

THE ENZYMES OF THE PITCHER LIQUOR OF THE SARRACENIACEÆ

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PROTEASE

A series of experiments on the occurrence of a protease in the pitcher liquor of *Sarracenia flava* was described at the general meeting of the American Philosophical Society in 1917.¹⁶ These experiments have been continued and extended to include the other North American representatives of the family. Each species has been studied separately. Tests have been made on liquor from closed pitchers and on filtered liquor from open pitchers. In the field experiments, carmine fibrin was used as the substrate. This substrate and others were used in laboratory experiments. As a rule, the test for the presence of a protease by means of carmine fibrin was made in an acid medium containing 0.2 percent hydrochloric acid, also in an alkaline medium containing 0.5 percent sodium carbonate; frequently it was also carried out without the addition of either acid or alkali; in every case, trikresol (0.2 percent) was used as a bactericide. Two reagents were prepared; one contained 2.2 percent hydrochloric acid and 2.2 percent trikresol; the other contained 5.5 percent sodium carbonate and 2.2 percent trikresol; by addition of 1 volume of a reagent to 10 volumes of pitcher liquor, the proper degree of acidity or alkalinity was imparted to the reaction mixture. Control or blank tests were carried out, using pitcher liquor which had been boiled, then cooled to the temperature of the atmosphere prior to addition of the reagents. The temperature of digestion was that of the atmosphere. Each separate test was usually made in duplicate, however, at times, in triplicate or even in quadruplicate. Unless otherwise stated, each test was made on a composite sample of liquor drawn from a number of pitchers.

With four species—*Darlingtonia californica*, *Sarracenia Sledgei*, *S. flava*, and *S. Drummondii*—edestan and casein were used as substrates in laboratory tests for the presence of a protease in the pitcher liquor. In these tests, the temperature of digestion was 37.5° C. The pitcher liquor had been preserved by addition on one-tenth its volume of a 2.2 percent aqueous solution of

trikresol; therefore 1.1 cc. of preserved liquor equalled 1.0 cc. of liquor as drawn from the pitchers.

In the experiments with *edestan*, a solution of that substrate was prepared by solution of 0.1 gram of edestin in 100 cc. of 0.1 percent hydrochloric acid by heating to boiling, then cooling to room temperature. In each experiment 1.1 cc. of preserved pitcher liquor were mixed with 2.0 cc. of edestan solution. After incubation for 1.5 hours, 0.5 cc. of a saturated aqueous solution of sodium chloride was added to the reaction mixture. If digestion of the substrate had not occurred, a cloudy precipitate of edestan formed; if partial digestion had occurred, a faint cloud formed; if the edestan had been completely digested, the solution remained perfectly clear. The controls in these experiments always yielded a cloudy precipitate.

In the experiments with *casein*, a freshly prepared solution of that substrate was used. A beaker, containing 0.1 gram of purified casein (caseinogen), 5 cc. of 0.1 normal sodium hydroxide solution and 25 cc. of distilled water, was placed on a wire gauze and heated until boiling occurred and the casein dissolved. The beaker and its contents were quickly cooled to room temperature. The solution was rendered neutral by addition of 0.1 normal hydrochloric acid, and was diluted to a total volume of 100 cc. In each experiment, 1.1 cc. of preserved pitcher liquor were mixed with 2 cc. of the substrate solution. The mixture was incubated at a temperature of 37.5° C., usually for 2 hours. Then 6 drops of a solution of acetic acid in dilute alcohol (1 part by volume of glacial acetic acid, 49 parts of distilled water and 50 parts of 95 percent alcohol) were added. If digestion of the substrate had not occurred, a cloudy precipitate of casein formed; if partial digestion had occurred, a faint cloud formed; if complete digestion had occurred the solution remained perfectly clear. The controls always yielded a cloudy precipitate. Whenever the pitcher liquor was so acid in reaction that a precipitate immediately formed on mixing it with the substrate solution, then the experiment was repeated using pitcher liquor which had previously been rendered neutral to litmus by addition of an aqueous solution of sodium carbonate.

Coagulated egg white was used as the substrate in certain experiments on the liquor from closed pitchers of *Sarracenia flava*, *S. Drummondii* and *S. Sledgei* and from open pitchers of *S. flava*. A cube of white of hard-boiled egg, 0.25 inch to the edge, was placed in 5 cc. of pitcher liquor; 0.2 percent of trikresol was used as a bactericide. This substrate was also used in the presence of acid and of alkali.

All experiments in which either carmine fibrin or egg white served as the substrate, were made in vials which were tightly closed with cork stoppers in order to prevent evaporation of their contents.

The action of the pitcher liquor upon the various substrates is described by species in the following paragraphs.

SARRACENIA FLAVA

The experiments on *Sarracenia flava*, reported in Table V, demonstrate that a proteolytic enzyme, acting on carmine fibrin, is present in the liquor of closed pitchers as well as in that of open pitchers. They further show that this enzyme is far more active in the presence of 0.2 percent hydrochloric acid than in the presence of 0.5 percent sodium carbonate.

TABLE V.—DIGESTION OF CARMINE FIBRIN BY PITCHER LIQUOR OF *SARRACENIA FLAVA*

Type of pitchers.	Reaction of medium.	Volume of pitcher liquor, cc.	Mass of carmine fibrin, gram.	Solution of substrate.	
				Marked in hours.	Complete in hours.
Closed.....	0.2% HCl....	10	0.20	3	12
		5	0.20	3	15
		5	0.20	3	26
		5	0.20	2-5	17
		5	0.20	< 16	< 27
		3	0.05	3-5	< 14
		3	0.05	8	< 14
		4	0.05	7	< 14
Open.....	0.2% HCl....	3	0.05	5	..
		10	0.20	2	8
		4	0.05	..	15
Closed.....	0.5% Na ₂ CO ₃ .	3	0.05	48	96
		3	0.05	67	76*
		4	0.05
Open.....	0.5% Na ₂ CO ₃ ..	4	0.05	144	†

The proteolytic enzyme had practically no action on carmine fibrin in the absence of added acid or alkali. In four separate experiments, from 3 to 10 cc. of liquor from *closed* pitchers were permitted to act upon carmine fibrin. In three separate experiments, from 4 to 10 cc. of liquor from *open* pitchers were permitted to act upon that substrate. In each experiment, either 0.05 or 0.20 gram of carmine fibrin was used, with 0.2 percent trikresol as a bactericide. The period of observation varied with the experiment, and ranged from 5 to 42 days. The substrate was not appreciably attacked in any of the seven experiments.

* Solution incomplete at end of 8 days.

† Solution incomplete at end of 40 days.

The edestan test, previously described, was applied to four samples of liquor from closed pitchers and to two samples of liquor from open pitchers. The substrate was completely digested in 1.5 hours by all six samples. A third sample of liquor from open pitchers showed almost complete digestion of the substrate when the period of digestion was extended to 2 hours. In an experiment on liquor from closed pitchers, the substrate was almost all digested on incubation at room temperature for 30 minutes, and was completely digested on incubation at the usual temperature (37.5° C.) for the same period of time.

The casein test, previously described, was also used. On digestion for 2 hours, the casein was partially digested by three of the four samples of liquor from closed pitchers, and by both samples of liquor from open pitchers.

In the experiments with coagulated egg white, four series of tests were made. In two series, only trikresol was added, and incubation occurred at temperatures of 49° to 52° C. and 37.5° C., respectively. In the third series sufficient hydrochloric acid was added to give a concentration of 0.2 percent of that acid, in the fourth series sufficient sodium carbonate to give a concentration of 0.5 percent of that salt; trikresol was used in both series, and incubation occurred in them at a temperature of 37.5° C. In these series, use was made of one composite sample of liquor from each type of pitcher, closed and open. The substrate was not attacked in any of the four series by liquor from either type of pitcher on digestion for a period of 120 hours.

SARRACENIA DRUMMONDII AND SARRACENIA SLEDGEI

The liquor from both closed pitchers and open pitchers of *Sarracenia Drummondii* and of *Sarracenia Sledgei* contains a protease which digests carmine fibrin in the presence of 0.5 percent sodium carbonate, as may be seen on reference to Table VI. Tests were also made to ascertain the activity of this enzyme in the presence of dilute acid, and also without the addition of either acid or alkali. Each experiment was made on a separate gathering of pitcher liquor.

Six experiments were made on liquor from *closed* pitchers of *S. Drummondii* in the presence of 0.2 percent hydrochloric acid, using 0.05 gram of carmine fibrin and from 2.0 to 4.2 cc. of liquor in each experiment. The period of observation ranged from 12 to 54 days. Four experiments were carried out with liquor from *closed* pitchers of *S. Sledgei* in the presence of 0.2 percent hydrochloric acid; the mass of carmine fibrin ranged from 0.025 to 0.05 gram, the

volume of liquor from 2.0 to 3.8 cc., the period of observation from 12 to 60 days. The substrate was not even partially dissolved in any of these ten experiments.

TABLE VI.—DIGESTION OF CARMINE FIBRIN BY PITCHER LIQUOR OF *SARRACENIA DRUMMONDII* AND *SARRACENIA SLEDGEI* IN AN ALKALINE MEDIUM

Species and Type of Pitcher	Volume of Pitcher Liquor, cc.	Mass of Carmine Fibrin, gram.	Concentration of Sodium Carbonate, percent.	Solution of Substrate Complete in hours.
<i>Sarracenia Drummondii</i> Closed.....	1.9	0.05	0.5	2.5
	3.0	0.01	0.5	3.5
	3.0	0.05	0.5	4.5
	2.0	0.05	0.5	< 18
	2.0	0.05	0.5	< 18
<i>Sarracenia Drummondii</i> Open.....	3.5	0.025	0.5	1.5
	3.0	0.01	0.5	< 14
	3.0	0.05	0.5	< 14
	2.0	0.05	0.5	< 18
	3.5	0.025	0.5	1.5
<i>Sarracenia Sledgei</i> Closed.	1.6	0.01	0.5	3.5
	2.0	0.05	0.5	< 18
	2.0	0.05	0.5	< 18
	3.5	0.05	0.5	< 14
<i>Sarracenia Sledgei</i> Open. . .	3.5	0.05	0.5	< 14
	3.5	0.025	0.5	1.5
	2.0	0.05	0.5	5.5
	3.0	0.01	0.5	< 11
<i>Sarracenia Drummondii</i> Open.....	3.0	0.01	0.25	< 11
	3.0	0.01	0.125	< 11
	3.0	0.01	0.0625	< 11
	3.0	0.01	0.03125	36
	3.0	0.01	0.5	3.5
<i>Sarracenia Drummondii</i> Closed.....	3.0	0.01	0.25	5 days
	3.0	0.01	0.125	32 days*
	3.0	0.01	0.0625	†
	3.0	0.01	0.03125	†

In the experiments with liquor from *open* pitchers of both species in the presence of 0.2 percent hydrochloric acid, the volume of pitcher liquor used ranged from 2.0 to 3.5 cc., the mass of carmine fibrin from 0.025 to 0.05 gram. Four experiments were made with liquor of *Sarracenia Drummondii*, the period of observation ranged from 12 to 57 days; the substrate was not attacked in three of the experiments; in the fourth experiment it was partially dissolved at the end of 31 days and practically completely dissolved at the end of 49 days. Three experiments were made with liquor of *Sarracenia Sledgei*, the period of observation ranging from 5 to 57 days; the carmine fibrin was not attacked in two experiments; in the third experiment it was partially dissolved in 37 days and practically completely dissolved in 57 days.

In those experiments which were made on the pitcher liquor without the addition of either acid or alkali, 0.2 percent trikresol was used as a bactericide,

* Partial solution.

† No solution at end of 50 days.

as in all the other experiments. Six experiments were carried out with liquor from *closed* pitchers of *Sarracenia Drummondii*, using from 1.9 to 3.0 cc. of liquor and 0.05 gram of carmine fibrin. In four experiments, which were under observation for periods of 50 to 55 days, the substrate was not attacked. In one experiment the carmine fibrin was completely dissolved in 7 days; in another experiment it showed marked solution at the end of 21 days. Two experiments were made with liquor from *closed* pitchers of *Sarracenia Sledgei*, using 2.0 cc. of liquor and 0.05 gram of substrate; in one experiment complete solution of the carmine fibrin occurred in 7 days, in the other experiment, marked solution in 21 days.

Four experiments were carried out with liquor from *open* pitchers of *Sarracenia Drummondii*; the volume of liquor ranged from 2.0 to 3.5 cc., the mass of carmine fibrin from 0.01 to 0.05 gram. In one experiment, solution of the substrate had not occurred at the end of 21 days; in the other three experiments, solution slowly occurred, being noticeable at the end of approximately 30 days and practically complete at the end of approximately 50 days.

Three experiments were conducted with liquor from *open* pitchers of *Sarracenia Sledgei*, using from 1.5 to 3.5 cc. of liquor and from 0.01 to 0.05 gram of carmine fibrin. In one experiment, solution of the carmine fibrin had not occurred in 9 days. In the other experiments, the substrate was practically completely dissolved in 37 days and 49 days respectively.

In these experiments, as in all the other experiments, digestion of the substrate did not occur in the controls.

The experiments with carmine fibrin demonstrate that the pitcher liquor of *Sarracenia Drummondii* and *S. Sledgei* contains a protease and that this enzyme acts best in an alkaline medium. The liquor from *closed* pitchers did not show proteolytic activity in the presence of 0.2 percent hydrochloric acid; and that from *open* pitchers showed this activity only occasionally and on prolonged digestion. As recorded in a preceding section, the liquor from *closed* pitchers of these species is distinctly acid to litmus. Probably the native acid plus the hydrochloric acid produced a degree of acidity at which the enzyme was inactive. When neither acid nor alkali was added to the pitcher liquor, the substrate, at times, was dissolved on prolonged digestion.

Since the protease acted best in an alkaline environment, experiments were made in which the concentration of sodium carbonate was varied, ranging from 0.5 to 0.03125 percent, while the mass of carmine fibrin and the volume

of pitcher liquor remained constant. The liquor was obtained from pitchers of *Sarracenia Drummondii*. The results are recorded in the bottom section of Table VI. With liquor from open pitchers, the substrate was completely dissolved during the night, in less than 11 hours, at all concentrations of sodium carbonate from 0.5 to 0.0625 percent, and was completely dissolved in 36 hours when the sodium carbonate had a concentration of 0.03125 percent. With liquor from closed pitchers, the time required for solution of the carmine fibrin increased as the concentration of the sodium carbonate decreased. In fact with the higher dilutions of that salt, even partial solution had not occurred at the end of 50 days. In this connection it should be noted that the acid present in the liquor from closed pitchers exerted a neutralizing effect on the sodium carbonate; the reaction mixtures containing 0.0625 and 0.03125 percent of that salt were respectively neutral and acid to litmus, while the others in the series were alkaline to litmus.

The edestan test and the casein test were applied to two samples of liquor from closed pitchers of *Sarracenia Drummondii*, two samples from closed pitchers of *S. Sledgei*, and one sample from open pitchers of *S. Drummondii*.

In the edestan test, after incubation for 1.5 hours, no digestion of the substrate had been produced by the liquor from closed pitchers, while a slight digestion had been produced by that from open pitchers. The edestan, which had been precipitated by the sodium chloride, was not further digested when incubation was continued for an additional period of 70.5 hours at a temperature of 37.5° C.

In the casein test, that protein was completely digested by all five samples of pitcher liquor on incubation for 2 hours at a temperature of 37.5° C. This test was also applied to a third sample of liquor from closed pitchers of *Sarracenia Drummondii*; the casein was completely digested in 8.5 hours.

Coagulated egg white was also used as a substrate with liquor from closed pitchers of both species. Two samples of liquor from *Sarracenia Drummondii* and one sample from *S. Sledgei* were permitted to act on egg white in the presence of trikresol without the addition of either acid or alkali. The action of each sample was studied at two temperatures, 37.5° C. and 49° to 52° C. The substrate was not attacked in any of these tests during a period of 120 hours. Other experiments were made in which the pitcher liquor was permitted to act upon the egg white at a temperature of 37.5° C. in the presence of either dilute acid or dilute alkali. The liquor from *S. Sledgei* had no action

on the substrate on incubation for 120 hours in the presence of 0.2 percent hydrochloric acid. In the presence of 0.5 percent sodium carbonate, it produced marked digestion of the egg white in 24 hours; solution was almost complete in 72 hours and complete in 120 hours. Both samples of liquor from *S. Drummondii* had a proteolytic action on the egg white in the presence of 0.5 percent sodium carbonate; incipient digestion was noted in 24 hours, marked digestion in 48 and 144 hours, and advanced digestion in 168 and 216 hours respectively.

SARRACENIA RUBRA

Opportunity did not occur to procure abundant material for study of the pitcher liquor of *Sarracenia rubra*. Experiments were made with a single sample of liquor from closed pitchers, using 1.3 cc. of liquor and 0.01 gram of carmine fibrin in each experiment. In the presence of 0.5 percent sodium carbonate and trikresol, the substrate was completely dissolved in 2 hours. In the presence of 0.2 percent hydrochloric acid and trikresol, partial solution was noted at the end of 9 days, complete solution at the end of 50 days. These results indicate that the pitcher liquor of this species contains a protease which acts best in an alkaline medium.

SARRACENIA MINOR

Field tests were made on one sample of liquor from closed pitchers and on one sample from open pitchers of *Sarracenia minor*. The action of each sample was studied on carmine fibrin in the presence of (1) 0.2 percent trikresol, (2) trikresol plus 0.2 percent hydrochloric acid, and (3) trikresol plus 0.5 percent sodium carbonate. Neither sample exerted any proteolytic action on the substrate on digestion for 30 days in the presence of trikresol without the addition of either acid or alkali. In the case of the *closed* pitchers, 1.6 cc. of liquor and 0.01 gram of carmine fibrin were used in each test; the substrate was partially dissolved in 6 hours and completely dissolved in 15 hours in the presence of 0.2 percent hydrochloric acid; it was almost completely dissolved in 9 days in the presence of 0.5 percent sodium carbonate. In the case of the *open* pitchers, 3.0 cc. of liquor and 0.05 gram of carmine fibrin were used in each test; the substrate was partially dissolved in 6 hours and completely dissolved in 15 hours in the presence of 0.2 percent hydrochloric acid; it was partially dissolved in 15 hours and almost completely dissolved in 27 hours in the presence of 0.5 percent sodium carbonate.

These results indicate that the pitcher liquor of *Sarracenia minor* (*S. variolaris*) contains a protease which acts best in an acid environment, although it also acts, though less rapidly, in an alkaline medium.

SARRACENIA PSITTACINA

Sarracenia psittacina was found in Walton County, Florida, in early May, in some abundance but not of maximum size; the new pitchers for the year were just open or about to open. Although these pitchers were obviously secreting liquor in small amounts, yet it was insufficient in quantity to be collected by means of a fine pipette. An attempt was made to secure a diluted pitcher liquor by introduction of approximately 0.5 cc. of water into each of 50 open pitchers which were free from captures; four hours later, the water was collected by means of a pipette, and the composite sample was used in tests for a protease. Four cc. of the collected water and 0.01 gram of carmine fibrin were used in each test. The reaction mixtures were kept under observation for a period of 4 months. No evidence of digestion was obtained in the presence of 0.2 percent trikresol, or in the presence of that bactericide plus 0.5 percent sodium carbonate, or in the controls. In the presence of trikresol plus 0.2 percent hydrochloric acid, the substrate, which had swollen in the usual manner, gradually decreased in volume, but had not dissolved at the end of 4 months. These results indicate that *Sarracenia psittacina* may possibly secrete into its pitcher cavity a protease which is active in the presence of 0.2 percent hydrochloric acid. However, conclusive experiments with this species must be deferred until it is found growing vigorously in an accessible locality.

SARRACENIA PURPUREA

With respect to the amount of liquor present in its closed pitchers, *Sarracenia purpurea* presents difficulties almost as great as *S. psittacina*. However, a pitcher of *S. purpurea* is occasionally found in which the secretion, usually present as a fine, perspiration-like beading on the inner wall of the pitcher, has collected into drops at its bottom.

Two experiments were made in which open pitchers of living plants were thoroughly flushed by a current of water under pressure, then emptied as completely as possible. From 10 to 15 cc. of water were introduced into each pitcher. After the lapse of a number of days, the water was removed by means of a pipette; and the composite sample was subjected to tests for a

protease, in the presence of acid and of alkali, and without the addition of either reagent.

In one experiment two pitchers were used, and the water remained in them for 15 days. In each test 6 cc. of the collected water was permitted to act upon 0.01 gram of carmine fibrin. The collected water almost completely digested the substrate in 57 days in the presence of 0.5 percent sodium carbonate and trikresol. In the other experiment seven pitchers were used, and the water remained in them for 10 days. Fourteen cc. of the collected water were permitted to act upon 0.20 gram of carmine fibrin in each test. The collected water produced marked digestion of the substrate in 40 hours, and completely dissolved it in 64 hours in the presence of 0.5 percent sodium carbonate and trikresol. The substrate was not dissolved in either experiment within the times stated, in the presence of 0.2 percent hydrochloric acid and trikresol, or in the presence of that bactericide without the addition of either acid or alkali.

Liquor from open pitchers was used in a series of digestion experiments in which carmine fibrin served as the substrate. On reference to Table VII it is seen that the liquor contained a protease which completely dissolved the substrate in a short period of time in the presence of 0.5 percent sodium carbonate and trikresol.

The experiment on May 23 was made on liquor freshly collected from open pitchers which had matured during the preceding season and remained green through the winter. The remaining experiments were made on material from pitchers maturing during the season in which the liquor was collected. On November 15 the liquid contents of the pitchers were frozen to cores of ice which were thawed when the samples were collected; therefore the enzyme had not been destroyed by freezing. These two experiments (May 23 and November 15) indicate the retention of proteolytic activity by the liquor throughout the life of the pitcher.

The digestion experiments made in the presence of 0.2 percent hydrochloric acid and trikresol (Table VII) show that the protease may be slightly active under these conditions.

The experiments of June 30, July 1, and July 14 also were carried out in the presence of 0.2 percent trikresol without the addition of either acid or alkali. The period of observation ranged from 2 to 61 days; the substrate was not even partially dissolved in any of these experiments. All of the ex-

periments on this species thus far described were made on material gathered from strong vigorous plants in Ocean County, New Jersey. Liquor was also collected from smaller and less vigorous open pitchers growing in Tolland County, Connecticut, late in July. Ten cc. of this liquor failed to even partially dissolve 0.01 gram of carmine fibrin in 36 days in the presence of 0.5 percent sodium carbonate and trikresol, or 0.2 percent hydrochloric acid and trikresol, or 0.2 percent trikresol without the addition of either acid or alkali.

TABLE VII.—DIGESTION OF CARMINE FIBRIN BY LIQUOR FROM OPEN PITCHERS OF *SARRACENIA PURPUREA*

Reaction of medium.	Date of collection.	Volume of pitcher liquor, cc.	Mass of carmine fibrin, gram.	Solution of substrate Marked in	Complete in
0.5% Na ₂ CO ₃ ...	May 23	25	0.20	..	87 hours
	June 5	2	0.01	72 hours	120 "
	June 30	10	0.05	8 "	48 "
	July 1	3	0.01	24 "	48 "
	July 14	25	0.20	..	42 "
	July 16	8	0.01	..	48 "
	November 15	25	0.20	87 "	135 "
0.2% HCl.....	June 5	2	0.01	42 days	..
	June 30	10	0.05	..	†
	July 1	3	0.01	..	‡
	July 14	25	0.20
	July 16	8	0.01	47 days	..

DARLINGTONIA CALIFORNICA

Study was made of the action of liquor from closed, plugged, and open pitchers of *Darlingtonia californica* on various substrates. Carmine fibrin and fibrin were used most frequently. From 5 to 15 cc. of pitcher liquor and from 0.01 to 0.20 gram of one of these substrates were used in each experiment, with 0.2 percent trikresol as a bactericide. Some tests were made in the presence of 0.2 percent hydrochloric acid, others in the presence of 0.5 percent sodium carbonate, still others without the addition of either acid or alkali. The period of observation was usually 10 days, frequently 30 days, occasionally 40 days. Field experiments were made at atmospheric temperature, laboratory experiments at a temperature of 37.5° C. A total of 57 such experiments were performed—19 with fibrin and 38 with carmine fibrin; no digestion of the substrate was produced by liquor from any of the three types of pitcher.

In the edestan test, the period of digestion was prolonged, ranging from 4 to 7 days. Digestion of the edestan was not produced by liquor from closed pitchers (2 samples), plugged pitchers (2 samples), and open pitchers (1

* No solution in 16 days.

‡ No solution in 48 hours.

† No solution in 61 days.

sample). Two other samples of liquor from open pitchers produced a very slight digestion of the edestan.

In the casein test, the period of digestion was prolonged, ranging from 1 to 4 days. No digestion of the substrate was produced by liquor from closed pitchers (1 sample) and plugged pitchers (2 samples). The casein was completely digested by two samples of liquor from open pitchers.

In two field experiments, solid casein was used as a substrate. Several milligrams of casein and 5 cc. of pitcher liquor were permitted to digest in the presence of 0.2 percent hydrochloric acid and trikresol. One experiment was made with liquor from closed pitchers, the other with that from open pitchers. The casein was not dissolved at the end of 18 days.

In another series of field experiments, approximately 0.25 gram of solid casein was suspended in 3 cc. of water and introduced into a closed pitcher which was then plugged in the manner already described. Ten pitchers were treated in this manner. When the pitchers were opened 5 days later, undissolved casein was found at the bottom of each pitcher.

The protean derived from castor bean globulin was also used as a substrate in one experiment on liquor from closed pitchers and in one experiment on that from open pitchers. In each experiment, 2 cc. of a 2 percent solution of the globulin in a 5 percent aqueous solution of sodium chloride were mixed with 2 cc. of pitcher liquor and 0.5 cc. of 0.1 normal hydrochloric acid; and sufficient trikresol was added to make the concentration of that bactericide 0.2 percent. The period of incubation was 6 days at a temperature of 37.5° C. The protean, which was precipitated on addition of the acid, was not even partially dissolved in either experiment.

In certain field experiments, coagulated egg white, raw egg white, raw beef, and cooked beef were introduced into pitchers; and study was made of the action of the pitcher liquor upon them.

Four cubes of white of hard-boiled egg—one-eighth inch to the edge—were introduced into each of ten vigorous pitchers which had just opened. Seven of these pitchers were cut open for examination at the end of 72 hours, the remainder at the end of 144 hours. The cubes were found submerged in the pitcher liquor, unchanged in appearance, and with sharp edges. This procedure was repeated with eight closed pitchers and with eight plugged pitchers. The cubes were removed from the pitcher liquor at the end of 24 hours, and found unaltered.

Raw egg white was diluted with water in the ratio of 1 to 9, and was introduced into ten mature plugged pitchers, 10 cc. of the dilution into each pitcher. Five days later the contents of the pitchers were withdrawn; the liquid was somewhat glairy, and yielded a voluminous precipitate on boiling.

Cubes of raw fresh lean beef—one-eighth inch to the edge—were introduced into eight closed, fourteen plugged, and four open pitchers. The pitchers were cut open from 5 to 7 days later; the cubes had not undergone any disintegration, having retained their shape and size. This entire experiment was repeated with cubes of cooked beef with the same result.

The weight of the evidence, comprised in all the foregoing digestion experiments, is that a protease, secreted by the plant, does not occur in the pitcher liquor of *Darlingtonia californica*. The only evidence of the presence of a protease was the slight digestion of edestan and the complete digestion of casein, both produced by certain samples of liquor from open pitchers in laboratory experiments in which extremely small amounts of substrate were used while the period of incubation was prolonged; it is quite possible that this digestion was produced by enzymes of bacterial origin. For none of the tests for the detection of a protease revealed the presence of such an enzyme in the liquor from either closed or plugged pitchers; and, with the exceptions just noted, none of the tests showed the presence of such an enzyme in the liquor from open pitchers.

STABILITY OF THE PROTEASE AT ROOM TEMPERATURE

A composite sample of liquor from closed pitchers of *Sarracenia flava* was divided into two portions: one portion was preserved by addition of sufficient trikresol to produce a concentration of 0.2 percent of that bactericide; both portions were then kept at the temperature of the room, and tested at intervals with respect to their action on carmine fibrin in the presence of 0.2 percent hydrochloric acid and trikresol. In each test, use was made of 0.05 gram of substrate. The requisite control tests were also made. The data have been collected in Table VIII. The initial test was made on the day of collection.

The results obtained with the liquor preserved with trikresol demonstrate conclusively that the protease in the pitcher liquor retained its activity for over a year under the conditions stated. It is of interest to note that the liquor, to which no bactericide had been added, also exhibited proteolytic

activity during a like period. These results are in harmony with the occurrence of a protease in the liquor obtained in the spring (May 23) from open pitchers of *Sarracenia purpurea* which had matured during the preceding season and remained green throughout the winter.

TABLE VIII.—DIGESTION OF CARMINE FIBRIN BY PITCHER LIQUOR OF *SARRACENIA FLAVA* KEPT AT ROOM TEMPERATURE

Pitcher Liquor	Period of keeping, days.	Volume of Pitcher Liquor, cc.	Solution of Substrate	
			Marked in hours.	Complete in hours.
Preserved with trikresol.....	0	4	7	<18
	18	3	15	24
	34	3	..	21
	54	3	24	<120
	67	3	7	<23
	37°	3	21	46
Without bactericide.....	0	4	7	<18
	18	3	24	40
	34	3	..	21
	54	3	24	<120
	67	3	7	<23
	37°	3	..	21

RETENTION OF PROTEOLYTIC ACTIVITY AFTER DILUTION OF THE PITCHER LIQUOR

It is a matter of common observation that beating rains enter the pitchers of most species of *Sarracenia*, necessarily diluting their normal liquid contents. As is well known, enzymes continue to act on dilution of their solutions. The following experiments were therefore undertaken to ascertain if the protease of the pitcher liquor obeys this general law, and continues to act on dilution of the liquor, a phenomenon which necessarily occurs in nature.

The liquor was collected from pitchers and diluted with sterile water in varying proportions. These dilutions were permitted to act on carmine fibrin in the presence of 0.2 percent trikresol. In each experiment, the reaction (acid or alkaline) was that which had been found most suitable for the protease of that particular species in the experiments already described.

The results, which have been collected in Table IX, demonstrate that the protease of the pitcher liquor retained its activity on dilution of the latter; appreciable masses of carmine fibrin were completely dissolved in a few hours even when the degree of dilution was as great as 1:17.

STIMULATION EXPERIMENTS

Separate studies were made of the influence of mechanical stimulation and of food stimulation upon the proteolytic activity of the pitcher liquor. The technic of stimulation has been described in the preceding paper.

TABLE IX.—DIGESTION OF CARMINE FIBRIN BY DILUTED PITCHER LIQUOR

Species of <i>Sarracenia</i>	Type of Pitcher.	Degree of Dilution of Pitcher Liquor.	Volume of Diluted Pitcher Liquor, cc.	Reaction.	Mass of Substrate, gram.	Solution of Substrate. Marked in hours.	Complete in hours.
<i>S. Drummondii</i> ...	Closed	1:2	3	0.5% Na ₂ CO ₃	0.01	..	3
		1:3	3	"	0.01	..	4
		1:5	3	"	0.01	..	4.5
		1:9	3	"	0.01	..	<18
		1:17	3	"	0.01	..	<18
<i>S. Drummondii</i> ...	Open	1:3	3	"	0.01	..	<10
		1:5	3	"	0.01	..	<10
		1:9	3	"	0.01	..	<10
<i>S. Drummondii</i> ...	Open	3:10	10	0.125% Na ₂ CO ₃	0.05	..	<10
		1:10	10	"	0.05	10	<3.5
		1:10	10	"	0.05	10	<3.5
<i>S. flava</i>	Closed	1:8	8	0.2% HCl	0.05	6	<18
<i>S. flava</i>	Open	1:8	8	0.2% HCl	0.05	15	48

Mechanical stimulation did not cause the secretion of a protease by *Darlingtonia californica*. With respect to proteolytic activity, liquor of mechanically stimulated pitchers of *Sarracenia Sledgei*, *S. flava*, and *S. Drummondii* did not differ from liquor of unstimulated pitchers of these species.

Attention has already been called to the remarkable response of the pitchers of *Darlingtonia californica* to stimulation by certain *foods* and *reagents*. As a result of the stimulation, the secretion of pitcher liquor was greatly increased. However, a protease was not present in the pitcher liquor of this species after stimulation by the following substances: Sterile milk, raw egg white, coagulated egg white, raw beef, boiled beef, meat broth, fibrin, and acetic acid, hydrochloric acid, and sodium hydroxide in the concentrations stated in the preceding paper.

SUMMARY OF PROTEASE STUDIES

The weight of the evidence is that a protease, secreted by the plant, does not occur in the pitcher liquor of *Darlingtonia californica*.

Sarracenia psittacina possibly secretes a protease active in the presence of dilute acid.

The other species of *Sarracenia* unquestionably secrete proteases. As is shown in one of the following papers, the liquor in closed pitchers is bacteriologically sterile, yet it contains a protease; therefore that enzyme is secreted by the pitcher. Proteolytic activity is exhibited by liquor from both closed pitchers and open pitchers.

The proteases of *Sarracenia flava* and *S. minor* act best in the presence of dilute acid, but also exhibit some activity in the presence of dilute alkali.

The proteases of *S. Sledgei*, *S. Drummondii*, *S. rubra*, and *S. purpurea* act best in the presence of dilute alkali, but also exhibit some activity in the presence of dilute acid. In our experiments, the dilute acid was 0.2 percent hydrochloric acid, the dilute alkali 0.5 percent sodium carbonate. The proteases of *S. Sledgei* and *S. Drummondii* also exhibit some activity without the addition of either acid or alkali.

As is shown in the preceding and following papers, the hydrogen-ion concentration of the liquor from individual pitchers almost always lies on either the acid or the alkaline side of true neutrality. This fact, and the more or less marked activity of the proteases in either an acid or an alkaline environment would indicate that the proteases are active in the liquor in the pitchers, and have a part in the digestion of the prey prior to absorption.

Neither mechanical nor food stimulation of the pitchers increased the proteolytic activity of their liquor.

The protease was active after dilution of the pitcher liquor, thereby duplicating conditions which occur in the native habitat of the Sarraceniaceæ.

When the pitcher liquor of *Sarracenia flava* was kept at room temperature, its protease retained its activity for a period of more than a year. The liquor in pitchers of *Sarracenia purpurea* exhibited proteolytic activity during the entire life of the pitchers.

It is of interest to note that proteases have been found in the pitcher liquor of genera of all three families of pitcher plants: *Sarraceniaceæ*, *Cephalotaceæ* and *Nepenthaceæ*. The work of previous investigators and of ourselves on the *Sarraceniaceæ* is described in this monograph. In previous publications, we have reviewed the work of other investigators on *Nepenthes*, the only genus of the *Nepenthaceæ*,^{64, 65} and have described our work on the occurrence of a protease in the pitcher liquor of this genus.⁶⁶ A protease, active in the presence of dilute hydrochloric acid, has recently been found by Dakin⁶⁷ in the pitcher liquor of *Cephalotus follicularis*, the only genus and species of the *Cephalotaceæ*.

OTHER ENZYMES

Tests for the presence of enzymes, other than proteolytic, were made on composite samples of liquor from pitchers of *Darlingtonia californica* and of *Sarracenia flava*. The pitcher liquor was collected in the field and was mixed with one-tenth its volume of 2.2 percent aqueous solution of trikresol. The sample was shipped to Philadelphia and was examined on its arrival.

The two genera were studied in different years; and all the conditions were not exactly the same in the two series of tests. However, in the tests for urease, diastase, and the inverting enzymes (maltase, emulsin and invertase or sucrase), the ratio of the volume of the pitcher liquor to the concentration of the substrate was kept constant by use of 1.0 cc. of a 1 percent aqueous solution of the substrate for each 1.1 cc. of the sample of pitcher liquor containing trikresol which actually represented 1.0 cc. of the liquor as collected. The temperature of incubation was always 37.5° C. The requisite blank or control experiments were always made using pitcher liquor which had been boiled, then cooled to the temperature of the atmosphere. Whenever necessary, after the pitcher liquor and the substrate had been mixed, sufficient 2.2 percent solution of trikresol was added to bring the concentration of that bactericide in the reaction mixture to 0.2 percent. Urea was used as the substrate for urease, alpha methyl *d*-glucoside for maltase, amygdalin for emulsin, sucrose (cane sugar) for invertase, and soluble starch for diastase. All these reagents were used as 1 percent aqueous solutions prepared by solution of a weighed amount of the substrate in the requisite volume of distilled water at room temperature. Tributyrin was used as the substrate for lipase and ethyl butyrate for esterase. In the tests for diastase and the inverting enzymes, the volume of the composite sample of pitcher liquor used was:—*Darlingtonia californica* 11.00 cc., *Sarracenia flava* 2.75 cc.; these volumes corresponded to 10.00cc. and 2.50 cc. respectively of the liquor as actually collected.

Composite samples of the liquor from closed pitchers of *Darlingtonia californica* and from open pitchers of that species were tested separately for the presence of urease, maltase, emulsin, invertase, and diastase. A composite sample of the liquor from closed pitchers of *Sarracenia flava* was tested for the presence of these five enzymes and of lipase and esterase.

Inverting enzymes.—After incubation for 4 days, both the determination proper and the control were tested for the presence of reducing sugar by means of Benedict's qualitative alkaline copper solution. Neither the determination proper nor the control reduced Benedict's solution in the tests for maltase, emulsin, and invertase in the liquor from closed pitchers of *Darlingtonia californica*, or in the tests for maltase and emulsin in the liquor from open pitchers of that species, or in the tests for maltase and emulsin in the liquor from closed pitchers of *Sarracenia flava*. The determination proper reduced Benedict's solution in the test for invertase in the liquor from open

pitchers of *Darlingtonia californica*, and in the test for that enzyme in the liquor from closed pitchers of *Sarracenia flava*, while the corresponding controls had absolutely no reducing action. The test for invertase was repeated on a second composite sample of liquor from open pitchers of *Darlingtonia californica*; the period of incubation was 12 days; the result was the same as that obtained with the first sample.

These results indicate that the closed pitchers of *Darlingtonia californica* do not contain any of the three enzymes:—maltase, emulsin, or invertase. The liquor in open pitchers of this species does not contain either maltase or emulsin but may contain invertase; the invertase may be of bacterial origin since not present in the liquor of closed pitchers. The liquor in closed pitchers of *Sarracenia flava* does not contain either maltase or emulsin, but does contain invertase.

Diastase.—The period of incubation was as long as 12 days. At intervals, aliquots of both the determination proper and its control were tested for the presence of reducing sugar by means of Benedict's qualitative alkaline copper solution, and for the color reaction with iodine dissolved in an aqueous solution of potassium iodide.

The experiments with the first composite sample of pitcher liquor from closed pitchers of *Darlingtonia californica* were indecisive. After incubation for 10 days, both the determination proper and its control reduced Benedict's solution, and both yielded a blue color with iodine. The test was also applied to a second composite sample of liquor from closed pitchers of this species, the period of incubation being 12 days. The determination proper produced a faint reduction of Benedict's solution, its control no reduction; both gave a blue color with iodine.

With the first composite sample of liquor from open pitchers of *Darlingtonia californica*, after incubation for 4 days, the determination proper gave a slight reduction of Benedict's solution, its control no reduction; after incubation for 10 days, the determination proper gave the reaction of erythro-dextrin (red color) with iodine, while its control still gave the blue color produced by starch. A second composite sample of liquor from open pitchers of this species was also tested; the period of incubation was 12 days. The determination proper markedly reduced Benedict's solution, and gave no color with iodine; its control had no action on Benedict's solution and yielded a blue color with iodine.

When the composite sample of liquor from closed pitchers of *Sarracenia flava* was subjected to this test, the result was exactly the same after incubation for 4 days and for 10 days. Neither the determination proper nor its control reduced Benedict's solution, and both yielded a blue color with iodine.

These results indicate that a trace of diastase may be present in the liquor of closed pitchers of *Darlingtonia californica*. Liquor from open pitchers of this species possesses a distinct diastasic activity, but it may be, at least in part, of bacterial origin. The liquor from closed pitchers of *Sarracenia flava* does not contain a diastase.

Urease.--In the tests for the presence of urease, use was made of 11.00 cc. of a composite sample of pitcher liquor from *Darlingtonia californica* or of 5.50 cc. of that from *Sarracenia flava*. These samples had been diluted slightly by addition of trikresol in the field; and the volumes used represented 10.00 cc. and 5.00 cc. respectively of actual pitcher liquor. After the pitcher liquor and the solution of the substrate had been mixed and the proper amount of trikresol solution added, the resulting solution was rendered neutral to methyl orange. After incubation, the solutions were alkaline, and were titrated with 0.1 normal hydrochloric acid using methyl orange as an indicator in order to measure quantitatively the produced alkali.

In the experiments with *Darlingtonia californica*, the period of incubation was 3 days. In each of the four titrations—the determination proper with liquor from closed pitchers and its control and the determination proper with liquor from open pitchers and its control—0.25 cc. of 0.1 normal hydrochloric acid was required to neutralize the alkali.

In the experiment with liquor from closed pitchers of *Sarracenia flava*, both the determination proper and its control required 0.50 cc. of 0.1 normal hydrochloric acid to neutralize the alkali produced during incubation for 4 days.

Since the alkalinity of the determination proper did not exceed that of its control in any of the tests, urease was not present in any of the samples of pitcher liquor examined.

Lipase and Esterase.—In testing for the presence of these enzymes in the liquor from closed pitchers of *Sarracenia flava*, 1 cc. of the substrate (tributyryl for lipase, ethyl butyrate for esterase) was mixed with 5.50 cc. of the composite sample of pitcher liquor as collected. The mixture was rendered neutral to phenolphthalein, and was then incubated for 4 days. The liberated butyric

acid was then titrated with 0.1 normal sodium hydroxide using phenolphthalein as an indicator.

In the test for esterase, both the determination proper and its control required 0.10 cc. of 0.1 normal sodium hydroxide in the final titration, therefore esterase was not present.

In the test for lipase, in the final titration with 0.1 normal sodium hydroxide, the determination proper required 0.50 cc. and its control only 0.40 cc. Therefore lipase was probably present.

Summary.—The preceding results lead to the following general conclusions:

The liquor from closed pitchers of *Darlingtonia californica* contained a trace of diastase, while maltase, emulsin, invertase, and urease were absent.

The liquor from open pitchers of *Darlingtonia californica* contained invertase and diastase while maltase, emulsin, and urease were absent. However, no invertase and only a trace of diastase occurred in the liquor from closed pitchers; and these enzymes may have been formed in the open pitchers by bacteria which are present in the contents of open pitchers but are absent from the liquor in closed pitchers.

The liquor from closed pitchers of *Sarracenia flava* contained invertase and probably lipase, while maltase, emulsin, diastase, urease, and esterase were absent.

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