BIOCHEMICAL STUDIES OF INSECTIVOROUS PLANTS

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I. The Work of Previous Investigators on Nepenthes

BY

Joseph Samuel Hepburn, A.M., M.S., Ph.D.

Voelcker (1) studied the composition of the pitcher liquor of *Nepenthes*. He describes the liquor from *unopened* pitchers as a clear, colorless liquid with a refreshing taste, an agreeable but not very pronounced odor, and an acid reaction to litmus. The acid was *not* volatilized by evaporation of the liquor to dryness.

The total solids of the liquor were determined by drying at 212° F. (100° C.), the ash by ignition of the solids at a red heat. Total solids are expressed as percent of the pitcher liquor, ash and volatile matter as percent of the total solids. The solids were yellow or cream colored, very hygroscopic, and readily soluble in water. Samples of liquor from different *unopened* pitchers gave the following values:—

	Total solids	Volatile matter (loss on ignition)	Ash
1.	0.92		
2.	0.91	25.86	74.14
3.	0.62	36.06	63.94
4.	0.27	32.92	67.08

* Through the kindness of Dr. John M. Macfarlane, these studies were conducted in the Botanical Laboratory and Nepenthes House of the University of Pennsylvania during 1914-1916. Papers that have appeared since that time have also been cited in the bibliographies.

The liquor from *unopened* pitchers contained potassium, sodium, magnesium, calcium, chlorine (hydrochloric acid), and organic acids; other bases, and sulphuric, phosphoric, oxalic, tartaric, and racemic acids were shown to be absent.

The liquor from *opened* pitchers was yellowish and not always clear; it was acid to litmus, and contained the same acids and bases as were present in the liquor of unopened pitchers. A determination of total solids gave 0.87 percent; the solids were yellow and readily soluble in water. Free volatile acids (including acetic and formic) were absent, for distillation of one-half ounce of the liquor to dryness yielded only distilled water as a distillate.

A further study of the organic acids and the ash was made on the residue obtained by evaporation to dryness of the mixed liquor collected from both unopened and opened pitchers. *The organic acids* were found to consist chiefly of malic acid, plus a little citric acid.

The ash had the following composition:—

Potassium chloride	76.31%
Sodium carbonate	16.44%
Calcium oxide	3.94%
Magnesium oxide	3.94%
Total	100.63%

Using the average value for the volatile matter of the total solids of the liquor of unopened pitchers (see above), and allowing for the carbonic acid content of the ash, the composition of the total solids was found to be:—

Organic matter (chiefly malic acid and a little citric acid)	38.61%
Potassium chloride	50.42%
Sodium oxide	6.36%
Calcium oxide	2.59%
Magnesium oxide	2.59%
Total	100.57%

The yellow color (e.g., of the total solids) is ascribed to a small quantity of "another organic matter."

Hooker (2) found that the pitcher liquor was always acid. The secretion of the liquor was not increased by the introduction of inorganic substances, but was increased by the introduction of animal matter into the pitcher. As substrates, Hooker used fibrin, raw meat, cartilage and cubes of egg white. He reported:—

"After twenty-four hours' immersion, the edges of the cubes of white of egg are eaten away, and the surfaces gelatinised. Fragments of meat are rapidly reduced; and pieces of fibrin weighing several grains dissolve and totally disappear in two or three days. With cartilage the action is most remarkable of all; lumps of this weighing 8 or 10 grains are half gelatinised in twenty-four hours, and in three days the whole mass is greatly diminished, and reduced to a clear transparent jelly. After drying some cartilage in the open air for a week, and placing it in an unopened but fully formed pitcher of *N. Rafflesiana*, it was acted upon similarly and very little slower."

All of the above experiments save the last were apparently conducted in opened pitchers. Experiments, made *in vitro* with pitcher liquor and fibrin, meat, and cartilage, gave a digestion of the substrates "wholly different" from that occurring in the pitchers. Hence the digestive action in the pitchers was not produced by the fluid first secreted by the pitchers.

From these and similar experiments Hooker concluded:—"It would appear probable that a substance acting as pepsine is given off from the inner wall of the pitcher, but chiefly after placing animal matter in the acid fluid; but whether this active agent flows from the glands or from the cellular tissue in which they are imbedded, I have no evidence to show." However, he attributed all three phenomena—secretion of the acid liquor, digestion (secretion of the digestive enzyme), and assimilation—to the glands, and decided that *Nepenthes* possess a true digestive process.

From the following experiments, it seemed probable that the temperature influenced the digestive action. When cartilage or fibrin was kept for six days in pitchers of *N. ampullaria* in a cold room, it was not digested; the substrate, however, was immediately acted upon when transferred to pitchers of *N. Rafflesiana* in a stove house.

If large amounts of substrate were introduced into a pitcher, digestion and absorption did not attain completion, and putrefaction was noted after the lapse of many days.

Tait (3) isolated an enzyme from the liquor of unopened *Nepenthes* pitchers, and also from the liquor of opened pitchers. His procedure

was as follows. The liquor was acidulated with dilute phosphoric acid, and a thin suspension of calcium carbonate in water was added until effervescence ceased; the precipitate of calcium phosphate, which adsorbed the enzyme, was permitted to stand for 24 hours, then was separated from the supernatent liquid by decantation, and was dissolved in very dilute hydrochloric acid. A saturated solution of cholesterol in a mixture of absolute alcohol and absolute ether was added to this solution. The precipitated cholesterol, which adsorbed the enzyme, was dissolved in absolute ether. The aqueous layer now contained the enzyme as a gray, amorphous, flocculent, suspended solid, which was partly soluble in distilled water, insoluble in boiling water, very soluble in glycerol, and produced a characteristic viscid change in a small quantity of fresh milk. Unfortunately the power of the enzyme to digest proteins was not tested; and its action on milk recalls that of rennin, rather than that of a true protease.

Tait permitted the liquor from four *virgin* pitchers of *N. phyllamphora* to act for 28 hours on cubes of albumen (volume of each cube 1 cubic millemeter). The substrate remained unchanged. But one sample of the liquor was acid; the other three samples were absolutely neutral; the enzyme described above was found in but one sample.

After the pitchers had opened, the liquor became changed in its reaction, and in its proteolytic power. "Fluid taken from pitchers into which flies have previously found their way is always very acid, has a large quantity of the ferment, and acts in a few hours on cubes of albumen, making them first yellow, then transparent, and finally completely dissolving them." The liquor in a pitcher, in which insects were undergoing digestion, was "very viscid and very acid."

Tait sums up:—"In the unopened pitcher the secretion is only faintly acid and not at all viscid. The secretion is increased therefore, ... , in quality after food has been taken in."

The work of von Gorup and Will (4) was largely carried out on the secretion of *N. phyllamphora*, Willd. and *N. gracilis*, Korth. Separate studies were made, *in vitro*, on (a) liquor from pitchers which were free from insects, and (b) liquor from pitchers which had been entered by insects and contained their remains. These may be spoken of as non-stimulated and stimulated pitchers respectively.

The liquor was almost colorless, faintly opalescent or entirely clear, odorless, without any distinct taste, and of varying consistency—some samples being thick, others being thin like water. The liquor from non-stimulated pitchers was neutral or, at the most, very faintly acid; that

from stimulated pitchers imparted a decidedly red color to litmus paper, and the color did not completely vanish on exposure to the air.

Liquor from Stimulated Pitchers

The insect residues were removed by filtration, and the action of the filtrate on certain substrates was studied. Ox-blood *fibrin* was swollen to a gelatinous mass in 0.2 percent hydrochloric acid, then was freed as completely as possible from the adherent acid by means of pressure. A flock of this fibrin was almost completely dissolved by the pitcher liquor in ¾ to 1 hour at a temperature of incubation of 40° C., and in 2 hours at a temperature of incubation of 20° C. The resulting solution was faintly opalescent. The time required for solution of the fibrin was reduced to ¼ hour by addition of several drops of 0.2 percent hydrochloric acid to the liquor. After digestion for two hours, the filtered solution remained clear on boiling, and was not precipitated by mineral acids or by acetic acid plus potassium ferrocyanide, but was precipitated by mercuric chloride, by tannin, and by phosphotungstic acid, and gave an excellent rose-red biuret reaction.

Little slices of *coagulated egg white* were digested with liquor to which 1 or 2 drops of 0.2 percent hydrochloric acid had previously been added. After 24 hours at 20° C., the slices were attacked at the edges, and were transparent; the filtrate from them gave a distinct rose-red biuret reaction.

On digestion at 20° C. with pitcher liquor and 1 or 2 drops of 0.2 percent hydrochloric acid, *raw meat* soon became transparent at the edges and somewhat swollen, and was partly dissolved without putrefaction. A further change was not noted after 48 hours. The filtered solution was not precipitated by boiling, nor by the addition of acetic acid and potassium ferrocyanide, was precipitated by mercuric chloride and by tannin, yielded a cloudy precipitate with phosphotungstic acid soluble in excess of the reagent, and gave a positive biuret reaction.

Legumin became transparent on the edges and somewhat swollen after digestion for 24 hours at 20° C. with pitcher liquor plus 1 or 2 drops of 0.2 percent hydrochloric acid; the filtered solution gave a very decided biuret reaction.

Gelatin, upon which pitcher liquor plus several drops of 0.2 percent hydrochloric acid had been poured, dissolved almost completely after 24 hours at ordinary temperature. The solution was filtered and concentrated to a small volume; it did not gelatinize, but remained a thick syrup; digestion had occurred with the production of gelatin-peptone, and the power to gelatinize had been destroyed.

The pitcher liquor did *not* contain a diastase. The liquor was mixed with a thin starch paste, and then incubated for 24 hours at 20° to 30° C. The starch was not hydrolyzed, the filtered solution was optically inactive, and did not reduce Fehling solution, hence did not contain reducing sugar.

Sufficient liquor from stimulated pitchers was not available to determine the nature of the free acid present in it. It is stated that hydrochloric acid may be excluded.

Liquor from Non-stimulated Pitchers

Gelatinous, swollen fibrin was washed until the acid reaction (due to hydrochloric acid) had almost completely vanished. Flocks of the fibrin, which were placed in the liquor, suffered no noticeable change within several hours at 20° to 30° C., and had not dissolved to the slightest extent at the end of 24 hours; the fibrin had contracted somewhat, and the filtrate from it gave a scarcely noticeable tinge of rose-red in the biuret test. However, the fibrin was almost completely dissolved in 1½ hours by pitcher liquor to which 2 or 3 drops of 0.2 percent hydrochloric acid had previously been added; the resulting solution behaved in every way as did the solution obtained by the action of the acid liquor from stimulated pitchers.

Swollen fibrin, carefully freed from adherent hydrochloric acid, was dissolved almost instantaneously at the ordinary temperature by liquor to which 3 or 4 drops of dilute formic acid had previously been added. The residue, which was scarcely noticeable, was removed by filtration, and the filtrate was carefully neutralized. The very slight precipitate which formed was collected on a filter; the filtrate gave none of the reactions of the true proteins, but did give an intense biuret reaction. When the formic acid was replaced by acetic acid, or by propionic acid, the fibrin was digested less rapidly, the rate of digestion now being about the same as in the liquor of the stimulated pitchers. At a temperature of 20° to 30° C., the fibrin was completely dissolved in 2 to 3 hours; the resulting solution contained mainly metaproteins and gave a very faint biuret reaction.

When the pitcher liquor was previously acidified with malic acid, the fibrin was almost completely dissolved in 10 minutes at ordinary temperature; the resulting solution gave a faint but distinct biuret reaction. After the digestion had been in progress for 2 hours, the precipitate on neutralization was very slight, and the biuret reaction decidedly more marked. When citric acid was substituted for malic acid, the fibrin was

dissolved in a considerably shorter time; after digestion for 2 hours, the resulting solution gave a very slight precipitate on neutralization, and gave a biuret reaction as intense as that obtained in the case of formic acid.

The solutions, obtained by digestion, contained much more protein (metaprotein) immediately after the fibrin had dissolved, than in later stages of the digestion. This protein gradually vanished, and was displaced by peptone as the period of digestion lengthened. From these phenomena, which were repeatedly observed, it followed that, apparently, peptone represented the second and not the first stage of the action of the enzyme.

Von Gorup and Will, who made the necessary control experiments on their reagents, do not hesitate distinctly to designate the acid liquor of the stimulated *Nepenthes* pitchers as a solution of plant pepsin. The neutral secretion of the non-stimulated pitcher, like pepsin in the absence of free acid, exerts no digestive action.

Vines (5) dehydrated pitchers of *Nepenthes* species (*N. hybrida* and *N. gracilis*) by means of absolute alcohol, converted their tissue into a pulp, and extracted this with glycerol in order to obtain a solution of the protease. As a substrate, he used fibrin which had been soaked in 0.2 percent hydrochloric acid until gelatinous. When the fibrin was incubated at 40° C. with the enzyme solution to which a few drops of 0.2 percent hydrochloric acid had previously been added, at the end of 8 hours the substrate showed signs of digestion and, after filtration, the solution gave a distinct peptone (biuret) reaction. Control experiments on fibrin plus enzyme solution, and on fibrin plus 0.2 percent hydrochloric acid, did not show digestion and did not give a biuret reaction. Hence the glands of the pitchers contained a proteolytic enzyme, which was soluble in glycerol and was active only in the presence of acid.

Pitchers were gathered from the same plants (of the species mentioned above) at the same time. Some pitchers were immediately treated as outlined above for the preparation of a glycerol extract. Other pitchers were first treated with dilute (1 percent) acetic acid for 24 hours, then the glycerol extract was prepared, using the procedure already described. In every set of experiments, the extract from the pitchers, which received the preliminary treatment with acetic acid, was higher in proteolytic power than the extract from pitchers not so treated. Thus the same volume of each extract was permitted to act on a pellet of swollen fibrin in the presence of 2 cc. of 0.2 percent hydrochloric acid at a temperature of 40° C. The fibrin pellets were similar. At the end of 6 hours, the

extract from pitchers, which had received the acid treatment, had completely dissolved the fibrin, while the extract from pitchers not so treated had but slightly attacked the fibrin. The solutions, obtained by filtration of the contents of each tube, always gave a positive response to the biuret test.

Vines states:—"These experiments seem to indicate that in the gland-cells of the *Nepenthes* pitchers, ..., the digestive ferment exists at first in combination with some other body, as zymogen—and that in plants, as in animals, this zymogen can be split up by the action of dilute acid, the free ferment making its appearance as a result of this decomposition."

In another report on this research, Vines (25) also described tests for the presence of diastase in the pitchers. The glycerol extracts of the pitchers were without action on starch, therefore, did not contain diastase. He also noted that the glycerol extract did not contain sugar.

During a further study of the proteolytic enzyme of *Nepenthes*, Vines (6) found that pitcher liquor plus 0.25 percent hydrochloric acid completely digested fibrin in the presence of bactericides such as potassium cyanide, chloroform, thymol. The fibrin was dissolved when mercuric chloride (approximately 0.5 percent) was used as a bactericide; however, the proteolysis was somewhat retarded. Fibrin was also digested by pitcher liquor to which had been added 1 drop of concentrated hydrochloric acid and sufficient hydrocyanic acid to render the concentration of the latter acid one percent. The liquor plus 0.25 percent hydrochloric acid partially digested coagulated egg albumen in the presence of potassium cyanide or thymol, the products of proteolysis being detected by the biuret test.

The enzyme and its proteolytic power were destroyed by the action of 1 percent sodium hydroxide for 1 hour, or of 5 percent sodium carbonate for three hours, at a temperature of 35 to 40° C.; the solutions were then neutralized, and tested for protease in the usual way, but digestion of the substrate never occurred.

The influence of the following concentrations of hydrochloric acid on the proteolysis was studied:—1%, 0.5%, 0.25%, 0.125%. The optimum acidity for digestion was found to be 0.25%.

The enzyme was isolated from 100 cc. of pitcher liquor by the following procedure. An equal volume of absolute alcohol was added, then phosphoric acid and lime water; the solution was neutralized with ammonium carbonate; the precipitate was collected on a filter, and permitted to drain over night. A portion of the precipitate was shaken with 10 cc. of 0.25 percent hydrochloric acid, and the solution was filtered; the

filtrate digested fibrin, while a control experiment on fibrin plus 0.25 percent hydrochloric acid gave no digestion. After the precipitate had been kept in a bottle with chloroform vapor for a month, the enzyme contained in it was still active.

Vines was unable to find a zymogen of the protease in the pitcher liquor, although the activation of a zymogen was suggested by the requirement that acid be added in order to produce proteolysis.

Glycerol extracts, prepared from the washed and dried tissue of relatively young, vigorous pitchers, contained a protease which acted in the presence of 0.25 percent hydrochloric acid, and retained its activity for as long as 2 months, but not indefinitely.

Vines concluded that the digestive action of the pitcher liquor is due to an enzyme. He detected albumose, but not peptone, as a product of the digestion, and suggests that peptone was formed and immediately split into other compounds. Wheat gluten was digested in the same manner as fibrin.

Most of these experiments were made on *N. mastersiana*. The liquor in the unopened pitchers of this species usually was distinctly acid.

In his next paper on the proteolytic enzyme of *Nepenthes*, Vines (7) studied the action of heat on the protease. The pitcher liquor was heated, then cooled; acid and fibrin were added, and digestion was carried out as in the previous study. Control experiments were made on the unheated pitcher liquor. Heating the liquor at 80° C. for 15 to 20 minutes did not destroy the enzyme, but the proteolytic power was decreased to a marked degree. Thus in one experiment the heated liquor completely digested the fibrin in approximately 4 days, while the unheated liquor in the control experiment required but 3 hours to digest the fibrin completely. When the liquor was held at 78° to 83° C. for 30 minutes, the enzyme was completely inactivated; no digestion occurred in 4 days, while the control experiment on unheated liquor gave complete digestion of the fibrin in 1½ hours. When the liquor was kept at 80° C. for 30 minutes, the enzyme was completely inactivated, and exerted no proteolytic action on fibrin after digestion for 1 week; the unheated control completely digested the fibrin in 5 hours. Boiling the liquor "for some seconds" partly inactivated but did not completely destroy the enzyme. It was necessary to subject the liquor to a temperature of 100° C. "for an appreciable time, say 3-5 minutes," in order to destroy the enzyme completely.

The destructive action of sodium carbonate on the enzyme was also studied. The degree of inactivation of the enzyme depended on the con-

centration of the alkali, the period of time during which it acted, and the temperature at which it acted. Sufficient solid sodium carbonate was added to the pitcher liquor to bring the salt to the desired concentration. After the resulting solution had been incubated at the desired temperature for the desired period of time, it was neutralized, then acidified with hydrochloric acid, and a digestion experiment was made in the usual way to determine the enzyme activity. The concentration of the sodium carbonate varied between 0.5 and 5 percent; it was permitted to act on the enzyme at a temperature of either 35° to 38° C. or 50° C. for a period of time varying between 30 minutes and 17 hours. Each control experiment on untreated liquor was incubated at the same temperature and for the same period of time as its determination proper; then its enzymic activity was determined.

The typical series of experiments in the following table may be quoted. The substrate in each experiment was 0.01 gram of fibrin.

Series	% of sodium carbonate	Time of incubation with sodium carbonate at 50° C.	Proteolytic activity subsequently
a	5	1½ hours	no digestion in 6 days
	1	1½ hours	digestion complete in 4 days
	Control		digestion complete in 3½ hours
b	5	1½ hours	no digestion in 5 days
	1	1½ hours	no digestion in 5 days
	Control		digestion complete in a few hours
c	1	1 hour	no digestion in 4 days
	Control		digestion complete in a few hours

"On comparing the results of *a*, *b*, and *c*, it would appear that treatment with 1% Na_2CO_3 for one hour at a temperature of 50° C. is an approximate index to the stability of the enzyme."

The pitcher liquor lost its acid reaction and its coloration, when passed through a Berkefeldt filter. "It still retains some digestive power, but is far less active than unfiltered liquid, the period of digestion being more than doubled." Filtration through a Berkefeldt filter caused a marked loss of the enzymic power of solutions of pepsin (from the stomach of the pig) and of salivary ptyalin. Hence the partial loss of proteolytic power by the pitcher liquor was due to the retention by the

filter of a true proteolytic enzyme, and not to the retention of proteolytic bacteria, *i.e.*, the liquor owed its proteolytic power to a true enzyme.

Experiments (usually, though not invariably, made on washed, unopened pitchers) showed that "under certain circumstances, previous treament with acid causes the glands of the pitcher to yield a more active glycerin-extract, or to yield an active extract when otherwise the extract would be inactive; and it can only be concluded that this must be due to the presence of a zymogen in the glands from which the enzyme is liberated on treatment with acid." The zymogen was best converted into active enzyme by treatment of the glandular tissue with acid for a short time at a relatively high temperature, say ¾ hour at 50° C. Action of the acid for a longer time at lower temperature, not only activated the enzyme but also extracted it from the glands. In the activation experiments, 0.25 percent hydrochloric acid was used; 0.5 percent acetic acid apparently was less satisfactory. One function of the high acidity of the liquor in unopened pitchers probably is to activate the zymogen.

Peptone and leucine were recognized among the products of digestion.

Vines concludes that the enzyme is derived from a zymogen which is present in the gland cells. The enzyme is a tryptic ferment, requires an acid medium for its action, and resembles the proteases of germinating seeds with respect to the reaction of the medium, which it requires, and the products, which it forms.

In his final paper on the proteolytic enzyme of *Nepenthes*, Vines (8) applies to it the name "Nepenthin." Fibrin and Witte peptone were subjected to a somewhat prolonged digestion at 38.5° C. with pitcher liquor, to which hydrochloric or citric acid had been added. The following are typical experiments:—

I. 10 grams moist fibrin
50 cc. 0.3% hydrochloric acid
50 cc. pitcher liquor (from *N. Mastersiana*)
Period of incubation 18½ hours

II. 1 gram Witte peptone
0.2 gram citric acid
10 cc. distilled water
40 cc. pitcher liquor
Period of incubation—from noon until morning of the next day.

After digestion, the resulting solutions gave the tryptophane reaction (a violet or pink color with chlorine water), which is stated to be characteristic of tryptic digestion only.

The solutions, obtained by digestion, were evaporated to dryness, the residue was extracted with absolute alcohol, and the alcohol was removed by evaporation. This residue gave a biuret reaction. Hence tests could not be made for *free* tyrosin among the products of the proteolysis, for the tyrosin reactions would have been given by the combined tyrosin groups of the albumose and peptone, which were present in the residue from the alcoholic solution.

Dubois (9) studied the pitcher liquor of the following species of *Nepenthes:—coccinea, distillatoria, Hookeriana, hybrida, maculata, phyllamphora, Rafflesiana.* Before the opening of the operculum, the pitcher liquor of all these species was limpid, slightly viscid and slightly acid. In opened pitchers, the liquor generally was thick, contained whole insects, and, at times, emitted a strong odor of putrefaction.

When liquor was removed from closed pitchers, which were about to open, and was immediately placed in contact with cubes of coagulated albumen, then incubated at the temperature of the atmosphere, or at a temperature of 35° to 40° C., the albumen was not attacked; the liquor remained limpid at the end of several hours. It was then filtered; the filtrate contained no peptone. The experiment was repeated by transferring the liquor from closed pitchers to Pasteur culture tubes which contained albumen cubes. The results were the same as before; the angles of the cubes remained absolutely intact, and neither microorganisms nor putrefaction were present at the end of several days.

Liquor from pitchers, which had been open for but a short time, was still clear. However, it attacked cubes of egg-white, quite rapidly at ordinary temperatures, and very rapidly at the temperature of the incubator. The cubes became swollen, transparent and gelatinous, and lost their angles; the liquor became viscid, and a distinct odor of putrefaction developed in some of the tubes. The liquor contained numerous microorganisms of different species and, after filtration, gave some of the reactions of peptone. Many open pitchers contained insects, which were in process of putrefaction and not in process of digestion.

The manner in which coagulated egg albumen behaved in the presence of the pitcher liquor—contaminated or not contaminated by microorganisms—led Dubois to the following conclusions:—

(1) That the liquor does not contain any digestive substance (enzyme) comparable to pepsin, and that *Nepenthes* are not carnivorous plants.

(2) That the phenomena of disintegration or pseudo-digestion, observed by Hooker, were due without any doubt to the activity of micro-

organisms which came from without the pitcher, and were not due to a secretion of the plant.

Couvreur (26) supported the conclusions of Dubois, and maintained that the digestive phenomena observed by Vines were due to the action of the reagents on each other, and not to the presence of a protease in the pitcher liquor of *Nepenthes*.

Tischutkin (27), in a paper on *Pinguicula*, commented on the work of Von Gorup and Will. He considered that the protease, which these investigators found in the pitcher liquor of *Nepenthes*, was entirely of bacterial origin.

Among the insectivorous plants studied by Tischutkin (10) was *Nepenthes Mastersi*. The pitchers were stimulated by means of small, sterile cubes of albumen. Even 24 hours later, the liquor of the pitchers contained myriads of bacteria, as was regularly shown by the direct microscopic examination. The bacteria were isolated by cultures on weakly acid nutrient gelatin and were tested for their peptonizing power; several species were always found which dissolved small, sterile albumen cubes fairly rapidly in an acidified menstruum.

The following experiments were also carried out. Incisions were made in the side wall of pitchers, which had not yet opened and therefore contained no bacteria; the liquor was removed with a pipette and conveyed to test glasses which contained water and small cubes of albumen (1 cube in each glass); in some glasses the water was neutral, in other glasses it had been acidfied. The experiments were carried out with antiseptic precautions. The results showed that the pitcher liquor did not contain a peptonizing enzyme, for the substrate remained unchanged after incubation for 48 hours at 37.5° C. In order to overcome the possible objection that the pitchers had been too young, the experiments were repeated in a modified form. Openings were made in the wall of pitchers, which had not yet opened; small, sterile cubes of albumen were introduced into the cavities of the pitchers; the openings were closed; and the plants were permitted to remain undisturbed. When the pitchers opened, 4 days later, the albumen cubes were found unaltered; their angles had *not* been rounded off; the liquor had a strongly acid reaction and contained no peptone; and bacteria were present "in very slight number." When the liquor of these pitchers was placed in test glasses and treated anew with small cubes of albumen, it dissolved the cubes only after 4 to 5 days had passed, *i.e.*, at a time when the bacteria had already multiplied to a considerable degree.

Tischutkin maintained that the digestion of protein in the liquor of insectivorous plants is produced exclusively by the vital activity of micro-organisms, which are always present in the secretion of the fully developed plant, entering from the air and also with the bodies of the insects, etc. The rôle of the insectivorous plants is therefore limited; they secrete a medium favorable for the activity of the peptonizing micro-organisms, and they make use of the products of this activity.

Goebel (11) made an elaborate research on the pitcher liquor. Pitchers of *Nepenthes paradisiaca* (a hybrid) contained a clear, colorless, tasteless fluid free from insects. Fibrin flocks were placed in the pitchers, and also in water as a control. In both cases, after 6 days, the flocks had been disintegrated into little shreds, and innumerable bacteria were present. The liquor, in all the experiments, was either neutral or very faintly alkaline, and contained no peptone. The liquor from the pitchers gave no color with Nessler's reagent; the solution from the flasks, which contained water and fibrin, gave a strong yellow color with this reagent. Hence, in the pitchers, the nitrogenous compounds produced by the proteolysis of the fibrin were absorbed, either as ammonia or as some other compound.

Liquor was removed from the pitchers, in which the fibrin had been digested, and was sown on nutrient gelatin. The gelatin became liquefied to a marked degree in 2 days and acquired a green fluorescence which is characteristic of *Bacillus fluorescens liquefaciens*. Neither bacteria nor moulds were found when liquor from unopened pitchers was inoculated into the gelatin.

Liquor was collected from unopened pitchers of *N. paradisiaca*; it exerted very little digestive action after 0.2 percent hydrochloric acid had been added, forming very little peptone; therefore very little enzyme was present.

The absorption of ammonia by the pitcher was demonstrated by the following procedure. A definite volume of aqueous solution of ammonia (containing 1 part of ammonia in 20,000 parts of solution) was placed in a pitcher, an equal volume of the solution was placed in a glass vessel in the *Nepenthes* house as a control. Twenty-four hours later, the volume of liquid within the pitcher was practically unchanged. However, Nessler's reagent produced no precipitate with the contents of the pitcher, and formed a thick precipitate with the control experiment; therefore ammonia had been absorbed by the pitcher. Further experiments demonstrated that one-half of the ammonia was absorbed during the first three hours, and that all the ammonia had been absorbed at the end

of 6 hours; in these experiments Nessler's reagent was used for the colorimetric determination of ammonia.

Pieces of meat, the size of grains of rye, were introduced into pitchers; after 2 days, the contents of 3 pitchers were acid in reaction, those of 5 pitchers neutral in reaction; after another week had passed, the contents of but 1 pitcher had an acid reaction which, however, was not very marked.

A vigorous plant of *Nepenthes paradisiaca* was cultivated in a glass chamber at a temperature of 20° to 25° C. in an atmosphere saturated with moisture. The plant bore 3 pitchers. The oldest of the pitchers was brownish and no longer vigorous, and contained a small amount of neutral liquor. A wasp was introduced into the liquor and soon died; 3 days later the liquor had an alkaline reaction; bacteria and infusoria were present in abundance. The second pitcher contained an acid liquor, in which a small fly was present. This liquor dissolved fibrin (which had been stored in glycerol, washed, and strongly swollen) in 1 hour; after 3 hours, soluble protein was absent, but peptone was present; the temperature of incubation was 25° C. Another fibrin flock and 0.2 percent hydrochloric acid were added, and the temperature of incubation was changed to 16° to 18° C.; the flock dissolved in 40 minutes; this solution was sown on nutrient gelatin, and bacteria were not found.

The youngest pitcher was still closed. Inoculation of its contents into nutrient gelatin gave no growth. The liquor was neutral in reaction and mucilaginous. A flock of swollen fibrin and 0.1 percent formic acid were added to the liquor; the flock was completely digested in 12 hours; even after 8 days, bacteria were absent as was shown by a nutrient gelatin culture, which was made in duplicate. It was also found that 0.1 percent formic acid prevented the development of putrefactive bacteria in an approximately 0.5 percent peptone solution, which was exposed in the open air for 8 days, and that only a few mold-threads developed. When the acid had not been added to the peptone solution, clouding and an unpleasant odor soon appeared, due to the development of innumerable bacteria.

Goebel interprets these observations:—

"These facts in themselves suffice to refute the acceptance of a bacterial digestion. They show that, in the *Nepenthes* hybrid studied, even in the unopened pitcher, a peptonizing enzyme is present which exerts an energetic digestive action on the addition of acid. Normal pitchers, into which an insect falls, very soon secrete formic acid. With excessive feeding, of course, the enzyme action is insufficient and putrefaction

may occur even in otherwise normal pitchers; it is probable, but not certain, that an increased secretion of enzyme occurs in the opened pitchers in the presence of stimulating substances." However, an increased secretion could not be detected in a pitcher stimulated by a fibrin flock, and compared with an unstimulated pitcher as a control; but one such experiment was made.

The tests for a protease in the glands of the pitcher-cover, which secrete nectar, were entirely negative. Flocks of fibrin were fastened over the glands by means of filter paper, which was kept moist. Digestion of the fibrin did not occur.

The glycerol extract of the pitchers contained too little enzyme and showed no proteolytic activity. Apparently the procedure of Vines (5) for the detection of enzymic activity was followed.

The secretion of unopened pitchers of *Nepenthes paradisiaca* was neutral in reaction. The liquor of unopened pitchers of *Nepenthes Mastersiana* was strongly acid; when fibrin was introduced into these pitchers, it was dissolved in 3 days, and cubes of albumen were strongly attacked; the protein was peptonized in the pitcher, and no bacteria were present.

A pitcher of *Nepenthes Sedeni* contained a strongly acid liquor and dissolved fibrin in 25 hours. Similar observations were made with a pitcher of *Nepenthes robusta*. Cultures demonstrated the absence of bacteria. The filtered solutions from the pitchers plus 0.1 percent formic acid digested meat fibres in 5 hours at a temperature of 35° C.

Goebel states that free formic acid could be shown to be present in the secretion (pitcher liquor) of the species of *Nepenthes* studied by himself. The total acidity of different pitchers, calculated as formic acid, was:—0.036%, 0.025%, 0.021%. All bacteria are not killed by this degree of acidity.

The strongly acid pitcher-liquor of *Nepenthes Mastersiana* was sown on non-acid nutrient gelatin, and on nutrient gelatin rendered acid by the addition of 0.2 percent tartaric acid. After 8 or 9 days, no growth was noted, or else a few bacteria and molds, which were doubtless due to air-contamination.

Goebel considered that a true enzymic digestion occurred in normal pitchers of *Nepenthes*, in which the liquor had not been diluted by water; this dilution often occurs in greenhouses. The enzyme was classified as a peptonizing enzyme, not identical with pepsin, and different from the pancreatic protease. Bacterial digestion could occur only in the liquor

of enfeebled pitchers, which possessed a neutral or alkaline reaction; it was recorded as much slower than the normal digestion, although its products could be absorbed, in part at least, by the plant, provided the putrefaction had not injured the pitcher. Goebel stated that the pitchers of enfeebled plants frequently contain only water, and that such plants may give results like those obtained by Dubois and by Tischutkin.

The absorption of peptone by the pitchers was demonstrated. Six pitchers of *N. Mastersiana* were washed out; and a known volume of a 0.1 percent solution of peptone, containing 0.1 percent formic acid, was placed in them. At the end of 66 hours, the pitchers were emptied. Resorption and evaporation of the liquid had been but slight, it was clear, colorless, free from bacteria, and contained only a trace of the mycelium of mold. The formic acid content had not decreased; however, but a faint trace of peptone remained, and an enzyme could not be detected. Hence the peptone had been absorbed. These pitchers again secreted liquor, and digested small pieces of meat.

Clautriau (12) conducted experiments on plants of *Nepenthes melamphora* in their native habitat in Java. The liquor in non-stimulated pitchers was neutral to litmus. When an unopened pitcher was merely shaken, its liquor had a more or less acid reaction on the following day. The reaction became acid after fine glass tubes, 1 to 2 cm. in length, were dropped through the lid into the pitcher, or after 2 or 3 drops of tincture of litmus were added to the liquor within the pitcher. Hence slight stimulation sufficed to cause the appearance of acid. The strongest acid reaction in the stimulated pitchers was about equal to that possessed by a solution, obtained by diluting 2 cc. of slightly fuming hydrochloric acid with sufficient water to render the final volume 1 liter. The liquor contained in solution a substance which was apparently thermolabile; this substance caused insects to become wet and to sink. The liquor did not contain a poison which kills insects. The insects were finally digested, leaving only a residue of chitin; putrefaction did not occur. The pitchers were sensitive to the presence of even exceedingly small quantities of antiseptics, such as formaldehyde, chloroform, spirit of camphor, essence of peppermint and of lemon, in the pitcher liquor; these reagents caused a cessation of the secretion of acid and of digestion, and the pitchers died in a few days.

When coagulated egg-white was introduced into the pitchers, digestion and absorption occurred. If but a small quantity of egg-white was used, absorption equalled digestion; proteolytic products did not remain in the pitcher, and a substrate for bacteria was lacking. If a large

quantity of egg-white was used, the unabsorbed products of digestion accumulated, and the pitcher was invaded by bacteria.

When a sterile solution of egg-white, prepared as follows, was introduced into the pitchers, digestion occurred; and bacterial invasion and putrefaction were rarely noted. Ten cc. of white of egg and 90 cc. of water were shaken together, to break up the membranes in the white and to dissolve the albumin. The solution was filtered and 0.1 milligram of ferrous sulphate was added, *i.e.*, 2 drops of a freshly prepared, 0.1 percent solution of ferrous sulphate. If the egg had not been fresh, more iron was added, but never an amount in excess of 1 milligram of ferrous sulphate. The solution was then boiled; it usually remained clear and limpid, but at times showed a faint opalescence. After conducting a digestion experiment with this solution as the substrate, the undigested albumin was removed by the following procedure. An alkaline salt was added to the solution, which was then acidified very quickly, and the albumen was coagulated with heat.

In most vigorous pitchers, all the protein was digested at the end of two days. Thus 5 cc. of the solution of egg-white, described above, was introduced into a pitcher. At the end of two days the pitcher liquor gave no precipitate on neutralization, and on boiling in the presence of salts or of acids; it yielded merely a trace of a precipitate with the following reagents:—potassium mercuric iodide, acetic acid and potassium ferrocyanide, phosphomolybdic acid.

The plant itself played an important rôle in the digestion. Experiments were made *in vitro* with liquor from both unopened and open pitchers, using chloroform as a bactericide. Absolutely no digestion of the substrate occurred. In one experiment, liquor, which possessed digestive power while in the pitcher, digested the substrate *in vitro*; the albumen disappeared, and much albumose (proteose) and possibly a little peptone were formed. Separation of the pitcher from the plant during the course of digestion inhibited the digestion of the albumin.

Clautriau also conducted experiments at Brussels on *Nepenthes* which had been cultivated in greenhouses.

He used the following technique in studying the products of the proteolysis. After digestion, the solution was neutralized with dilute sodium hydroxide solution in order to separate the syntonin (acid metaprotein), which was collected on a filter. To the filtrate were added an equal volume of saturated solution of sodium chloride and a trace of acetic acid; the solution was boiled to coagulate the albumin, which was then removed by filtration. The filtrate was saturated, while hot, with

ammonium sulphate, first while acid, then while alkaline in reaction; the resulting solution was permitted to stand; and the albumoses (proteoses) collected, and were removed by filtration. In the filtrate, the ammonium sulphate was removed by means of barium carbonate, and the excess of barium was removed with sulphuric acid. The solution was then filtered and concentrated; it contained no albumose, for it did not form a precipitate with potassium mercuric iodide, or with acetic acid and potassium ferrocyanide; it contained peptone, for it responded to the biuret test, and gave precipitates with tannin, phosphotungstic acid, and phosphomolybdic acid.

The liquor in a pitcher of *Nepenthes Mastersiana*, containing insects, was filtered and used in digestion experiments *in vitro*. Three cc. of the liquor and 20 drops of albumin (egg-white) solution, prepared as described above, were permitted to react for 3 days, with and without the addition of 1 drop of hydrochloric acid (1 drop contained 0.01 gram of hydrochloric acid), in the presence of camphor as a bactericide. The albumin was completely digested to peptone in both the presence and the absence of the hydrochloric acid, while a blank experiment, heated for 10 minutes at 100° C. at the beginning of the experiment, contained no peptone. The digestion *in vitro* by the liquor plus hydrochloric acid was far more rapid at 37° C. than at 20° C.

Experiments were made *in vitro* with the liquor of non-stimulated (unopened) pitchers of *Nepenthes coccinea* and of a *Nepenthes* similar to *N. phyllamphora*. Albumin was the substrate, and hydrochloric acid was added. After digestion in the incubator for 5 or 6 days, syntonin and a little albumose were present, but no peptone, and enzyme action probably had not taken place. However, Clautriau hesitated to advance the proposition that the secretion of the enzyme, like that of the acid, is the result of stimulation, although the two experiments were concordant.

The products of proteolysis were rapidly absorbed by the pitcher. Successive and abundant additions of albumin were supported perfectly, without inconvenience. In four days, the pitcher of *Nepenthes Mastersiana*, mentioned above, digested 2.5 cc. of albumin solution, and completely absorbed the products. Then 10 cc. of albumin solution (nitrogen content determined by the Kjeldahl method as ammonia equalled 14 cc. of 0.1 *N* sulphuric acid) were introduced into the pitcher; 7 days later the liquor contained nitrogen equal to but 2.8 cc. of 0.1 *N* sulphuric acid. Another 10 cc. portion of albumin solution was added; at the end of 7 days the nitrogen content of the liquor equalled but 2.7 cc. of 0.1 *N*

sulphuric acid. For the third time, 10 cc. of albumin solution were placed in the pitcher, and its protein was also digested. It should be noted that a portion of the nitrogen, found in the liquor after the albumin had been digesting for a week, was due to the enzyme and to the chitin of insects.

Digestion also took place in the pitcher of *Nepenthes coccinea*. In but two instances was peptone found in the pitchers after digestion of albumin, once with a plant of *Nepenthes coccinea*, and once with a plant of *Nepenthes* from Borneo similar to *N. phyllamphora*. The peptone, it is stated, is diffusible, and probably is absorbed.

Clautriau classified the protease of the pitcher liquor as a pepsin, since it acted only in an acid medium, and formed true peptone as the ultimate product of its action. Leucine, tyrosin, and other crystalline compounds were not found among the products of proteolysis.

An amber color, becoming red with alkali, was very frequently noted in the liquor after digestion. It was ascribed to the presence of tannin derived from the glands, and not to the presence of tryptophan.

The pitcher liquor did not contain an amylase, for it had no action on starch paste, when the mixture of liquor and paste was digested for 5 days.

Stimulation was found necessary to excite an *abundant* secretion of both the acid and the protease. The glands, which secrete both the acid and the enzyme, are said to absorb the digested protein. The microchemical reactions of the proteins were more intense in the region of the glands, hence it was concluded that the peptone was absorbed by the glands and stored as protein.

Since the liquor, obtained from healthy pitchers of *Nepenthes melamphora* in its natural habitat, failed to produce proteolysis *in vitro*, it was suggested by Clautriau that the pitchers of this variety possibly absorb either albumin or albumose. He also pointed out that, if too many insects accumulate in a pitcher, they may be decomposed by bacteria without injury to the plant, which probably utilizes the ammonia and amino acids formed by the bacteria.

Clautriau looked on the digestion in the pitcher as a source of nitrogen, and possibly of mineral food, for the nutrition of the plant.

Fenner (13) used *Nepenthes Rafflesiana* Jack in his study of the proteolysis in the pitchers of *Nepenthes*. He states that the innumerable glands of the pitcher lie in niches which open downwards, and are from one-half to two-thirds covered by a projecting, roof-like, epidermal structure. Wet insects, climbing up the wall to escape from the pitcher,

come under the "roof" of a gland, and at the same time come in contact with the gland itself, and adhere to it. The gland now secretes a slight quantity of a sticky mucilage, which is more viscous than the other secretion of the glands—the pitcher liquor, that is found even in unopened pitchers. The mucilage dissolves that part of the insect-body which can be utilized by the plant. When the surface of the gland becomes dry by absorption, the undissolved insect-residue falls from the gland, and is usually washed down by the pitcher liquor, either as its level rises or through the swinging motion of the pitcher, or its leaf. The resulting sediment consists for the most part of chitin. The glands on the border of the level of the pitcher liquor come in contact with the insects in this manner, and the cells of these glands show aggregation-phenomena in consequence of the absorption of organic substances, while the cells of glands above the level of the liquor, or beneath its level and merely washed by it, usually have unclouded contents.

When a number of gnats were placed in a pitcher, which was just opening, they swam on the surface of the liquor and came in contact with the glands at its level. The pitcher was emptied, a portion of the glandular region was dried, and some gnats were placed on the dry place; the secretion occurred only after the course of 4 to 6 hours, was slight in amount and insufficient to digest the insects, but dried up about them. When an insect was wet with the pitcher liquor and then brought on the dry place in the glandular region, very soon secretion of the mucilage, digestion, and absorption took place, so that only the residue of chitin was left at the end of 5 to 8 hours. The liquor, used for wetting the insect, must not have been diluted by water; since water may enter the pitcher during the watering of the plant, the liquor should be taken from a pitcher from which water has been excluded by means of a cotton plug.

Greenhouse plants are abnormal in that they usually contain only a slight quantity of liquor, secreted by the glands; as a consequence the greatest portion of the glandular region is not wet by the liquor. Fenner used suitable control experiments in his research.

From his own experiments, and from the observations of Goebel(11) (see above), Fenner draws the following conclusions:—

(1) Normal pitchers, in which insects are found, contain a faintly acid liquor (formic acid according to Goebel), which acts upon the glands as a chemical stimulant when insects, wet with it, come in contact with the glands.

(2) The liquor thus gives rise to digestion, so that insect bodies, which are saturated with it, can rapidly be dissolved and absorbed if they come

upon the glands of the pitcher wall beneath the roof-like epidermal structures.

(3) Insects, which enter the pitcher liquor, can be completely dissolved with the exception of their chitin plates; the latter are found as a sediment.

Fenner considered it a question for further study whether (a) the dissolved products of the digestion were withdrawn from the liquor and absorbed by the glands, or (b) their solution in the liquor was absorbed as such by the glands. Apparently the same glands, that secrete the pitcher liquor and the digestive mucilage, absorb the products of digestion.

Abderhalden and Teruuchi (28) studied the action of the pitcher liquor of *Nepenthes* on the dipeptide glycyl-*l*-tyrosin. This peptide is quite soluble in water, and is not cleaved by pepsin-hydrochloric-acid but is readily split by trypsin into its components, glycine (glycocoll) and *l*-tyrosin; the latter compound is very difficultly soluble in water, and precipitates.

Liquor was obtained from pitchers with closed lids, and from open pitchers which appeared to contain no very large quantity of condensation water. It was viscous, neutral in reaction, and exerted a very slow but distinct proteolytic action on fibrin flocks; after digestion for 3 days, the fluid was removed by filtration and then gave a distinct biuret reaction.

One gram of glycyl-*l*-tyrosin was dissolved in 10 cc. of liquor, collected from several pitchers, and toluene was added as a bactericide. The solution was kept at room temperature for 7 days; a slight cloudiness occurred, but no precipitate formed. Then the solution was transferred to an incubator; the opalescence did not increase; and a precipitate did not separate, even when the solution was evaporated to half its original volume.

The conclusion was drawn that the protease of *Nepenthes* is not a trypsin. "It therefore seems that the flesh-eating plants, *Nepenthes*, do not act through a trypsin-like enzyme. We do not venture to record our finding as a certainty, since from lack of material, it was not possible for us to repeat the experiment under different conditions."

Robinson (14) introduced various solutions into pitchers of *Nepenthes distillatoria* and noted their action upon the pitchers. A dilute (M/1024) solution of potassium nitrate had exerted no injurious action at the end of 9 days, but the pitcher began to wither at the end of 12 days. The nutrient solution of Sachs (calcium nitrate 6 grams, potassium nitrate

1.5 grams, dipotassium phosphate 1.5 grams, magnesium sulphate 1.5 grams, ferrous sulphate a trace, water 6000 cc.) caused a withering of the tissues on about the eighth day. When a dilute solution of Liebig's meat extract was introduced, and the plants then observed for a period of two weeks, the contents of the pitchers did not become foul, and the pitchers did not decay. A 10 percent solution of glucose, kept in the pitchers for 4 days, apparently had no harmful effect on the plant; the solution retained its power to reduce Fehling solution.

Robinson also made tests concerning the secretion of enzymes by the pitchers. A 10 percent solution of sucrose, which had been in the pitchers for 4 days, failed to reduce Fehling solution, whence it was inferred that an invertase is not secreted by the pitchers. A thin starch paste was kept in the pitchers for 4 days; it then had no reducing action on Fehling solution, and "the iodine test showed that the starch granules in the paste had not been broken down"; therefore the pitchers do not secrete an amylase (diastase).

Neutral olive oil and water were mixed in the proportion of 0.4 cc. of oil and 9.6 c.c. of water. After the mixture had been thoroughly shaken, it was introduced into pitchers, and permitted to remain in them for a period of from 4 to 7 days, then removed and titrated with 0.01 *N* potassium hydroxide solution, using phenolphthalein as an indicator. Control experiments were carried out (a) with the emulsion of oil *in vitro*, and (b) with the emulsion plus toluol in the pitchers. The results indicated that hydrolysis of the oil did not occur, and that the pitcher does not secrete lipase. Tap water, which had been kept in the pitchers for 1 day, was used in an experiment with ethyl butyrate; 2 cc. of this water and 4 drops of the ester were allowed to react at room temperature for 24 hours; the hydrolysis of the ester was determined by titration with 0.01 *N* fixed alkaline hydroxide, using phenolphthalein as an indicator. Enzymic cleavage of the ester was not detected, hence an esterase was not present.

Jenny Hempel (15) found that "the sap of the stimulated pitcher of *Nepenthes* gives values for the hydrogen ion concentration greater than 10^{-7}, but unstimulated pitchers give no definite value."

Shibata and Nagai (16) noted the presence of flavone in the leaves and the flowers of *Nepenthes phyllamphora* Willd. More flavone was present in *N. phyllamphora*, growing in the open on the island of Yap, than occurred in *N. Mastersiana*, growing under glass at Tokyo.

Pfeffer (17) has suggested that the insectivorous plants derive both nitrogen and phosphorus from their prey.

Nepenthes have found application in medicine. "The water abstracted from their leaf pitchers is an article of commerce in the East Indies. The leaves and root of *N. Boschiana*, Krthls. are especially employed, chiefly as an astringent."(18)

II

A Study of the Protease of the Pitcher Liquor of Nepenthes

BY

Joseph Samuel Hepburn, A.M., M.S., Ph. D.

Broadly speaking, two hypotheses exist concerning the mechanism of the proteolytic digestion within the pitchers of *Nepenthes*. One view is that digestion results from the action of a protease, secreted by the pitchers. The other view is that digestion is due to bacterial action. A third factor to be considered is the autolysis of the tissues of the dead insects.

In the present study, the volume of the liquor secreted by a single pitcher was always so small, that liquor could not be obtained from the same pitcher both before and after stimulation. Very rarely indeed did two pitchers mature on the same plant at the same time, thereby permitting a comparative study of the liquor from both stimulated and non-stimulated pitchers of the same plant. Differences due to individual plants could not be entirely eliminated, but the problem was attacked by several methods for the study of proteolysis, and a number of experiments were made according to each method; the results obtained by all the methods lead to the same general conclusions.

Material for this research has been obtained from the following species and hybrids of *Nepenthes*, grown in the Nepenthes House of the University of Pennsylvania:—*ampullaria, atrosanguinea, Chelsonii, Claytonii, Dominii, Dyerinana, gracilis, Hamiltoniana, Henryana, Hookeriana, Mastersiana, mixta, Morganiana, paradisae, Rafflesiana pallida, rufescens, splendida, Wittei.*

Pitchers were always selected prior to opening. They were closely watched; and the mouth of each pitcher was closed with absorbent cotton as soon as the lid opened; the entrance of insects was thereby prevented; and possible contamination of the pitcher liquor by the tissue enzymes of the digested prey was entirely excluded.

When the liquor from non-stimulated pitchers was studied, it was used as soon as possible after the opening of the pitcher.

When liquor from stimulated pitchers was desired, recourse was had to mechanical stimulation by chemically inert substances. In some experiments, the glands of the pitcher were stroked repeatedly with a camel's hair brush, and the cotton plug was then inserted; the liquor was removed for study on the following day. In other experiments, several small, round, solid glass beads, such as are used in fractionating columns, were inserted into the newly opened pitcher; the cotton plug was introduced; and the pitcher and its contents were shaken thoroughly at intervals during one or more days, taking care not to wet the cotton and thereby lose liquor; the liquor was finally removed for study.

In *all* the tests for the presence of a protease, a bactericide was used in order to exclude completely the action of micro-organisms. In some experiments, sufficient solid sodium fluoride was added to the mixture of pitcher liquor and substrate to render the final concentration of the fluoride 1 percent. In other experiments, a sufficient volume of a concentrated (2 percent.) aqueous solution of trikresol was added to render the final concentration of that bactericide 0.2 percent. This concentration of trikresol was found satisfactory by Graves and Kober (19) in certain of their experiments with proteases. When the mixture of pitcher liquor and substrate was diluted to a definite volume, the concentrated trikresol solution was added before the dilution to the final volume was made.

In each experiment, a blank or control determination was carried out, using pitcher liquor which had previously been boiled, then permitted to cool to the temperature of the room; the control was made in exactly the same manner, in all other respects, as the determination proper. The control or blank was always compared with the determination proper, and due allowance was thus made for the possible action of any thermostable catalyst present in the pitcher liquor, and also for any action of the reagents on each other.

The following reactions for the detection of a protease were used:—

(1) The formol-titration of Sörensen.

(2) The digestion of:—

(a) carmine fibrin,

(b) edestan,

(c) protean derived from castor bean globulin,

(d) ricin (Jacoby).

(3) The cleavage of glycyltryptophane.

The temperature of incubation was 37°C., unless otherwise stated.

Formol-titration

The following substrates were used:—ovalbumen, fibrin, edestin, ovomucoid, Nährstoff-Heyden, and Witte peptone. The fibrin was prepared from ox blood; the ovomucoid was obtained by the procedure of Eddy (20); the Nährstoff-Heyden, according to Gotschlich (21), was a mixture of different albumoses.

After incubation, any insoluble protein was removed by filtration, and was washed on the filter; the combined filtrate and washings were made neutral to phenolphthalein. If metaprotein separated, it was filtered out and washed on the filter; the filtrate and washings were again made neutral to phenolphthalein—if necessary—and one-half of their volume of formol (40 percent. formaldehyde), previously rendered neutral to phenolphthalein, was added. The basic amino group in the amino acid molecule was thereby converted into its methylene derivative by condensation with the formaldehyde, and no longer neutralized the acidic carboxyl group in the same molecule. This carboxyl group now functioned as an acid, giving the solution a reaction acid to phenolphthalein. This acidity, due to amino acids, was immediately titrated with standard fixed alkaline hydroxide, using phenolphthalein as the indicator, and *served as a measure of the proteolysis*. Usually 0.1 *N* sodium hydroxide was used for the titrations; however, 0.05 *N* sodium hydroxide was used when it was expected that the proteolysis would be slight on account of the small volume of pitcher liquor used.

The following experiments were made with the liquor from *stimulated* pitchers.

Ovalbumen (0.05 gram) was digested with 15 cc. of pitcher liquor for 3 days. After the addition of formol, the determination proper required 0.15 cc., the blank experiment 0.00 c.c. 0.05 *N* sodium hydroxide for the neutralization of the amino acids.

Fibrin (0.05 gram) was digested with 15 cc. of pitcher liquor for 14 days; on titration after the addition of formol, the determination proper required 0.45 cc., the blank 0.00 cc. 0.1 *N* sodium hydroxide.

Ovomucoid (0.05 gram) was dissolved in 10 cc. of water, then incubated with 5.5 cc. of pitcher liquor for 6 days. The formol titration was:- determination proper 0.10 cc., blank 0.00 cc. 0.1 *N* sodium hydroxide.

Nährstoff-Heyden (15 cc. of a 1 percent. aqueous solution) was mixed with 15 cc. of pitcher liquor, then incubated for 3 days. The formol titration was:- determination proper 0.30 cc., blank 0.10 cc. 0.1 *N* sodium hydroxide.

Witte peptone (25 cc. of a 1 percent. aqueous solution) was mixed with 25 cc. of pitcher liquor, then incubated for 4 days. The formol titration was:— determination proper 4.60 cc., blank 1.85 cc. 0.1 *N* sodium hydroxide.

A solution of edestan was prepared by dissolving 0.1 gram of edestin in 15 cc. of 0.1 *N* hydrochloric acid, previously diluted to 50 cc. with water. The pitcher liquor (8 cc.) was mixed with 25 cc. of this solution (equal to 0.05 gram of edestin), and then incubated for 25 days. The formol titration was:— determination proper 0.90 cc., blank 0.00 cc. 0.1 *N* sodium hydroxide.

When liquor from *non-stimulated* pitchers was used, the following results were obtained.

In three experiments, the period of incubation was 4 days. In the first experiment, 10 cc. of pitcher liquor and 0.05 gram of ovalbumen were used; in the second experiment, 12.5 cc. of pitcher liquor and 25 cc. of a 1 percent. aqueous solution of Nährstoff-Heyden; in the third experiment, 12.5 cc. of pitcher liquor and 25 cc. of a 1 percent aqueous solution of peptone (Witte). In all three experiments, the formol titration of the determination proper was the same as that of the blank, showing that enzymic cleavage of the substrates had not occurred.

A solution of edestan was prepared by dissolving 0.1 gram of edestin in 15 cc. of 0.1 *N* hydrochloric acid, previously diluted with water to a volume of 25 cc. In one experiment, 25 cc. of the edestan solution were mixed with 11.5 cc. of pitcher liquor; sufficient water was added to make a total volume of 50 cc., and the solution was digested for 28 days. In another experiment, 9 cc. of the edestan solution were added to 8 cc. of pitcher liquor; sufficient water was added to make a total volume of 25 cc., and the solution was incubated for 21 days. In both experiments, both the determination proper and the blank remained neutral, after neutral formol had been added in the formol titration, hence digestion with the production of soluble proteolytic products had not occurred.

Carmine Fibrin

The directions of Grutzner (22) for the use of this reagent were somewhat modified. The carmine fibrin was washed with water to remove the glycerol, in which it had been preserved, then was permitted to

swell in 0.2 percent. hydrochloric acid, to which sufficient trikresol had been added to produce a 0.2 percent. solution of that bactericide. The swollen, gelatinous carmine fibrin was placed in the pitcher liquor; sufficient hydrochloric acid (0.6 percent. solution) and trikresol (2 percent. solution) were added to make a concentration of 0.2 percent. of each of these reagents in the resulting solution. *The temperature of incubation was that of the room.* The occurrence of digestion was made known by two phenomena— (1) the flocks of carmine fibrin decreased in size and finally dissolved completely; and (2) the carmine was thereby liberated, dissolved, and imparted a red color to the solution.

In the *preliminary* series of experiments, the carmine fibrin was swollen in one mass, and a definite volume of the gelatinous reagent was used in each experiment. Three experiments were made in each of which the liquor from a single, *stimulated* pitcher was used. In the first experiment, the pitcher liquor (1.5 cc.) completely dissolved 0.1 cc. of carmine fibrin in 13 hours. In each of the other experiments, 4 cc. of pitcher liquor were permitted to act on 0.5 cc. of carmine fibrin; in one of these experiments, the substrate was partially dissolved in 15 hours, and completely dissolved in 24 hours; in the other experiment, the substrate was markedly digested in 48 hours, and completely dissolved in 6 days. In still another experiment, 4.75 cc. of liquor, collected from several *stimulated* pitchers, completely dissolved 0.25 cc. of carmine fibrin in 26 hours.

Liquor (1.5 cc.) from a single *non-stimulated* pitcher completely dissolved 0.1 cc. of carmine fibrin in 13 hours. The liquor (4.75 cc.) from several *non-stimulated* pitchers produced marked digestion, but not complete solution, of 0.25 cc. of the same substrate in 31 hours.

In the *final* series of experiments, the liquor from a *separate* pitcher was used in each experiment, and the carmine fibrin (0.2 gram) for each experiment was weighed out into a separate tube.

One set of experiments was conducted on liquor from *stimulated* pitchers. The carmine fibrin for each experiment was swollen in its tube in the usual manner, then was placed in the pitcher liquor, and hydrochloric acid and trikresol were added as described above. The time required to dissolve the swollen substrate was:—

Pitcher A, 3.5 cc. liquor, 48 hours.
" B, 2.5 cc. " 72 "
" C, 2 cc. " 93 "
" D, 3.5 cc. " 111 "
" E, 1 cc. " 133 "

Another set of experiments was made with liquor from *non-stimulated* pitchers; *unswollen* carmine fibrin was used, and *no acid* was added to the reaction-mixture. The pitcher liquor was placed on the substrate, and sufficient trikresol (2 percent. solution) was added to give a concentration of 0.2 percent. of the bactericide. Six experiments were made, the volumes of pitcher liquor being 1.0, 1.5, 2.5, 3.0, 3.5 and 5.5 cc. respectively. Even after 70 days, the substrate was absolutely unattacked. The supernatent liquid had assumed a very faint pink tinge, no more marked than that of a blank experiment.

In a third set of experiments, unswollen carmine fibrin was incubated with liquor from *non-stimulated* pitchers, in the presence of both hydrochloric acid and trikresol, as described above. Two experiments were made, the volumes of pitcher liquor being 2.5 and 1.0 cc. respectively. The substrate was markedly digested, in the first experiment in 16 hours, in the second experiment in 52 hours.

Edestan

The solution of edestan, used in these experiments, was prepared by dissolving 0.1 gram of edestin in 15 cc. of 0.1 *N* hydrochloric acid, previously diluted to 25 cc. with water. After the mixture of edestan solution and pitcher liquor had been incubated under the conditions stated below, it was neutralized with 0.1 *N* sodium hydroxide, using phenolphthalein as the indicator.

Liquor from *stimulated* pitchers was used in the following experiments:

The pitcher liquor (20 cc.) was mixed with 25 cc. of edestan solution; the mixture was diluted to a volume of 50 cc. with water, and was incubated for 14 days. Then the solution was neutralized. The determination proper gave absolutely no precipitate, showing that proteolysis had occurred, and that both the protean, edestan, and the meta-protein, which is one of the first products of proteolysis, had been converted into simpler proteolytic products. In the blank, on the other hand, a voluminous precipitate formed.

In a second experiment, 1 cc. of pitcher liquor and 2 cc. of the edestan solution were mixed; and the mixture was diluted with water to a volume of 5 cc., then incubated for 8 days. On neutralization, the determination proper failed to give a precipitate, while the blank yielded a voluminous precipitate. Hence the edestan had been digested.

Experiments were also made, using the liquor from *non-stimulated* pitchers:—

In one experiment, 1 cc. of liquor, 2 cc. of the edestan solution, and sufficient water to render the total volume 5 cc., were mixed; and the mixture was incubated for 8 days. On neutraliztion, a voluminous precipitate formed in the blank; the precipitate, which formed in the determination proper, was about one-half as great as that in the blank, showing that partial digestion of the edestan had occurred.

In another experiment, a mixture of 4 cc. of pitcher liquor and 1 cc. of the edestan solution was incubated for 13 days. On neutralization, the blank yielded a voluminous precipitate, the determination proper, a precipitate but one-tenth as great as that in the blank. Hence partial digestion of the edestan had occurred.

Protean derived from castor-bean globulin

The castor-bean globulin, used in these experiments, was presented by Dr. Isaac F. Harris, to whom I am also indebted for the outline of its preparation. Ground castor-beans were extracted with gasoline to remove the oil, then were extracted with a 10 percent. sodium chloride solution. This solution was filtered, and the clear filtrate was dialyzed. The globulin, which deposited, was dissolved in a 10 percent. solution of sodium chloride; the solution was filtered and dialyzed. The globulin, which separated, was washed with water, alcohol, and ether, and was desiccated.

A 2 percent. solution of this globulin in a 5 percent. solution of sodium chloride was used as a reagent for proteolytic enzymes; the solution was filtered, if necessary. When the clear solution of the globulin was mixed with the pitcher liquor and 0.5 cc. of 0.1 *N* hydrochloric acid was added, a cloudy precipitate of the protean derived from the globulin formed. On incubation, if a proteolytic enzyme, active in the presence of hydrochloric acid, was present, the insoluble protean was digested and converted into less complex, soluble compounds, which dissolved; and the cloud gradually became less dense, and finally disappeared.

The following experiments were made on liquor from *stimulated* pitchers.

The pitcher liquor (2.5 cc.) was incubated with 2 cc. of the globulin solution and 0.5 cc. 0.1 *N* hydrochloric acid. Proteolysis was marked on the third day, advanced on the seventh day, and almost complete on the twelfth day.

The experiment was repeated using 0.5 cc. of pitcher liquor, 4 cc. of the globulin solution, 1 cc. 0.1 *N* hydrochloric acid, and 4.5 cc. of water (to secure the same concentration of substrate and of acid as in the pre-

ceding experiment). The proteolysis was marked on the fourth day, and was almost complete on the ninth day.

Liquor from *non-stimulated* pitchers was used in all of the following experiments.

The liquor (0.6 cc.) was digested with 2 cc. of the globulin solution 0.5 cc. 0.1 *N* hydrochloric acid, and 1.9 cc. of water. No proteolysis had occurred at the end of 14 days.

In another experiment, 2.5 cc. of the liquor, 2 cc. of the substrate solution, and 0.5 cc. 0.1 *N* hydrochloric acid were mixed and incubated; the protean was completely digested in 14 hours.

Two experiments were made, using 1 cc. of liquor, 2 cc. of the substrate solution, 0.5 cc. 0.1 *N* hydrochloric acid, and 1 cc. of water. The protean was almost completely digested in one of these experiments in 29 hours, and was completely digested in the other experiment in 48 hours.

It should be noted that liquor from a separate pitcher was used in each experiment in which this protean served as the substrate.

Jacoby's Ricin

This test was carried out with the reagents prescribed by Jacoby (23). A solution of 1 gram of ricin (Jacoby) and 1.5 grams of sodium chloride in 100 cc. of water was prepared, and filtered if necessary. The pitcher liquor (1 cc.) and the ricin solution (3 cc.) were mixed; 1 cc. of 0.56 percent. hydrochloric acid was added; and the resulting mixture was incubated. In the presence of a protease active in this concentration of hydrochloric acid, the cloudy precipitate, which forms on the addition of the acid, is dissolved during subsequent incubation. The reactions involved are essentially the same as those described above for the castor-bean globulin.

Liquor from a *stimulated* pitcher was used in one experiment. The cloudy precipitate underwent a marked proteolysis in two days.

Liquor from a *non-stimulated* pitcher was used in another experiment. The cloudy precipitate was partially digested in two days, but had not been entirely digested at the end of 1 week.

Glycyltryptophane

Liquor (10 cc.) from *stimulated* pitchers was incubated with 2 cc. of an aqueous solution of the dipeptide, glycyltryptophane (so-called Ferment diagnosticum). In this series of experiments only, toluene was used as a bactericide. After incubation, the test for free trypto-

phane was made in the usual way with dilute acetic acid and bromine vapor; the production of a red color showed the presence of free tryptophane, and cleavage of the dipeptide.

In the first experiment, the period of digestion was nine days in the incubator; the test for free tryptophane was negative.

In a second experiment, the period of digestion was 21 days in the incubator, followed by 7 days in the room. A distinctly positive test for free tryptophane was obtained.

General Summary

The following conclusions may be drawn from the experiments reported.

The *formol titration* showed that the liquor from stimulated pitchers produced proteolysis of ovalbumen, fibrin, ovomucoid, Nährstoff-Heyden, and Witte peptone, while the liquor from non-stimulated pitchers lacked proteolytic power. This method also showed that, in the presence of very dilute hydrochloric acid, edestan was digested by the liquor from stimulated pitchers, but not by that from non-stimulated pitchers.

Carmine fibrin was dissolved by the liquor from both stimulated and non-stimulated pitchers, in the presence of 0.2 percent. hydrochloric acid. This substrate was not dissolved by liquor from non-stimulated pitchers in the absence of acid.

In the presence of very dilute hydrochloric acid, the pitcher liquor produced proteolysis of *edestan*; digestion proceeded more rapidly in liquor from stimulated pitchers than in liquor from non-stimulated pitchers.

The *protean derived from the globulin of the castor bean* was usually dissolved by the liquor from both stimulated and non-stimulated pitchers, in the presence of very dilute hydrochloric acid. The same statement may be made concerning Jacoby's *ricin*.

The liquor from stimulated pitchers apparently hydrolyzed glycyltryptophane, provided the period of incubation was sufficiently long.

The liquor from *stimulated* pitchers possessed proteolytic power in both the absence and the presence of acid.

The liquor from *non-stimulated* pitchers exerted no proteolytic power in the absence of acid, but possessed such power in the presence of acid.

Further study is required to determine the manner in which stimulation imparted *active* proteolytic power to the pitcher liquor. Possibly stimulation gave rise to a change in the reaction (hydrogen ion concentration) of the liquor and thereby created a favorable environment for

the activity of an enzyme already present; or stimulation may have produced the activation of a zymogen present in the liquor; or it may have increased the secretion of protease by the glands of the pitcher.

In the presence of acid, liquor from stimulated pitchers digested certain substrates more rapidly than did liquor from non-stimulated pitchers; this was especially true of edestan.

The proteolytic enzyme of the pitcher liquor undoubtedly plays a highly important rôle in the digestion of insects within the pitcher.

III

A Bacteriological Study of the Pitcher Liquor of Nepenthes

By

Joseph S. Hepburn, Ph. D. and E. Quintard St. John, M. D.

Since certain investigators (9-10) have attributed the digestive action of the pitcher liquor of *Nepenthes* to the activity of micro-organisms, it has seemed desirable to study the bacterial content of the pitcher liquor, and the proteolytic power of its bacteria.

Description of the Media

Bacterial counts were obtained by sowing the pitcher liquor—undiluted, and in several successive dilutions—on plain nutrient agar, incubating the plates at 37°C., and counting the colonies in the usual manner.

For the study of the proteolytic activity of the bacteria of the pitcher liquor, certain protein media were used, following, to a large extent, the directions of Crabill and Reed (24). The basis of these media was a stock solution which contained magnesium and ferrous sulphates, dipotassium phosphate, and potassium chloride. For solid media a stock agar was prepared by addition of 2 percent. of agar to this solution. The protein solid media were obtained by addition of approximately 1 percent. of *one* of the following proteins to the stock agar:—casein, egg albumen, carmine fibrin, edestin, ricin (Jacoby), *protein* (prepared from aleuronat). After these media had been sterilized, the proteins were present as suspended, insoluble particles. Whenever proteolytic bacteria were present in the pitcher liquor plated on such media, their colonies gradually digested and dissolved the suspended particles over which they grew.

In a few experiments, plates of plain nutrient gelatin were also sown with the pitcher liquor, in order to detect the presence of liquefying (proteolytic) micro-organisms.

To test for the liberation of tryptophane and the formation of indol, a liquid medium was prepared, containing 0.4 gram *protein* (from aleuronat), 20 cc. 0.1 *N* sodium hydroxide solution, and 80 cc. of the stock solution of inorganic salts already mentioned. The resulting suspension of protein was placed in tubes (10 cc. to a tube), and was sterilized. The protein gave a purple color with glyoxylic acid and sulphuric acid (reaction of Hopkins and Cole), and therefore contained a tryptophane group in its molecule.

The formation of basic compounds (e.g. ammonia) from simple organic compounds of nitrogen was also studied, using as substrates:—glycocoll (an amino acid), acetamide (an acid amide), asparagin (which is both an amino acid and an acid amide), and ammonium lactate (an ammonium salt of an organic acid). For this study, recourse was had to solid media, prepared by addition of *one* of the compounds just named to the stock agar. One percent. of asparagin was used, and the other compounds in molecular concentration equal to that of the asparagin. One-half percent., by volume, of a two percent. solution of rosolic acid in sixty percent. alcohol was added to each medium as an indicator. These media were always sterilized by the discontinuous method. The production of basic compounds by bacteria growing on these media was indicated by a red color of the medium beneath and surrounding the colony. Sterile plates of the rosolic acid media were always poured as controls, to be used in determining the changes in color in the experiments proper.

In inoculating all of the special media just described, 1 cc. of the undiluted pitcher liquor was sown into each plate or tube.

Lactose bile-salt broth was used to test for the presence of members of the colon-aerogenes group of bacteria.

The temperature of incubation was always 37°C., except for the gelatin plates which were kept at 20° C.

Sources of the Pitcher Liquor Examined

In the majority of the experiments, the liquor was obtained either from unopened pitchers or from active, open pitchers containing insect remains. A few experiments were conducted on liquor from pitchers partly opened and not yet invaded by insects. *The liquor from each pitcher was studied as a separate experiment.*

Unopened Pitchers

The prolonged midrib or tendril, which carries the pitcher, was severed at the end of the basal part of the lamina; and that portion of the tendril, which interfered with the subsequent manipulation, was removed. Sterile scissors were always used in cutting the plant tissues. The top portion of the pitcher was rapidly passed through the flame, and was then cut off. The cut edge of the pitcher was then rapidly flammed; and the liquor was immediately withdrawn by means of a sterile pipette, and plated in the usual way on plain nutrient agar. The liquor from 12 unopened pitchers was studied in this manner, and was invariably found to be *sterile*, as was shown by the absence of colonies on the plates after incubation for 4 days.

Opened Pitchers

The liquor was removed from opened pitchers with sterile pipettes, placed in sterile test tubes, and immediately plated.

Partly opened pitchers, free from insects, were used in two experiments. The liquor from each of these pitchers contained a goodly number of bacteria which grew on plain nutrient agar.

The remaining experiments were conducted on liquor from open, active pitchers, containing insect remains. The number of bacteria present in 1 cc. of liquor was determined in each of five pitchers with the following result:—

Pitcher 1	450,000
2	8,000,000
3	1,200,000
4	1,900,000
5	48,000

The morphology of these bacteria was studied. Smears were made from several colonies, which differed in physical appearance, and were stained with Loeffler's alkaline methylene blue. All the micro-organisms were rod-like, and therefore belonged to the family of the *Bacteriaceae*. A few of the organisms contained spores; none of them produced gas when inoculated into lactose bile-salt bouillon.

The liquor in an old pitcher, which was becoming brown at the top, contained 104,000 bacteria per cc.

Gelatin. In two experiments, gelatin plates were poured. The bacteria grew and completely liquefied the gelatin in 48 hours.

Action on Special Media

After the plates of the special media had been inoculated, they were held in an incubator at 37°C., and were examined at intervals as stated below, until drying of the media rendered further observation useless.

Casein agar. Eight experiments were made using this medium. In seven experiments, growth, but no proteolysis, had occurred at the end of 3 days; digestion of the casein had begun by the fifth day, had become more marked by the ninth day, and still more marked by the twelfth day. In the eighth experiment, bacterial colonies failed to develop.

Egg albumen agar. Two series of experiments were made with egg albumen agar as the substrate. The first series included eight experiments; colonies had appeared in three experiments by the third day and in a fourth experiment by the ninth day. The plates in the other experiments remained sterile. Digestion of the albumen was not noted, even at the end of 12 days.

In a second series, which consisted of seven experiments, growth of the bacteria occurred during the first five days of incubation, but proteolysis of the albumen had failed to develop at the end of 14 days. Possibly, in both series, sufficient ovomucoid (a non-coagulable protein) was present in the dried egg albumen to supply the bacteria with the necessary carbon and nitrogen.

Carmine fibrin agar. Six experiments were conducted on carmine fibrin agar. Washed, unswollen carmine fibrin had been used in the preparation of the medium, and the flocks were rather large. Colonies developed by the third day, and showed a marked tendency to grow over the flocks. Proteolysis had not become apparent on the fifth day. On the ninth day digestion of the fibrin was distinctly under way.

Edestin agar served as the medium in four experiments. Growth of the bacteria, and possibly incipient proteolysis of the edestin, occurred by the third day. No further change was noted on the fifth day. The digestion of the edestin had advanced somewhat by the ninth day, and was still more marked on the twelfth day.

Ricin agar was used as the medium in three experiments. Colonies developed in one experiment by the third day, and in another experiment by the fifth day. Digestion of the ricin had begun in both experiments by the ninth day, and had become very marked by the twelfth day. Bacterial growth failed to occur in the third experiment.

Protein agar. Agar, containing protein from aleuronat, served as the medium in eight experiments. On the third day colonies were present in all the experiments, and proteolysis had probably begun in six

experiments. On the fifth day distinct evidences were noted of incipient digestion of the protein in all eight experiments; this digestion was more marked on the ninth day, and still more marked on the twelfth day.

Each pitcher did not always contain sufficient liquor to permit a complete set of experiments on all six of the agar media which contained suspended proteins. However, a general tendency existed that, if the micro-organisms present in the liquor grew on one of these media, they grew on all of the media, and usually exerted a proteolytic action on all the proteins.

Asparagin rosolic acid agar. This medium was used in three series of experiments. In the first series which included seven experiments, colonies had developed by the fifth day, and a red (alkaline) color had been imparted to the medium. By the fourteenth day, the medium had become yellow in color (acid in reaction).

The second series consisted of eight experiments. Colonies appeared on all the plates by the third day. The entire medium next became alkaline in reaction; this change had occurred on from the third to the fifth day. While the colonies themselves remained alkaline, the medium finally became acid in reaction; this change had taken place in over one-half of the experiments by the ninth day, and in the remaining experiments by the twelfth day.

The third series included seven experiments, in six of which good growth of the bacteria and an alkaline reaction of the medium were apparent by the third day. The medium had become acid in reaction in one of these experiments by the tenth day. In the seventh experiment of this series, growth had not occurred by the third day, but both colonies and the alkaline reaction of the medium had developed by the tenth day. The liquor from this series of seven pitchers was also sown on glycocoll rosolic acid agar, acetamide rosolic acid agar, and ammonium lactate rosolic acid agar; the results are given below.

Glycocoll rosolic acid agar. In six experiments, good growth of the bacteria had occurred, and an alkaline reaction had been imparted to the entire medium at the end of three days; in one of these experiments, about one-half the total area of the agar had become acid in reaction by the tenth day. In the seventh experiment, bacterial growth did not occur.

Acetamide rosolic acid agar. In six experiments, good growth of the bacteria and an alkaline reaction of the medium were noted by the third

day. On the tenth day, the medium was still alkaline in three experiments, and had become distinctly acid in two experiments, while the change from an alkaline to an acid reaction was almost, but not entirely, complete in the sixth experiment.

In the seventh experiment, colonies were absent on the third day, but had developed by the tenth day, and had caused the entire medium to assume an alkaline reaction.

Ammonium lactate rosolic acid agar. Six experiments were characterized, on the third day, by good growth of the bacteria and an alkaline reaction of the medium. On the tenth day, the reaction of the medium had begun to change from alkaline to acid in five of these experiments.

In the seventh experiment, growth of the bacteria had not occurred by the third day, but had taken place by the tenth day, and the medium had then become alkaline in reaction.

In the set of seven experiments, which were studied on all four rosolic acid agars—asparagin, glycocoll, acetamide, and ammonium lactate—the odor was also recorded. The plates were quite frequently characterized on the third day by an odor recalling that of ammonia or amines. This odor was rarely noted on the tenth day.

Protein liquid medium. The bacteria of the pitcher liquor were permitted to act on the suspension of protein (from aleuronat) in the stock solution of inorganic salts. Two series, of 8 experiments each, were made. In each experiment, 1 cc. of pitcher liquor was added to a tube of the medium.

In the first series of experiments, the test for indol was made after incubation for three days, and again after incubation for ten days. The test was always negative.

In the second series of experiments, neither indol nor *free* tryptophane was present after incubation for twelve days.

Lactose bile-salt bouillon. In two experiments, 1 cc. of pitcher liquor was sown in lactose bile-salt bouillon; gas developed within 72 hours, showing the presence of organisms of the colon-aerogenes group in both pitchers.

The liquor from several pitchers was mixed, and five tubes of the bouillon were inoculated, using 1 cc. of the mixture, and of its 1:10, 1:100, 1:1,000, and 1:10,000 dilutions, respectively. Gas developed in all five tubes within 72 hours, hence it may be stated that at least 10,000

micro-organisms of the colon-aerogenes group were present in 1 cc. of the pitcher liquor examined.

General Summary

The following conclusions are based on the bacteriological experiments.

The liquor taken aseptically from unopened pitchers was found to be sterile.

The liquor in partly opened pitchers, which were free from insects, contained a goodly number of bacteria.

Liquor from open, active pitchers, containing insect remains, had a bacterial count of from 48,000 to 8,000,000 per cc. These organisms were rods (*Bacteriaceae*). The bacteria in the liquor from such pitchers liquefied gelatin, and grew on agar in which the sole source of nitrogen and carbon was either a protein (casein, egg albumen, carmine fibrin, edestin, Jacoby's ricin, protein from aleuronat), or a simple organic compound of nitrogen (glycocoll, acetamide, asparagin, ammonium lactate). The bacteria usually digested the protein in the medium, but the rate of digestion was exceedingly slow. The bacteria decomposed the simple organic compounds of nitrogen; an odor recalling that of ammonia and amines was frequently produced; the medium became alkaline in reaction; later on, this reaction changed to acid, but the bacterial colonies themselves remained alkaline. These bacteria did not liberate tryptophane nor produce indol in their action upon protein (from aleuronat). The pitcher liquor, on the average, contained at least 10,000 micro-organisms of the colon-aerogenes group per cc.

The following conclusions are supported by the results of our studies on the protease and the bacteria of the pitcher liquor.

The slowness, with which bacterial digestion of the protein occurred, shows that bacteria play but a secondary rôle in the digestion of the insects in the pitcher. The leading role in the digestion is played by the protease of the pitcher liquor.

The bacteria live in symbiosis with the *Nepenthes* plant, drawing their nutrition from the digested insects, and assisting somewhat in the digestion of the insects.

Needless to remark, the tissue enzymes of the insects may produce autolysis of their tissues, and thereby assist in the digestion.

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IV.

Occurrence of Antiproteases in the Larvae of the *Sarcophaga* Associates of *Sarracenia flava*

By
Joseph Samuel Hepburn and Frank Morton Jones

The parasitic intestinal worms of man and the domestic animals contain antiproteases (antipepsin and antitrypsin), which effectively prevent the digestion of the parasite by the proteolytic enzymes in the digestive fluids of the host. This is especially true of *Ascaris* (1).

The pitcher liquor of *Sarracenia flava* contains a proteolytic enzyme (2). The larvae of certain species of *Sarcophaga* (*sarraceniae* Riley, *Rileyi* Aldrich, and *Jonesi* Aldrich) habitually occur in the pitchers of *Sarracenia flava*, where they are constantly bathed in the digestive liquor of the pitcher. This phenomenon suggested the examination of *Sarcophaga* larvae from *Sarracenia* pitchers for the presence of antiproteases. Live larvae obtained from open pitchers of *Sarracenia flava* were used in the study. Two series of experiments were made.

In the first series, 16 larvae (total weight 1.86 grams) were crushed, and ground with sand and 4.5 cc. of distilled water. The turbid solution and suspended tissue were removed by decantation, and were mixed with sufficient 95 percent alcohol to render the final concentration of the alcohol 60 percent. The precipitate which formed was collected on a filter and dried over calcium chloride in a dessicator. The filtrate was mixed with alcohol until the concentration of the latter was 85 percent; the precipitate which formed was negligible, although the antiproteases should have separated at this point.

The thought, that possibly the rather tough larval tissue had not been ground sufficiently to liberate the antiproteases, led to an examination of the first precipitate for these antienzymes. When thoroughly dry, the precipitate was separated from the filter paper, ground intimately with glass powder, and then triturated with 10 cc. of distilled water. A supernatant liquid was obtained by centrifugation; 2.5 cc. of this liquid and 2.5 cc. of a 1 percent solution of pepsin in 50 percent glycerol were mixed; and sufficient hydrochloric acid and trikresol were added to produce a concentration of 0.2 percent of each of these reagents. The resulting solution was allowed to stand for 2 hours at room temperature to permit the pepsin and the antipepsin (if present) to combine. A

control experiment was made in which 2.5 cc. of physiological salt solution were substituted for the solution derived from the larvae. Carmine fibrin (0.2 gram, weighed, then swollen in 0.2 percent hydrochloric acid) was added to both the experiment proper and the control, and both were then incubated at room temperature. In the control, the carmine fibrin was completely dissolved in 1.75 hours. In the experiment proper, the carmine fibrin was not dissolved at the end of 12 days, but had been completely dissolved at the end of 17 days. Therefore antipepsin, an antiprotease, was present in the larvae, since the solution derived from the larvae markedly retarded the peptic digestion.

In the second series of experiments, 82 larvae (total weight 8.30 grams) were used. From the same gathering of larvae a number were bred to the adult fly, and proved, by examination of the male genitalia, to be *Sarcophaga sarraceniae* Riley, the first recognized *Sarcophaga* associate of *Sarracenia*. The larvae were ground with glass powder to an intimate mixture, which was thoroughly triturated with distilled water. The pasty mass was subjected to a pressure of 50 kilograms per square centimeter in a Buchner press; 48 cc. of press juice were obtained. The press juice was so cloudy that the edestan and casein tests could not be applied in the examination for antiproteases, and only carmine fibrin was used as a substrate.

Antipepsin. In the experiment proper, 12 cc. of press-juice and 12 cc. of a freshly prepared 0.2 percent aqueous solution of pepsin were mixed and allowed to stand at room temperature for 30 minutes to permit the pepsin and the antipepsin (if present) to combine. Sufficient hydrochloric acid (2 percent) and trikresol (2 percent aqueous solution) were then added to make the concentration of each 0.2 percent in the resulting solution; lastly, 0.2 gram of carmine fibrin was added. A control experiment was carried out in exactly the same manner as the experiment proper, save that 12 cc. of distilled water were substituted for the press-juice. The temperature of incubation was that of the room. In the control experiment, the carmine fibrin was completely dissolved in 45 minutes; in the experiment proper, it was partly dissolved in 14 hours and completely dissolved in 17 hours.

Antitrypsin. In the experiment proper, 12 cc. of press-juice and 12 cc. of a freshly prepared 0.2 percent aqueous solution of pancreatin (owing its proteolytic power to trypsin) were mixed, and held at room temperature for 30 minutes to permit the trypsin and the antitrypsin (if present) to combine. Sufficient 4 percent solution of sodium carbonate and 2 percent solution of trikresol were added to make 0.4 percent of

the former and 0.2 percent of the latter reagent in the final solution; then 0.2 gram of carmine fibrin was added. A control experiment was made exactly like the experiment proper, except that 12 cc. of distilled water were substituted for the press juice. The incubation was made at room temperature. In the control experiment, the carmine fibrin showed signs of incipient digestion in 45 minutes, and had completely dissolved in 14 hours. In the experiment proper, the carmine fibrin was only partly dissolved at the end of 17 hours, but was completely dissolved at the end of 22 hours.

Since the press juice markedly retarded the digestion of carmine fibrin by both pepsin and trypsin, both antipepsin and antitrypsin were present in the larvae of *Sarcophaga sarraceniae*.

Thermo-stability of the antiproteases. The experiments proper were also carried out as described above, except that the 12 cc. portions of press juice were boiled and cooled to room temperature, then used without filtration. The protein in the press juice was coagulated by the heat on boiling. The digestion of carmine fibrin by both pepsin and trypsin was retarded to about the same extent as when unboiled press-juice was used. The coagulated protein of the press juice was dissolved completely by pepsin and by trypsin (pancreatin) only after digestion at room temperature for 7 to 8 days, the coagulum remaining long after the carmine fibrin had disappeared. These results indicate that the antiproteases—antipepsin and antitrypsin—of the larvae were thermostabile. They also indicate that the coagulated protein of the press-juice either adsorbed antiprotease and thereby resisted digestion, or else was in itself not readily digestible.

The methods used in the preceding experiments were based on those described by Fischer (1) and by Wohlgemuth (3) The trikresol served as a bactericide.

In this study, antiproteases have been found in the larvae of the *Sarcophaga* associates of the pitcher plant, *Sarracenia flava*. The larvae of other species of *Sarcophaga*, and of several other dipterous genera, are likewise able to live and escape digestion in an environment rich in proteolytic enzymes; probably these larvae also contain antiproteases which protect them from digestion. Thus *Sarcophaga haemorrhoidalis* Fall. can live in the human intestinal tract; Haseman (4) has recently published a detailed account of a series of cases of intestinal myiasis in man, due to the presence of the larvae of this species in the intestines; Aldrich (5) gives an additional and similar case in which the parasite

was also positively identified as *S. haemorrhoidalis*, and cites other records where this species may have been the one concerned.

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