The Proteolytic Enzyme of Nepenthes (III).

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THE occasion of my reversion to this subject is the publication of researches by the late Georges Clautriau (2) which were concluded but a short time before his lamented decease. The paper is of such importance, and is moreover so relatively inaccessible to English readers, that it will not be out of place if I give a short account of its contents.

Clautriau was, I believe, the first to investigate the physiology of the pitchers of *Nepenthes* in the native habitat of the plant. The species which he so studied was *N. melamphora*, growing at a height of 1500–2200 metres near the laboratory which has been erected at Tjibodas on Mount Gedeh, a volcano in the island of Java. The proteid material used in his digestion-experiments was a 10 per cent. solution of egg-albumin rendered incoagulable on boiling by the addition of a small quantity of ferrous sulphate. In consequence of the altitude at which the experiments were performed, the temperature never exceeded 28° C.: digestion was therefore slow, and the experiments prolonged.

The liquid in the unstimulated pitcher was found to be colourless, tasteless, almost odourless, and slightly viscid. On stimulation of the pitcher, the liquid became acid; and when digestion had taken place, it acquired an odour resembling

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that of honey, and often an amber colour which Clautriau attributes to tannoid substances derived from the tissue of the pitcher, rather than to any chromogen such as is formed in tryptic digestion along with amido-acids.

The acidity of the liquid was the subject of special attention. The reaction of the liquid had been found by previous observers to be sometimes acid and sometimes neutral: even in unopened pitchers it is often acid, as I have pointed out (11.*a*, p. 575), a fact which is not readily accounted for. Clautriau made the interesting observation that acidity is caused, not only by the introduction of any foreign body, but also by mechanical stimulation of the pitcher, whether open or unopened. It suffices to shake a pitcher vigorously to induce acidity of the contained liquid in the course of a few hours.

Incidentally attention is drawn to the remarkable fact, well known in other pitcher-plants such as *Sarracenia*, that living insect-larvae, more especially those of the mosquito, are to be found in the pitchers. This fact, Clautriau rightly argues, cannot be accepted as evidence against the digestive activity of the pitcher-liquid: it is rather to be taken as an indication of special adaptation of the larvae. It is a fact belonging to the same physiological category as the presence of living parasites in the digestive tract of animals, and as the indigestibility of the gastric mucous membrane by its own secretions. It is, however, clear that the pitcher-liquid can contain no actively toxic or anaesthetic substances. Clautriau observed, in fact, that insects are very slowly killed when immersed in the liquid.

With regard to the actual process of digestion, Clautriau found that the albumin which he introduced into vigorous pitchers entirely disappeared within two days. The examination of the liquid at this stage showed that it gave no precipitate on neutralization, or on boiling in the presence of acids or of neutral salts: nor was any precipitate caused by ferrocyanide of potassium and acetic acid, by double iodide of mercury and potassium, or by phosphomolybdic acid.

Hence it would appear that the liquid contained no proteid matter of any kind. The conclusion at which Clautriau arrives is that the introduced albumin is rapidly attacked, and that the products of digestion are absorbed as quickly as they are formed. The proteolytic action is, moreover, exerted by an enzyme secreted by the plant, and is not attributable, as Dubois and Tischutkin suggested, to microbes. A further observation of special importance is that when a pitcher is separated from the plant, digestion is at once arrested.

In order, if possible, to trace the various stages in the proteolytic process, Clautriau instituted a series of experiments *in vitro*, the tubes being placed by the side of the plants in the open. In all cases but one, the albumin in the tubes underwent no change: in the exceptional case, a quantity of albumose, with perhaps some peptone, was formed. This negative result he attributes, with considerable probability, to the relatively low temperature. Nevertheless the experiments are of interest, in that they suggest some specific influence of the pitcher upon the digestive process.

On his return to Europe, Clautriau pursued his researches on plants (mostly *N. Mastersiana*) grown in hot-houses. As regards digestion in the pitcher itself, he found, as in his previous experiments in Java, that although the albumin which he introduced disappeared, he could, as a rule, find no proteids in the resulting liquid. For example: 15 c.c. of a watery solution containing 2·5 c.c. of the incoagulable albumin were poured into a pitcher: four days afterwards the liquid was found to have been partially absorbed, the remainder being viscid and amber-coloured, as is usually the case after digestion: but it contained no albumin, syntonin, albumose, or peptone. However in two such experiments he was able to detect the presence of peptone.

These experiments were supplemented by others *in vitro*, of which the following is a detailed example: 3 c.c. of filtered pitcher-liquid were placed in each of three tubes A, B, C, and to each 20 drops of a solution of incoagulable albumin were added: to B was also added one drop of

a dilute solution of hydrochloric acid (amounting to 0·01 c.c. HCl), and the same addition was made to C after it had been heated for about ten minutes in a water-bath at 100° C.: the three tubes were set to digest for three days at 37° C., camphor being used as an antiseptic. The examination of the contents of the tubes then showed that A contained no albumin or syntonin, and only doubtful traces of albumoses, whence it was concluded that here peptonification had been complete: the same result was obtained with B: whilst in C there was no albumin, but a great deal of syntonin, a small quantity of albumoses, and no peptone, results which are entirely attributable to the action of the introduced acid. Commenting on these facts, Clautriau makes the remark that the addition of HCl to the pitcher-liquid, which has been usual in experiments of this kind, is clearly unnecessary, since peptonification took place in the tube A without added acid.

In other similar experiments at a lower temperature (about 20° C.) he found that digestion proceeded very much more slowly. He then raises the question as to whether or not it is actually the case that, as asserted by von Gorup-Besanez (3) and others, the liquid of young unopened pitchers is quite as active as that of open pitchers, provided that it be acidified with HCl. He is inclined to take the opposite view, having on two occasions failed to obtain peptonification with liquid from unopened pitchers.

To the important question as to the nature of the proteolytic enzyme of the pitcher-liquid, Clautriau gives the answer that it is a pepsin: that is, an enzyme acting on the higher proteids in an acid medium, giving rise to peptones, but incapable of decomposing proteids into non-proteid substances such as leucin and tyrosin. He briefly criticizes the view which I have expressed (11 *b*, p. 555) that the enzyme is not peptic, but tryptic, in its action.

In conclusion, I would briefly mention his interesting observations on the absorption of the products of digestion in the pitchers. Using a solution of methylene-blue, he found that on introducing it into a living pitcher, the colouring-matter

readily penetrated into the glands whilst the epidermal cells stained but slowly. This differentiation was still more marked when methylene-blue was introduced into the pitcher together with some albumin, in which case the colouration was seen to extend into the tissues beyond the glands. At the same time the cytoplasm of the glands showed marked aggregation. It appears, therefore, that the glands not only secrete the digestive fluid, but are also the agents in absorption.

I should have been glad had it been possible for me to do no more than to express my appreciation of so much valuable work in a subject which has long interested me, to confirm the accuracy of the observations, and to recognize the validity of the conclusions. There are, however, certain points connected with Clautriau's experiments *in vitro* upon which I am reluctantly compelled to join issue.

In the first place, I take exception to his inference that the addition of HCl to the pitcher-liquid, as practised by other observers, is useless. It is perfectly true that if the pitcher-liquid be naturally acid, it will digest proteid without any addition of HCl. I have frequently found this to be the case: but I have also found that if a small quantity of HCl or an organic acid, such as citric, be added, digestion proceeds very much more rapidly, so that instead of extending over days, as did Clautriau's experiments, a few hours suffice. Moreover, neutral pitcher-liquid will not digest at all. Furthermore, the experiment upon which Clautriau bases this opinion is inconclusive, because no observations were made to determine the relative rate of digestion of the albumin in tubes A and B: had this been done, it is probable that the albumin in the acidified tube B would have been found to disappear some time before that in tube A.

Secondly, with regard to the doubt which Clautriau expresses as to the presence of the enzyme in the liquid of unopened pitchers, my experience entirely confirms the accuracy of the statement made by von Gorup-Besanez that this liquid is very active when properly acidified.

But the really important divergence between Clautriau's

conclusions and my own is as to the nature of the proteolytic enzyme of the pitcher-liquid, as manifested by the products of its activity. I need not attempt any reply to his criticisms beyond the remark that they appear to be based upon an imperfect acquaintance with my papers. The question at issue is simply one of fact: is the enzyme a pepsin or a trypsin?—a question of some moment, for if it be a pepsin it will be the first instance of such an enzyme having been definitely proved to occur in plants. We agree that the enzyme produces peptones; but here, Clautriau asserts, its activity ceases; whilst I have endeavoured to prove that it proceeds to the further stage of forming leucin, tyrosin, and other substances characteristic of tryptic digestion. Clautriau's evidence is of a negative character: he states that he has failed to detect leucin or tyrosin, but the investigation made with this particular object in view was by no means exhaustive. On the other hand, I have pointed out (11 *a*, p. 580) that there is to be found among the products of digestion a substance which presents some of the characteristics of leucin, though the presence of tyrosin was not detected.

It may be fairly urged that the evidence which I have adduced is inconclusive; and that the only absolutely convincing proof would be the separation of leucin, and of tyrosin too, in sufficient quantity to admit of ultimate analysis. This proof I am not able to give, and for the reason that I have not, so far, been able to carry on digestion-experiments on a sufficiently large scale. Comparing my results with those of Martin (6) in papaïn-digestion, and those of Chittenden (1) in bromelin-digestion, I am led to infer that the enzyme of *Nepenthes*, which may be conveniently termed *nepenthin*, produces leucin and tyrosin in smaller quantity than does that (papaïn or papayotin) of the Papaw (*Carica Papaya*, L.) or that (bromelin) of the Pine-Apple (*Ananas sativus*, Schult.); just as these enzymes, in turn, are less active than animal trypsin. This remark applies especially to tyrosin, which is always produced less abundantly than leucin in tryptic digestion;

hence Martin was unable to find any crystals of tyrosin in his experiments with papaïn, though he ascertained its presence among the products of digestion by Millon's reaction.

Short of the actual separation of these substances, there is no qualitative means of detecting them in the presence of peptones and albumoses which, of course, always occur in digestion-liquids. I have endeavoured to precipitate these proteids by means of absolute alcohol ; but the albumoses or peptones formed in nepenthin-digestion are remarkably soluble in alcohol, so that when the residue obtained by evaporating to dryness a digestion-liquid is extracted with absolute alcohol, the solution still gives the biuret reaction, showing that albumoses or peptones have been taken up. Tyrosin, it is true, gives characteristic colour-reactions: but these are also given by peptones and are useless when a mixture has to be dealt with.

If I am not in a position, at present, to establish my contention by such direct evidence as to the separation of leucin and tyrosin in measurable quantity would afford, I can at any rate adduce indirect evidence of a convincing character. As long ago as 1831, it was observed by Tiedemann and Gmelin (10) tha on the addition of chlorine-water to the liquid resulting from a pancreatic (tryptic) digestion, after acidification, the liquid acquires a colour varying, according to its concentration, from pink to violet ; when concentrated, there is a violet precipitate. This colouration is due to the presence of a substance which, together with leucin, tyrosin, and other bodies, is a product of tryptic, as distinguished from peptic, proteolysis. The substance in question is a chromogen, termed *proteinochromogen* by Stadelmann (9), but better known by the name *tryptophan* given to it by Neumeister (8) ; and its presence affords a ready means of distinguishing tryptic from peptic digestions.

I have found that the liquid resulting from a somewhat prolonged digestion of fibrin by the pitcher-liquid of *Nepenthes*, in the presence of either hydrochloric or citric acid, gives the

tryptophan-reaction. The following is an example of the experiments :—

A mixture was prepared, consisting of 10 grms. moist fibrin (preserved in dilute glycerin), 50 c.c. of 0·3 per cent. HCl, and 50 c.c. pitcher-liquid (*N. Mastersiana*) : this was placed in the incubator (temp. 38·5° C.) at 4 p.m. : next morning at 10.30 a.m. most of the fibrin was found to have been dissolved : a portion of the liquid was poured off, boiled, and filtered : the filtrate gave a good tryptophan-reaction.

The tryptophan-reaction is not, however, exclusively associated with the action of tryptic enzymes, for tryptophan has been found among the products of the disruption of the proteid molecule effected by boiling proteids with baryta-water, or by bacterial putrefaction. But in the absence of all sign of putrefaction, such as the odour of indol and skatol, in my experiments, it must be concluded that the production of the tryptophan was due to the action of the enzyme, and that nepenthin-digestion is tryptic in its nature.

As might be expected, I have also obtained the tryptophan-reaction in liquids resulting from the digestion of fibrin by both Pine-Apple juice and papaïn.

In order to further demonstrate the tryptic nature of nepenthin, I have made experiments as to its action on albumoses and peptones, using as the proteid material the preparation which is well known as Witte-peptone ; with the result that the digested liquid gives the tryptophan-reaction. The following are the details of one experiment :—

A mixture was prepared consisting of 1 grm. Witte-peptone, 0·2 grm. citric acid, 10 c.c. dist. water, 40 c.c. Nepenthes-liquid : placed in incubator at noon, temp. 38·5° C. ; next morning the liquid gave marked tryptophan-reaction.

I have also found that Pine-Apple juice and papaïn, under similar conditions, produce tryptophan from Witte-peptone. All the digestion-experiments have been controlled in various ways ; such as using boiled fibrin, in which case the digestion is very much delayed ; or making blank experiments with boiled *Nepenthes*-liquid, or without any at all.

My results make it apparent that the three enzymes,

nepenthin, bromelin, and papaïn (or papayotin), have essentially the same proteolytic action, which is tryptic: though, as I have already pointed out, they seem to differ in activity, bromelin being the most active, nepenthin the least. There is, however, a further difference between them as regards the media in which they are capable of acting. Nepenthin is only active in an acid liquid, and digests when as much as 0·25 per cent. HCl has been added. On the other hand, bromelin, according to Chittenden, and papaïn, according to Martin, are less active in acid than in neutral liquids; and their action is altogether inhibited when the liquid contains about 0·1 per cent. of free HCl. Furthermore, both bromelin and papaïn can digest in liquids which are alkaline to an extent not exceeding 1 per cent. Na_2CO_3. Of papaïn-digestion Martin says that it is essentially a neutral one; and in view of Chittenden's results, the same description may be extended to bromelin-digestion. It must, however, be borne in mind that both bromelin and papaïn have to act, in the plants in which they respectively occur, in liquid containing organic acid. There can be no question as to the acidity of the tissues of the Pine-Apple; and the fresh latex of the Papaw, like all other latices (see Molisch, 7), is acid. Moreover, I have found by experiment that papaïn can digest fibrin, to the extent of producing tryptophan, in liquids containing up to 0·5 per cent. of citric acid. In this respect these enzymes resemble animal trypsin, which, though most active in an alkaline liquid (1 per cent. Na_2CO_3), can digest proteid in a liquid which is neutral or even slightly acidified with an organic acid.

Including pepsin in the survey, the proteolytic enzymes under consideration may be classified as follows, according to the reaction of the media in which they act most vigorously, so far as is known :—

a. Active only in acid liquids { Pepsin, Nepenthin.

b. Most active in neutral liquids { Bromelin, Papaïn.

c. Most active in alkaline liquids Trypsin.

If, however, these enzymes be grouped according to their mode of action, the three vegetable enzymes may be associated with trypsin, since they induce that disruption of the proteid molecule which is marked by the formation of tryptophan and other non-proteid bodies such as leucin and tyrosin: on the same ground they would be distinguished from pepsin. But it must be borne in mind that the chemistry of peptic proteolysis is probably by no means so simple as it has been thought to be. Although it has been generally accepted that pepsin does no more than to hydrolyse the higher proteids into peptones, there have long been physiologists, like Hoppe-Seyler, holding the view that non-proteid crystallizable substances, such as leucin, tyrosin, and others, are formed in prolonged peptic digestion. The most recent researches in this direction, those of Zunz (12) and of Lawrow (5), go to prove that such substances are in fact among the products of peptic digestion, without, however, proving their identity with the non-proteid products of tryptic digestion. Lawrow asserts that he has detected leucin in a prolonged (two months) self-digestion of a pig's stomach: but, on the other hand, he failed to find tyrosin. In no case, so far as I am aware, has tryptophan been found in peptic digestion; and until that has been done, the tryptophan-reaction may be taken as the distinguishing criterion between the action of trypsin and that of pepsin. What exactly the relation between peptic and tryptic digestion may be, cannot be determined until a great deal more is known as to the chemical details of both processes. It is not impossible that they may be found to differ, not in kind, as is now generally assumed, but only in degree: in which case the vegetable enzymes enumerated above would form a series intermediate in properties between pepsin on the one hand and trypsin on the other.

The evidence afforded by the tryptophan-reaction strengthens the suggestion which I have already ventured to make (11 *b*, p. 555), that all known proteolytic enzymes of plants are tryptic, though some of them, such as that of *Drosera*, still await investigation.

This suggestion gains in interest when it is borne in mind that tryptic digestion is of general occurrence in the animal kingdom, and is apparently the sole process in many invertebrates. It is not improbable that it may be expanded into the proposition that tryptic digestion is a property of all living organisms, and that it is the more primitive form of the digestive process.

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