone medium were added; to one tube, (#I), I cc. of water and five drops of toluene were added; to the other tube,(#2) I cc. of tissue suspension and 5 drops of toluene were added. The tubes were then placed in a water bath at 30-40 degrees C. for three hours; removed, and tested as follows: five drops of vanillan solution were added to each of the tubes; 2 cc. of concentrated sulphuric acid were then added slowly by pouring down the wall of the tube. A violet ring appeared between the acid and the rest of the liquid. This is supposed to show the presence of tryptophane, but, as both digest and control gave the

same reaction, the test failed to show anything. It was discovered later that this test was not accurate in the presence of the thymol in the peptone medium and the alcohol in the vanillan solution. The thymol itself gives a violet ring under these conditions.

Another set of tubes ~~were~~ was prepared in the same way as the tubes above. After standing for twenty-four hours at room temperature, they were tested as follows: to each tube was added, drop by drop, one cc. of a 2% solution of P-dimethylamidobenzaldehyde. Then ten drops of concentrated HCl were added drop by drop. Tryptophane, if present in sufficient quantities, gives a blue color under this treatment. No such reaction was obtained.

Three pairs of tubes were prepared in the same manner as those above, and, after standing for twenty-four hours at room temperature, were tested by the bromine water method,(2). This test was made in the following way. The tubes were made slightly acid with HCl. A saturated solution of bromine water was added very slowly drop by drop. The bromine water throws the peptone out of solution as a light yellow, finely divided precipitate that settles out very slowly or not at all. After 4 drops of bromine water had been added to each tube, the digest was much clearer than the control, and showed a slight violet tinge. The greater clearness of the digest would seem to be due to the fact that part of the peptone had been broken down into substances not precipitated by bromine water. The violet tinge is an indication of the presence of tryptophane

in the digest.

The addition of bromine water was continued drop by drop. When between 20 and 25 drops had been added, the digest tube began to develop a decided orange tint; while the control maintained its original light yellow color. This difference was accentuated by adding more bromine water until about 40 drops had been added to each tube. The addition of more bromine water produced no further change in the tubes. The development of the orange color in the digest tube gives further indication of the presence of tryptophane in the digest.

Eight additional tests were made in the same manner as the one above described; one of them with a tissue suspension of Hydra fusca, and, in each case, the final result was the same as that in the above experiment. In none of these tests was a violet color noted upon the addition of only 3 or 4 drops of bromine water. This part of the reaction is very difficult to observe because of the yellow precipitate of peptone. And again, if the slightest excess of bromine water is added at this point, or if the bromine water is added too rapidly, the delicate violet color will not appear. The final colors, however, are easily distinguished, and are accentuated in the tubes by standing for one half to two hours. If tryptophane is present in large quantities, the addition of bromine water finally gives a violet red color. These experiments indicate the presence of trypsin in the tissues of Hydra viridis and Hydra fusca.

Several attempts were made to test for trypsin in the tissues of Hydra viridis microscopically. A green hydra was crushed to a pulp on a hollow ground slide. A drop of peptone

medium was then added. After a few minutes observation under the microscope, a drop of bromine water was added. The peptone was thrown out of solution as a yellow precipitate. The hydra tissues showed a faint pink tinge. This observation was intended as a control. Another slide was prepared in the same manner. It was allowed to stand at room temperature for one hour. A drop of bromine water was then added. Examination under the microscope failed to show any difference between this slide and the control. It was later found that not enough time had been given to the digest for it to show positive results.

The Hydra tissue suspension was now tried for its digesting power on fibrin prepared by Gutzner's Method,(2). The tissue suspension was prepared in N/10 NaOH. Two pairs of tubes were prepared in the following way. Seven cc. of water were added to each of the tubes. To each of two of these tubes, were added I cc. of tissue suspension and five drops of toluene. To each of the other tubes, as controls, were added I cc. of water and 5 drops of toluene. All the tubes were made slightly alkaline to litmus with NaOH. A small piece of colored fibrin was placed in each tube, and the tubes were left at room temperature.

The colored fibrin in the digest tubes began to lose color in less than thirty minutes. In six hours the color had been entirely dissolved from the fibrin in the digest tubes. The fibrin in the digest tubes had become transparent, and its edges had a frayed appearance. The fibrin in the controls was almost as bright in color as when placed in the tubes. It

showed a very little fraying along the edges and very little loss of color. After twenty-four hours, the fibrin in the digest tubes showed a much lower specific gravity than that in the controls. The fibrin in the controls still retained most of its original color, while that in the digest tubes was perfectly transparent.

A test for trypsin was now made using the gelatin method of Dernby (3) and Bodansky and Rose, (I). This test was made in the same way as that for pepsin with a few essential changes. The gelatin was made just alkaline to litmus with NaOH. When portions were tested at intervals by placing in an ice bath, they were first made acid with HCl, as alkalinity of it interferes with the setting of the gelatin. The same system for expressing results, used in testing for pepsin, is here used. The results of the test were as follows:

Time	24hrs.	48hrs.	72hrs.	96hrs.	I20hrs.	I44hrs.	I68hrs.
#I control	0	0	0	0	0	0	0
#2 digest	0	I	2	2+	3	3	3+

These results indicate the presence of trypsin in the bodies of Hydra viridis and perhaps Hydra fusca.

Trypsinogen- It is the opinion of authorities on the subject (2) that trypsin does not occur as such in the secreting cells or glands of the digestive tract, but as an inactive substance to which the name, trypsinogen, has been given. The trypsinogen, upon activation by certain secretions in the presence of lime salts, becomes trypsin. Trypsinogen itself is inactive and must be activated before it can act as

a digestive enzyme.

It was thought by Dr. Kepner of this laboratory that certain secreting cells around the mouth of the hydra secreted a substance that acted as an activator to some substance in the enteron. An attempt was made to determine the accuracy of this theory in the following way.

The distal ends of one hundred green hydras were cut off and only the basal ends used in preparing a tissue suspension in the usual way. The suspension obtained was divided equally between two test tubes. Enough water was added to each tube to bring the volume up to 3 cc. The solutions were made slightly alkaline to litmus with N/I0 NaOH, and three drops of toluene were added to each tube as a preservative. One of the tubes was placed in boiling water for ten minutes. A small piece of colored fibrin was now placed in each tube, and the tubes were left at room temperature. In I5 minutes the fibrin in the digest tube showed the beginnings of solution, while that in the control tube remained unchanged. After one hour the fibrin in the digest tube showed decided solution, while very little, if any, change could be seen in that of the control tube.

If the digestive enzyme had been present in the form of trypsinogen, solution would have taken place very slowly in the digest, or not at all. In this experiment it took place rapidly. Thus the test failed to show any evidence of trypsinogen, but did show the presence of trypsin in the tissue suspension as prepared.

Rennin- This is the principal coagulating,, digestive

enzyme. It is found as one of the digestive enzymes in the stomachs of many of the higher animals, and is also found in some of the lower animals. Bodansky and Rose, as pointed out before, found it in the bodies of some of the more complex Coelenterates. Under proper conditions it coagulates milk within a few minutes. It works best in a neutral, or very weakly acid solution.

Several tests were made to determine whether, or not, rennin occurs in the body of Hydra. Neutral tissue suspensions were added to fresh milk in test tubes. Toluene was added to prevent bacterial action, and the tubes were allowed to stand at room temperature. Three sets of tubes were prepared in this way. In all three cases coagulation took place in the tubes containing the tissue suspensions in 36-40 hours. In one case a slight bit of coagulation took place in one of the controls. Had rennin been present in appreciable amount, coagulation would have taken place within a few hours at the most. Pepsin, which also coagulates milk slowly, seems to have been the sole coagulating agent in these tests. No evidence of rennin in the body of Hydra viridis is given by these tests.

Amylase- This is a general term that includes all the starch splitting enzymes found in animal bodies. Such an enzyme is sometimes called animal diastase. Amylase works best in a slightly acid medium,(PHequal 4.6-6.4), but will work fairly well in a neutral medium. Tests were made to determine whether, or not, amylase occurs in the hydra.

Grains of arrowroot starch were placed in neutral tissue suspensions and examined from time to time under the microscope

for evidences of solution, or digestion. These observations were carried out over a period of several days, but, in no case, was solution observed. The tissue suspension seemed to have no effect whatsoever on whole starch grains. Calkins, in his Biology, states that starch grains fed to hydra are thrown out unaltered.

The action of Hydra tissue suspension on dilute starch paste was next tested. Tubes were prepared with dilute starch paste made from arrowroot starch, and tissue suspensions, with enough toluene added to prevent bacterial action. The contents of the tubes were examined at intervals by removing a few drops; placing them on a glass slab, and adding a drop of iodine solution. Any digestion of starch would produce dextrin which would give a wine red color with iodine. In the first set of tubes, the control showed a slight production of dextrin in twenty-four hours. Why this happened was not discovered. In the second set of tubes, both the digest and the control developed a trace of dextrin in twenty-four hours. The reason for this was also not discovered unless the temperature at which the tubes were incubated caused the hydrolysis. They were incubated at 37 degrees C.

Another test was made in the following way. Four cc. of dilute starch paste were added to each of two test tubes. One cc. of green hydra tissue suspension was added to one tube, and one cc. of water to the other tube which was to serve as a control. A few drops of toluene were added to each tube as a preservative, and both tubes were made slightly acid with HCl. Portions were removed from the tubes at intervals and tested

with iodine solution as in the preceding experiments. At the end of thirty-six hours, no trace of starch digestion had yet appeared.

Following this same method, another experiment was made using a tissue suspension of Hydra fusca. No trace of a starch splitting enzyme was observed up to thirty-six hours, the time extent of the experiment. Thus no evidence of a starch digesting enzyme was observed in either Hydra viridis or Hydra fusca. It is the opinion of the author, however, that some kind of an amylolytic enzyme exists in Hydra because of the abundance of glycogen in the body of the Hydra.

Lipase- This the fat-splitting enzyme found in both plants and animals. It acts upon neutral fats splitting them up into fatty acids and glycerine. It works best in a distinctly alkaline medium, (PH equal 9).

Tests were made for lipase in both Hydra viridis and Hydra fusca. The method of making these tests has been used for some time and is as follows: The tissue suspensions used were prepared in the same way as those for the later tests for pepsin and trypsin. Neutral fat was prepared by dissolving butter fat, or cooking compound, in ether and shaking this solution with a solution of sodium carbonate. The ether layer was then drawn off with a pipette and poured into Petri dishes. The ether was evaporated off at a little above room temperature. The deposited fat was carefully warmed to the melting point and evenly distributed over the dish.

Drops of tissue suspension were placed about over the surface of the fat after it had been allowed to solidify, and

the dish was covered and allowed to stand at room temperature for twenty-four hours. The dish was then filled with M copper sulphate solution for ten minutes; the copper sulphate solution poured off, and the surface of the fat gently washed with water. If lipase is present in the tissue suspension, blue-green spots are left on the surface of the fat where the tissue suspension droplets stood. The blue-green spots are due to the insoluble copper soap formed from the fatty acids that were produced from the fat by the lipase of the tissue suspension. This process will not work for temperatures above the melting points of the fats being used for plating.

Four Petri dishes were prepared , one with Crisco and three with butter fat. Tissue suspension from Hydra fusca was placed in one of the dishes containing butter fat. In the other three dishes, tissue suspension from Hydra viridis was placed. The dishes stood for twenty-four hours at room temperature. They were then treated by the process described above. In every case blue green spots were left on the surface of the fat where the drops of tissue suspension had stood.

These experiments apparently establish the fact that a fat-splitting enzyme, lipase, occurs in the bodies of Hydra viridis and Hydra fusca.

SUMMARY

1. The digestive enzymes, pepsin, trypsin, and lipase occur in Hydra viridis.

2. The digestive enzymes, lipase, and perhaps trypsin occur in Hydra fusca.

3. No rennin was found to occur in Hydra viridis.

4. No starch digesting enzyme was found in either Hydra viridis or Hydra fusca. This does not mean that no such enzyme exists in these animals. The author just failed to obtain any direct evidence of the presence of such in them.

5. Trypsin was not found to occur as trypsinogen in Hydra viridis in the one experiment made to determine its presence or absence.

BIBLIOGRAPHY

I. The Digestive Enzymes of Coelenterates, by M. Bodansky and W.C. Rose, Amer. J. of Physiol., 1922, Vol.62.

2. Biochemical Catalysts in Life and Industry, by Effront, (translated by Prescott, 1917), John Wiley and Sons, New York City.

3. Autolysis of Animal Tissues, by K.G.Dernby, J. of Biol. Chem., 1918, Vol. 35, page 179.