

The Proteases of Plants (VI).

BY

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THE latter part of my last paper (21) on this subject was devoted to an account of experiments upon ungerminated Hemp-seed (*Cannabis sativa*), made with the object of strengthening the evidence in favour of the view, to which I had given expression in several previous papers of this series, that 'vegetable trypsin' is not, as is commonly held, a single substance, but is a mixture of at least two proteases. Of these, the one is only capable of peptonizing the higher proteins (fibrin, albumin, &c.): the other has no action upon these proteins, but actively splits peptones and albumoses into amino-acids and other non-proteid nitrogenous substances. That is to say, that 'vegetable trypsin' is a mixture of a *peptase* (peptonizing protease) with an *ereptase* (peptolysing protease), and possibly of more than one of each of these two kinds of enzymes.

The method adopted in those experiments enabled me to prepare from Hemp-seed, solutions, of which some digested fibrin but were without action on peptones, that is, were merely peptonizing solutions; others were without action on fibrin, but split up peptones (as indicated by the tryptophane-reaction)—that is, were merely peptolysing solutions. Thus it was shown that two distinct proteases, having different solubilities, can be extracted from Hemp-seed; and there is no reason to doubt that these two proteases exist separately in the seed.

Having obtained this result with Hemp-seed, I proceeded to apply the same method to various structures and substances, in the hope of obtaining confirmatory results; but I soon found that it was only applicable to material which, like seeds, is rich in proteid substances. It became necessary, therefore, to find another method which should be of more general application; and, as the result of many attempts, the method sought for was found in the course of some experiments with papain, as described in the following pages.

PAPAIN (or Papayotin).

The latex of the Papaw (*Carica Papaya*) has long been known to act proteolytically. The first record of the digestive action is to be found in Griffith Hughes's 'Natural History of Barbados' (1750); it is there

stated that the milky juice of the fruit 'is of so penetrating a nature that if the unripe fruit, unpeeled, be boiled with the toughest old salt meat it will soon make it soft and tender; and if hogs are for any considerable time fed with it, especially raw, it is said that it will wear off all the mucous slimy matter which covers the inside of the guts, and would in time, if not prevented by a change of food, entirely lacerate them'. A similar account was given by Patrick Browne in his 'Civil and Natural History of Jamaica' (1756). He says: 'Water impregnated with the milky juice of this tree is thought to make all sorts of meat washed in it very tender; but eight or ten minutes' steeping, it is said, will make it so soft that it will drop in pieces from the spit before it is well roasted, or turn soon to rags in the boiling.'

The latex was first investigated scientifically by Wurtz and Bouchut (1). Having extracted the latex with distilled water, they mixed the aqueous extract with ten times its volume of alcohol: the considerable precipitate formed was collected in a filter and dried. To the white powder thus obtained they gave the name *papaïn*, and regarded it as the digestive enzyme of the latex. They observed that an aqueous solution (0.2 per cent.) of this powder readily digested fibrin at 40° C., whether the liquid were neutral, slightly alkaline (KHO) or acid (0.2 per cent. HCl). They found the ultimate products of the fibrin-digestions to be peptones. On this account they considered *papaïn* to be allied to animal pepsin, but to differ from it in that it digests in neutral, acid, and alkaline medium, whereas the action of pepsin is limited to an acid medium. Their ultimate view is expressed in the following words:—'Il circule réellement dans les différentes parties de cet arbuste un suc presque neutre qui a toutes les propriétés de la *pepsine*, sauf que celle-ci est acide, et n'agit qu'étant acide; et quelques propriétés de la *pancréatine*, ce qui en fait une sorte de *pancréatine végétale*.'

After a considerable lapse of time, the investigation of *papaïn* was resumed by Martin (2). From the analysis of 'commercial *papaïn*', he came to the conclusion that *papaïn* consists of a mixture of two proteids, a globulin and an albumose, and that the ferment-action is associated with the albumose. Having proved the formation of leucin and tyrosin in the course of digestion of fibrin and albumin, Martin concluded (3) that *papaïn* is the only vegetable ferment which has as yet been proved to act like trypsin, and then its normal action takes place in a neutral medium. Thus it was shown that *papaïn* not only digests the higher proteins, but also hydrolyses the peptones: it both peptonizes and peptolyzes.

Several years after Martin's papers had appeared, I made some experiments on *papaïn*-digestion (14), and found, by means of the tryptophane-test, that *papaïn* actively digests peptones in neutral solution, more actively in the presence of acid (best in 0.5 per cent. citric acid), and less actively in

the presence of alkali (0.5 per cent. Na_2CO_3), results that were confirmed by some further observations which were published about a year later (16). Returning again to the subject (18), I was struck by the fact that the digestion of fibrin and that of peptone were not always similarly affected by changes in the experimental conditions; and consequently expressed the opinion that papaïn may be a mixture of two proteases, the one fibrin-digesting but not peptolytic, the other peptolytic but not fibrin-digesting. In a supplementary paper, containing some further facts concerning papaïn-digestion amongst others, I suggested that the fibrin-digesting enzymes should be classed as *peptases*, and the peptolysing enzymes as *ereptases*.

The mode in which I have endeavoured to establish these views, in the case of papaïn by separating the peptase and the ereptase, is based upon the observation frequently made in the course of my work, that extracts of plant-material made with 2 per cent. NaCl-solution digest fibrin more actively than similar extracts made with distilled water. This fact suggested the possibility of there being a fibrin-digesting enzyme (peptase) present, which is less soluble in distilled water than the peptone-digesting enzyme (ereptase). If this be so, it should be possible to wash out all the ereptase, by treating the material with relatively large quantities of distilled water, leaving some, at any rate, of the peptase behind. I tried this repeatedly with papaïn, as well as with other material, but without success; however much water I used, the washings, as shown by experiment, continued to contain ereptase: in fact the ereptase in the material seemed inexhaustible.

It occurred to me eventually that extraction of papaïn with NaCl-solution, instead of with water, might lead to the desired result, and this proved to be the case. I found that when papaïn was extracted with 20–25 times its volume of 2 per cent. NaCl-solution and the liquid filtered off, either the residue contained no ereptase, or any remaining in it could be washed out by means of water; and further, that this residue treated with 2 per cent. NaCl-solution, yielded, after filtration, a liquid which digested fibrin but was without action on peptone. By these means, however, only the fibrin-digesting enzyme is separated; the ereptase not being obtained in pure solution. The method is illustrated in detail by the following note of an experiment:—

10 grms. of commercial papaïn mixed thoroughly in a mortar with 200 cc. 2 per cent. NaCl-solution: after standing for an hour or so, the mixture was placed on a filter: filtration was slow (it would have been better if 250 cc. of the NaCl-solution had been used).

Filtrate 1. The filtered NaCl-extract was clear, light yellow, acid; it gave a dense ppt. on boiling, and on the addition of nitric acid (HNO_3): it also gave a distinct tryptophane-reaction.

Digestive properties. 40 cc. of the filtrate digested 0.2 grm. fibrin within five hours, and also digested Witte-peptone, as shown by the tryptophane-reaction.

The papaïn-residue (about 5 grms.) was removed from the filter, triturated in the mortar with 200 cc. dist. water, and the mixture filtered.

Filtrate 2. The filtered H_2O -extract was clear and neutral, gave no ppt. or turbidity on boiling, or on adding HNO_3 , and only a slight xanthoproteic reaction.

Digestive properties. 40 cc. of the filtrate failed to digest 0.2 grm. of fibrin in forty-eight hours, and had no action on Witte-peptone.

Hence it appears in this case, that, since ereptase is readily soluble in water, the papaïn-residue contained none of it: that, in fact, the whole of the ereptase had been dissolved out in the extraction with NaCl-solution.

The papaïn-residue, after the extraction with H_2O , was extracted with 120 cc. of 2 per cent. NaCl-solution; the mixture was allowed to stand for a short time, and was then filtered.

Filtrate 3. The filtered NaCl-extract was colourless and neutral: it gave no turbidity on boiling, but a slight turbidity on adding HNO_3 , a faint xanthoproteic reaction, no biuret-reaction, no tryptophane-reaction.

Digestive properties. 35 cc. of the filtrate were put into each of three bottles, Nos. 1, 2, 3, the liquid placed in No. 2 having been previously boiled; to each bottle was added 0.2 grm. fibrin; and to Nos. 1 and 2, 0.2 grm. of Witte-peptone.

Within twenty-four hours the fibrin had completely disappeared in Nos. 1 and 3, remaining unaltered in No. 2. The contents of No. 1 gave no tryptophane-reaction: those of No. 3 gave a ppt. on boiling and on adding HNO_3 , and a good biuret-reaction, but no tryptophane-reaction.

It is clear that the NaCl-extract, filtrate 3, contains a fibrin-digesting (peptonizing) enzyme, but no peptone-splitting (peptolysing) enzyme.

A further experiment was made with the same papaïn-residue. It was extracted once more with 200 cc. 2 per cent. NaCl solution and filtered.

Filtrate 4. The neutral filtrate gave no turbidity on boiling or on adding HNO_3 , and but a slight xanthoproteic reaction.

Digestive properties. An attempt was made to ascertain in what way, if any, the digestive properties of the fibrin-digesting enzyme are affected by the reaction of the medium.

35 cc. of the filtrate were put into each of five bottles, Nos. 1-5: to one bottle (No. 2), 0.2 grm. of Witte-peptone was added, just to make sure that no ereptase had been extracted; to all the others, 0.2 grm. of fibrin: the contents of No. 3 were acidified to 0.1 per cent. HCl, those of No. 4 to 0.5 per cent. citric acid, and those of No. 5 were made alkaline to 0.5 per cent. Na_2CO_3 .

After twenty-four hours' digestion, the fibrin had disappeared in No. 5 (alkaline), and was much broken up in No. 1 (normal), whilst it was unaltered in Nos. 3 and 4 (acid). The contents of Nos. 1 and 5 gave ppt. on boiling and on adding HNO_3 , a strong xanthoproteic and a good biuret-reaction, but no tryptophane-reaction.

After forty-eight hours' digestion, the contents of No. 2 gave no tryptophane-reaction, thus proving the absence of ereptase: the fibrin in Nos. 3 and 4 was still quite unaltered.

The foregoing experiments demonstrate the isolation from papain, by this method, of a solely peptonizing enzyme, peptase, in the form of an active NaCl-solution. It is clear, however, that a great deal of this enzyme is lost in the preliminary treatment of the papain with 20–25 times its bulk of 2 per cent. NaCl-solution: for, as stated above, this first extract actively digests fibrin. This consideration led me to devise a supplementary method which makes use of this extract, as explained in the following experiment:—

10 grms. of 'raw papain', merely the dried latex as imported, were ground to powder, and were well triturated with 150 cc. of 5 per cent. NaCl-solution: the mixture was placed on a filter, and the filtrate dropped into 300 cc. of alcohol, where a dense ppt. was formed. The ppt. was then collected on a filter: it was subsequently extracted with 200 cc. of distilled water, and the mixture was put on a filter.

Filtrate 1. The filtered H₂O-extract is of a brown colour, the colour having developed during filtration; it is clear and neutral; it gives ppt. on boiling, dense ppt. on adding HNO₃, and strong xanthoproteic reaction: a portion of it, boiled and filtered, gives good biuret-, but no tryptophane-reaction.

Digestive properties. The filtrate was found to digest both fibrin and Witte-peptone within eighteen hours.

The residue was now extracted with 100 cc. 2 per cent. NaCl-solution, and the extract filtered.

Filtrate 2. The filtered NaCl-extract was clear, colourless, neutral, gave a turbidity on boiling and on adding HNO₃, and a distinct xanthoproteic reaction: a portion of it, after being boiled and filtered, gave no biuret- or tryptophane-reaction.

Digestive properties. The experiment is worth giving in full. 25 cc. of the filtrate were put into each of four bottles, the liquid put into No. 2 having been previously boiled; to each bottle were added 0.2 gm. fibrin and the same quantity of Witte-peptone; to No. 3 citric acid was added to 0.3 per cent.; and to No. 4, HCl to 0.05 per cent.

After twenty-four hours' digestion, the fibrin had disappeared in No. 1 (neutral); it was unaltered in No. 2 (boiled) and in Nos. 3 and 4 (acid), and remained so during twenty-four hours' further digestion. No tryptophane-reaction was given by the contents of any of the bottles.

Hence the solution contained only a fibrin-digesting enzyme, the action of which was arrested by acidity.

Thus the results obtained by the investigation of the NaCl-extract of papain agree with those obtained by the investigation of the papain-residue.

The material used in these experiments was chiefly what is known as 'Commercial Papain'; the more important results were verified by experiments with 'Raw Papain'—that is, the dried latex as imported, kindly supplied to me by Messrs. Thomas Christy & Co., of London.

It was thus shown that the digestive properties of the latex, as here described, are not due to, nor are materially affected by, the processes of purification by which commercial papain is prepared from the raw latex.

I may add that neither form of papain is completely soluble in water or NaCl-solutions; after extraction, a considerable insoluble residue remains, which, in the case of commercial papain, consists largely of some form of coagulated protein, as Martin has already noticed. It should also be recorded that all the digestions took place at 39° C., and that HCN was the antiseptic.

Turning, now, to the consideration of the bearing of the new facts that I have described, the first effect of them will be the disuse, as being no longer necessary, of the name 'papain' or 'papayotin'). The name, in its original strict sense, was applied to what was considered to be a single substance; but now that this supposed tryptic protease has been shown to be a mixture of two proteases the name has lost its significance, and, if used at all, might be applied to the dried latex, whether raw or refined.

A point of great importance is the relation between the digestive activity of the papain-enzymes and the reaction of the medium. As already mentioned, Wurtz and Martin both found that digestion of fibrin or albumin (peptonization) was most active when the medium was neutral. Martin showed further that digestion is even more active in a feebly alkaline medium (0.25 per cent. Na_2CO_3), and continues, though with diminishing activity as the alkalinity is increased, even when the medium contains 1 per cent. Na_2CO_3 ; and that it is arrested in a medium which is acid to the small extent of 0.05 per cent. HCl. The few observations that I have made on this point in the course of these experiments confirm these results. Thus (p. 4) I found that the NaCl-solution of peptase digested fibrin when neutral or alkaline (0.5 per cent. Na_2CO_3), but did not do so in the presence of 0.1 per cent. HCl, or of 0.5 per cent. citric acid. In another experiment (p. 5) digestion was prevented by 0.3 per cent. citric acid, and by 0.05 HCl.

The results of some earlier experiments on this very point (19) had led me to form a rather different opinion from that just stated. The conclusion drawn from them was that the reaction-range for fibrin-digestion by papain extended from an alkalinity = at least 1.5 per cent. Na_2CO_3 to an acidity = 0.3–0.5 HCl. The discrepancy on this point between the results of my present and of my previous experiments is, I believe, to be accounted for on the ground that in the latter the solution used contained more protease; and further that it also contained a good deal of protein in solution, which would protect the protease to some extent from the action of the acid, whereas, in the former, the amount of protein was very small.

The question of the relation between the enzymes and the proteins of papaw-latex naturally arises here. It has been already mentioned (p. 2) that Martin considered the digestive action to be associated with the albumose. My experiments seem to show that the fibrin-digesting enzyme is associated with the globulin of the latex, inasmuch as it is more soluble in

NaCl-solutions than it is in water; probably the pure peptase would be insoluble in pure water. But it must be pointed out that in several experiments the active peptase-solution gave but a faint xanthoproteic reaction, indicating a very small quantity of protein.

The demonstration of the presence of two distinct proteases in papain would be more complete had I succeeded in preparing, not only extracts that were exclusively peptonizing, but also extracts that were exclusively peptolysing. I have endeavoured to obtain solutions of the latter kind at various stages of the methods here described for obtaining those of the former kind. For instance, I have treated the papain residue, after extraction with 2 per cent. NaCl-solution, with water, and obtained solutions which peptolysed Witte-peptone actively, but also had some digestive action on fibrin; similarly, the alcoholic precipitate of the NaCl-solution, when treated with water, gave a solution that peptolysed actively, but also peptonized. The only liquid that I have found to be exclusively peptolytic is the alcoholic liquid filtered off from the alcoholic precipitate of the NaCl-extract. I found that some of this liquid, diluted with an equal bulk of water, acted upon Witte-peptone so as to cause a distinct tryptophane-reaction, but had no effect upon fibrin. Ereptase was clearly present; but it is not so clear that peptase was absent, though probably it was; for the amount of alcohol present was sufficient, as I found by control-experiments, to prevent the peptonization of fibrin in liquids known to contain peptase. I anticipate, however, that it will be possible to make use of the solubility of ereptase in fairly strong (above 60 per cent.) alcohol in devising a method for its isolation.

YEAST (*Saccharomyces Cerevisiae*).

The investigation of the proteolytic activity of Yeast began in 1889 with Salkowski's observation that, if Yeast be kept in chloroform-water, the liquid eventually contains leucin and tyrosin which can only have been formed by the digestion of its own proteins. Ten years later (1898–1900) Hahn and Geret began the publication of a series of papers (22, 23) bearing upon the subject; they found that leucin and tyrosin were formed in the self-digestion of the expressed juice of Yeast; that the juice digested fibrin, egg-albumin, casein, with the formation of leucin, tyrosin, and tryptophane among the products; and that digestion was most active in acid medium (0.2 per cent. HCl). The name 'endotrypsin' or 'endotryptase' was given by them to the proteolytic enzyme, on account of the nature of its digestive activity. It was, and still is in fact, regarded as a form of 'vegetable trypsin'.

My own observations on Yeast began in 1901, when I tested its digestive action in the course of my search for tryptophane as a constant product of the proteolysis of plants (14). The result of these first experiments was that tryptophane was found in Yeast-digestions when the medium was neutral or acid, but not when it was alkaline.

A year later, I returned to the study of Yeast-digestion, and pursued it in greater detail. The material used was chiefly dried Yeast. The results which I obtained were these (17)—

- (1) dilute watery extracts of Yeast digested Witte-peptone but not fibrin ;
- (2) dilute extracts of Yeast made with NaCl-solution (2 per cent.) readily digested both fibrin and Witte-peptone ;
- (3) peptolysis and peptonization were influenced in the same manner, but not in the same degree, by the addition of acid or alkali.

These results raised the important issue that may be best expressed in two questions : (1) Is there, as is generally held, a single protease in Yeast, or is there more than one ? (2) In the latter case, how many proteases are there, and what is their nature ?

My answer to these questions was that 'the two digestive processes—that is, the digestion of fibrin (peptonization) and the digestion of Witte-peptone (peptolysis)—are not effected by one and the same protease. On the contrary, the facts described in this paper indicate the presence of two proteases ; the one exclusively peptolytic, readily soluble in water [ereptase] ; the other exclusively peptonizing, less soluble in water, but readily soluble in 2 per cent. NaCl-solution [peptase]'.

After completing the investigation of the Hemp-seed and of papaïn, with the results already described, I turned yet once more to Yeast, to see if it might not be possible here also to effect the separation of the two enzymes, of which the distinct individuality had been so strongly suggested by my previous experiments.

These experiments had, in fact, already demonstrated that, on making a dilute extract of Yeast with distilled water, and filtering it, a solution is obtained which has no action upon fibrin, but readily digests Witte-peptone with the formation of tryptophane. Clearly such a solution contains only ereptase. This being so, what remained to be done in the new experiments was to obtain a solution from Yeast which would digest fibrin but not Witte-peptone, a solution which should, in fact, contain peptase.

I have now, after many and long-continued experiments, succeeded in preparing such a solution from both fresh and dried Yeast. In illustration of the method employed, I give a description of an experiment made with fresh Yeast.

About a litre of yeast was obtained from the brewery : it was placed upon a filter to allow the beer to drain off, and it was washed by running water through it. After allowing it to drain until no more water dropped from it, the solid residue was of the consistence of thick paste and amounted to about nine large table-spoonfuls. This was removed from the filter and thoroughly mixed with 500 cc. 5 per cent. NaCl-solution ; to the mixture was added chloroform enough to give the strength of chloroform-water (0.5 per cent.), and it was left standing all night in a covered jar kept in a cold room. It was noticed next morning that the mixture in the jar had frothed up

considerably, as if it had been fermenting: this occurred in all experiments with fresh Yeast.

The mixture was put on a filter next morning, the filtrate dropping into a vessel containing 1 litre of alcohol. A portion of the filtrate was collected for examination, it was found to be clear, yellow, strongly acid, giving marked turbidity on boiling, less turbidity on adding HNO_3 , strong xanthoproteic reaction, distinct biuret-, but no tryptophane-reaction: its digestive properties were that it acted quickly upon Witte-peptone, the digestion-liquid giving marked tryptophane-reaction in twenty-four hours, whilst its action upon fibrin was slower.

The filtrate dropping into alcohol gave rise to a copious precipitate: the mixture was put on a filter, and the alcoholic liquid drained off. When filtration was completed, the moist precipitate on the filter was found to weigh just over 15 grms.

The precipitate was now mixed with 200 cc. distilled water, and left standing for some hours. The mixture was then filtered: the water did not appear to have dissolved much, if any, of the precipitate, and the filtrate had no digestive action on either fibrin or Witte-peptone.

After having thus been washed with water, the precipitate was mixed with 150 cc. 2 per cent. NaCl -solution: after standing for some hours, the mixture was put on a filter.

The *filtered NaCl-extract* was neutral, opalescent, giving slight precipitate on boiling, and turbidity with HNO_3 , a slight xanthoproteic reaction, but no biuret.

60 cc. of the filtrate were taken for experiment: 30 cc. were put into a bottle (a) with 0.2 grm. of fibrin and 0.2 grm. of Witte-peptone; the other 30 cc. were boiled and filtered, the liquid being made up to the original quantity by adding distilled water, and put into a bottle (b) with fibrin and Witte-peptone as before: a few drops of HCN were added to each.

After twenty-four hours' digestion, the fibrin had almost entirely disappeared in (a); the liquid contents of it gave a considerable precipitate on boiling, somewhat less on adding HNO_3 , but no tryptophane-reaction: the fibrin in (b) was quite unaltered.

30 cc. of the filtrate were also put into each of three bottles; the contents of the first were acidified to 0.06 per cent. HCl , those of the second to 0.45 per cent. citric acid, and those of the third were made alkaline to 0.6 per cent. Na_2CO_3 : 0.2 grm. of fibrin was placed in each bottle, with a few drops of HCN .

At the end of seventy-two hours in the incubator the fibrin was apparently unaltered in all three bottles. It appears, therefore, that the reaction range of the peptase lies within the limits of acidity and alkalinity indicated in this experiment—a result which differs, as regards acidity, from that obtained by Hahn and Geret (see p. 7), the discrepancy being due, no doubt, to the presence of a considerable amount of protein in their liquids; but agrees fairly with my previous results (17, pp. 302–3).

By this method, which is the same as the supplementary method applied to the investigation of papain (see p. 5), it was possible to prepare a liquid which digested fibrin, but had no action on Witte-peptone; a liquid, therefore, which contained only peptase in solution.

The conclusion to be drawn from my experiments upon Yeast is that the cells contain two proteases, peptase and ereptase. The so-called 'endo-

trypsin' of Yeast, is, therefore, not a single protease, but a mixture of two ; consequently the term may now be dispensed with.

It will be seen that the results obtained with Yeast agree with those obtained with papaïn, and lead to the same conclusions.

REVIEW OF THE SUBJECT.

Now that my researches upon the proteases of plants have led to a definite conclusion, valid at least for the materials that have been subjected to experiments, I should like to retrace briefly the steps by which I have arrived at that conclusion.

Without attempting a complete history of the subject, the delivery of Sir Joseph Hooker's presidential address at the Belfast meeting of the British Association in 1874, and the publication of Darwin's book on 'Insectivorous Plants' in the following year, may be taken as the starting-point of the scientific investigation of proteolysis in plants. The first conclusion arrived at was that the secretions of insectivorous plants contain an enzyme similar to the pepsin of animals, inasmuch as it digests fibrin and the other higher proteins in acid medium.

The next step was the discovery by von Gorup-Besanez, about the same time, of the existence of proteolytic enzymes in ordinary, non-insectivorous, plants. The fact of the presence of leucin and asparagin in seedlings of a Vetch, when grown in darkness, suggested to him that these substances must be the products of changes in the reserve-protein of the seed effected by a digestive enzyme. Accordingly he examined the seeds of the Vetch, but whether germinated or ungerminated he does not say, and succeeded in extracting from them, by means of glycerin, an enzyme that converted fibrin into peptone in the presence of 0.2 per cent. HCl (4). He subsequently investigated the seeds of the Hemp (*Cannabis sativa*) and of the Flax (*Linum usitatissimum*), apparently ungerminated, as also Malt, arriving at much the same result as in the case of the Vetch (5). He sought in vain for leucin, trypsin, and asparagin among the products of the digestion of fibrin. Thus he failed to trace the leucin, asparagin, &c., found in seedlings to a digestive process ; and though he did not call the enzyme that he found by the name 'pepsin', he described it as 'peptonbildendes ferment'.

The first general idea as to the nature of the apparently wide-spread protease of plants was, therefore, what may be termed the *pepsin-idea* : that is, it was considered to be a peptonizing enzyme acting only in acid medium. However, facts soon began to come to light which gradually made this view untenable. The investigation of papaïn by Wurtz, already mentioned on p. 2, showed that here was an enzyme which digested fibrin not only in acid, but also in neutral and even alkaline media. Although Wurtz found nothing but peptone in the products of digestion, he realized that enzyme could not be a pepsin, so he regarded it as a kind of

pancreatin (i. e. trypsin). Martin's investigation of papain carried the matter a good deal further on in the same direction. He found leucin and tyrosin among the products of digestion, and ascertained that the presence of acid retarded, whilst that of alkali, within certain limits, accelerated, digestion; consequently he associated it, though very cautiously, with animal trypsin.

In this way the second general idea, what may be termed the *trypsin-idea*, of plant-proteases originated, the idea that these proteases not only peptonize fibrin and other proteins, but further split up the albumoses and peptones into amino-acids, such as leucin and tyrosin, and other nitrogenous substances. It was soon supported by a considerable amount of experimental evidence. Thus, Green found that from germinating seeds (Lupin, Castor-oil) extracts could be obtained which digested fibrin, in the presence of 0.2 per cent. HCl, leucin, tyrosin, and asparagin being formed during digestion (6, 7). Soon afterwards he showed that the Kachree Gourd (*Cucumis Melo* var. *utilissimus*) contains an enzyme which digested coagulated egg-albumen actively in alkaline (1.5 per cent. Na_2CO_3) medium, less actively in neutral, and least actively in acid (0.2 per cent. HCl); he found leucin among the products of digestion (8). Hansen's observations on the latex of the Fig (*Ficus Carica*) may be mentioned, though they are inconclusive (9). The latex digested fibrin in both acid (0.2 per cent. HCl) and alkaline (2 per cent. Na_2CO_3) medium; the products of digestion were not fully examined, but leucin and tyrosin were not found in the alkaline digestion. Chittenden (10), investigating the juice of the Pine-apple (*Ananas sativus*), observed that it digested fibrin most actively at its own natural acidity, and that leucin and tyrosin were products of digestion. He also found that the juice digested coagulated egg-albumen most actively when neutral in reaction, less actively at natural acidity, or at an alkalinity of 0.1 per cent. Na_2CO_3 , still less actively when made more alkaline or more acid (with HCl), and that digestion was arrested by an alkalinity=1 per cent. Na_2CO_3 , and (in an artificial solution) by an acidity=0.1 per cent. HCl.

Further evidence of this kind was afforded by my observations upon the digestive properties of the pitcher-liquid of *Nepenthes* (11, 12, 13, 1897-8, 1901). In these three papers I showed that the liquid has a 'tryptic' action, inasmuch as it not only peptonizes fibrin, but also splits the peptones into amino-acids, such as leucin and tryptophane. It was in the last of these three papers that I introduced the chlorine-reaction for tryptophane into the study of the proteolysis of plants. Although digestion was found to take place only in acid medium, I considered that the enzyme of the pitcher-liquid should be regarded as belonging to the trypsin-group (tryptases), on account of the products formed in digestion; and in the last of the three papers I went so far as to express the opinion 'that all known proteolytic

enzymes of plants are tryptic'. I extended my observations (14, March 1902) to other parts and secretions of plants, such as the juice of the Pine-apple (*Ananas sativus*), the latex of the Papaw (*Carica Papaya*) and of the Fig (*Ficus Carica*), Malt (*Hordeum sativum*), and Yeast (*Saccharomyces Cerevisiae*), and found in every case that tryptophane was one of the products of digestion, a proof that the digestion had been 'tryptic' in character, and that all the enzymes were active in acid medium. The conclusion that I drew from these facts was 'that the proteolytic enzymes of plants in general are essentially tryptic'; adding that 'this statement will hold good until definite evidence is adduced to prove the existence of a "peptic" enzyme'.

So far my investigations had been confined to parts or secretions of plants which were already known to have more or less definite digestive activity. I now turned my attention to the question of the possible presence of proteolytic enzymes in plants in which they had not yet been detected (15, Jan. 1903).

In the first set of these experiments, Witte-peptone was the material for digestion; the production of tryptophane was effected as the result of the action of the following substances: tissue of the Mushroom (*Agaricus campestris*); watery extract of Green Peas (*Pisum sativum*); extract of 'germ' of Wheat (*Triticum vulgare*); the expressed juice of the Melon (*Cucumis Melo*), of the Cucumber (*Cucumis sativus*), and of the Vegetable Marrow (*Cucurbita Pepo* var. *ovifera*); watery extract of the Banana (*Musa Sapientum*); the juice of the Tomato (*Lycopersicum esculentum*); the rind of the Apple (*Pyrus Malus*), and of the Orange (*Citrus Aurantium*); the juice of the Grape (*Vitis vinifera*); extract of the laticiferous shoots of *Euphorbia Characias*; the laticiferous leaves of the Lettuce (*Lactuca sativa*); the stems of *Dahlia* and of *Mirabilis*; the leaves of *Spinacia oleracea*, of the Cabbage (*Brassica oleracea*), of a Grass (*Holcus mollis*), of a Fern (*Scolopendrium vulgare*), and of several other plants; the bulbs of the Tulip, the Hyacinth, and the Onion (*Allium Cepa*); the tubers of the Potato (*Solanum tuberosum*) and of the Jerusalem Artichoke (*Helianthus tuberosus*); the roots of the Turnip (*Brassica Rapa*), the Tomato, the Vegetable Marrow, the Carrot, the Beet, and others.

Having shown that these various parts of plants possessed the property of digesting Witte-peptone, I proceeded, in the next place, to ascertain if any of them also possessed the power of digesting fibrin. In this way I was able to add to the list of plants known, at that time, to digest fibrin, the following: the juice of the Cucumber and of the Melon; the tissue of the Mushroom; the bulb of both the Tulip and the Hyacinth, but only in alkaline medium. I entirely failed to obtain any evidence of the capacity of ordinary foliage-leaves to digest fibrin.

The conclusions that I drew at the time are as follows: 'I have

succeeded in demonstrating that a proteolytic enzyme is widely distributed in plants ; and it may be inferred that it is much more generally present than I have shown it to be. The next point to be considered is the probable nature of the enzyme. In the previously known cases, the evidence goes to prove that the enzymes are allied to the trypsin of animals, since they both peptonize and proteolyse actively. Amongst the plants that I have recently examined, there are only two, the Melon and the Mushroom, which approach those previously known in their power of peptonization and proteolysis. Whilst all the others readily proteolysed Witte-peptone, their action on the higher proteins, so far as it was tested, was relatively feeble, and in some cases altogether wanting. It may be that the precise conditions favourable for peptonization were not afforded in the experiments : that is a point for future investigation. But taking the facts as they stand, it is an inevitable conclusion that if in some cases, such as the Melon and the Mushroom, the enzyme may be regarded as a vegetable trypsin, this view cannot be extended to the others. It seemed to me, at first, that I had come upon an altogether new type of enzyme, an idea that occasioned a certain amount of temporary misgiving as to the accuracy of my observations. But it was pointed out to me by my colleague, Professor Gotch, that within the last year (1901-2), Cohnheim had described an enzyme, formed in the mucous membrane of the small intestine, which actively proteolyses peptone and casein, but does not act upon the higher proteins. It is to this enzyme, termed 'erepsin' by Cohnheim, that the apparently new proteolytic enzyme of plants would correspond. It would appear, therefore, that plants form two distinct kinds of proteases, the one a trypsin, the other an erepsin ; and so far as the facts go, they indicate that the former is generally associated with depositories of proteid nutriment, such as seeds, fruits, bulbs, laticiferous tissue, the latter with ordinary foliage-leaves, stems, and roots'.

The discovery of the 'erepsin' of plants, or 'ereptase', as I prefer to call it, and the wide and general distribution of this substance, necessarily involved a modification of the 'trypsin-theory' of the proteases of plants previously mentioned, and to this extent : those plants whose tissues or juices peptonized fibrin, were considered to contain 'trypsin', with possibly ereptase as well ; those plants whose tissues or juices had no effect upon fibrin, but digested (peptolysed) Witte-peptone, as indicated by the tryptophane-reaction, were considered to contain ereptase only.

For some time (1903-4) I continued my researches from this point of view, adding to the number of plants that were found to have some kind of digestive activity, investigating also the conditions of the digestion-experiments with special reference to the effect of antiseptics upon the digestive processes, and giving the result of extracting the plant-material with NaCl-solution instead of distilled water. The experiments are described in two papers, published respectively in June 1903 (16), and in April 1904 (17).

Those in the latter paper were a more detailed re-examination of Yeast and of the Mushroom; and it is worth while to repeat here the conclusion to which I was led: 'It is suggested that the Yeast and the Mushroom contain two associated proteases, vegetable erepsin and vegetable trypsin . . .'

My next paper (18, Jan. 1905) contains an account of further experiments upon both new and old material, confirmatory of those already described. Perhaps the most important observation recorded here is the discovery that the leaves of *Phytolacca decandra*, unlike all the leaves (except those containing latex) previously examined, readily digested fibrin.

Another paper which followed closely (19, April 1905) upon the preceding, raised the question, 'What is the nature of the fibrin-digesting protease?' I had observed, when testing the action of various antiseptics upon the digestive processes, that sometimes the fibrin-digesting property of an extract or a tissue was destroyed by an antiseptic, whilst the peptone-splitting activity remained unimpaired, and *vice-versa*. This paper is a study of the action of acids and of alkalies upon the digestive processes in a variety of plant-material (papain, Pine-apple juice, Yeast, Mushroom, Malt, Hyacinth-bulb, pitcher-liquid of *Nepenthes*). My conclusions were as follows: 'The experiments detailed in the foregoing pages constitute a demonstration of the differential effect of varied reaction upon the proteolytic activities of the juices and extracts of certain representative plants. . . . On further consideration of these results, it will, I think, be generally admitted that the method employed does actually afford the means of realizing that separation of the proteolytic activities which I postulated as being essential to the investigation of the nature of the supposed "vegetable trypsin". I cannot interpret the evidence thus obtained otherwise than as indicating that peptolysis and fibrin-digestion are effected by two distinct proteases; that "vegetable trypsin" is, in fact, not a single protease, but a mixture of two; the one a peptolytic enzyme belonging to the ereptases, the other a peptonizing, fibrin-digesting, enzyme belonging to the peptases. . . . If it be admitted that two proteases, or two groups of proteases, exist in plants, the ascertained facts as to the distribution of the proteases in the vegetable kingdom may be succinctly stated in the following propositions: All plants that have been examined contain ereptase; in some of these plants the ereptase has been found to be associated with a larger or smaller proportion of a peptase; in no plant has a peptase been found to exist unassociated with ereptase'.

This altogether new view of the nature of the plant-proteases was a challenge to the idea of 'vegetable trypsin'; but the evidence was as yet insufficient to be convincing. The kind of evidence still required was the actual separation of the two kinds of enzymes, the peptase and the ereptase, so that from the same material there should be prepared extracts, one of which

would be purely peptonizing (i.e. containing peptase only), the other purely peptolysing (i.e. containing ereptase only).

Some further indirect evidence in favour of the new view was obtained by a series of experiments that I made with certain, chiefly starchy, seeds (*Phaseolus multiflorus* and *vulgaris*, *Vicia Faba*, *Lupinus hirsutus*, *Pisum sativum*, and *Zea Mais*). In discussing these experiments, I stated the position of the question as follows (20, April 1906): 'It must be admitted, however, that all the evidence that I have accumulated does not yet suffice to prove that there is no such thing as "vegetable trypsin". One point, at any rate, has become clear, namely, that "vegetable trypsin" is a mixture of enzymes, and that ereptase is one of the constituents. But the nature of the other constituent (or constituents), the fibrin-digesting protease, remains uncertain: it may be a tryptase, but it may also be a peptase. It is not, I think, going too far to suggest that the known facts make the latter suggestion the more probable—to transfer, in fact, the *onus probandi* to those who hold that the enzyme in question is a tryptase.'

Pursuing my researches on seeds, I next turned my attention to oily seeds, and found them to be much more proteolytically active than starchy seeds, even when ungerminated. Most of the experiments were made with ungerminated Hemp-seed (*Cannabis sativa*); but several others, such as those of the Mustard (*Sinapis alba*), the Hazel (*Corylus avellana*), the Castor-oil plant (*Ricinus communis*), and the Flax (*Linum usitatissimum*) were also examined.

In the course of my investigation of the Hemp-seed, I made an attempt, which eventually proved successful, to prepare solutions containing respectively the peptase and the ereptase which my experiments had shown to be present in the seed. It is unnecessary to repeat here the description of the process employed, which is fully given in the paper in which the account of the experiments was published (21, Jan. 1908). The really important result was the preparation, from the Hemp-seed, of a solution containing only a fibrin-digesting enzyme (peptase). I had frequently prepared extracts of various kinds of material which contained only ereptase; but never before had I, or indeed any one else, prepared from a part of a plant a solution of a peptase unmixed with ereptase.

This result seems to me to involve the final demolition of the 'vegetable trypsin' theory. But of course it requires reinforcing by similar results obtained with other material. This reinforcement is, to some extent, afforded by the results obtained with papain and with Yeast, described in the earlier part of this paper. It is, however, necessary that I should repeat most of the experiments that I have described in this series of papers, from the fresh stand-point and with the application of the new methods, so as to complete the confirmation of this view as to the nature of the proteases, and perhaps to come upon further developments of it.

The view that I propose in substitution for the 'vegetable trypsin' theory is that the proteases of plants belong to two groups, the *peptases* and the *ereptases*—a view that is now supported by a considerable body of evidence, both direct and indirect. It remains now to consider the respective properties of these proteases, so far as they are known.

The Ereptases are enzymes which are readily soluble in water, in watery solutions of neutral salts, and in alcohol up to over 65 per cent.; their digestive activity seems to be exclusively peptolytic, and to be especially associated with an acid medium. I am unable to give even an approximate reaction-range for plant-ereptase, because I have not yet made any experiments with it in pure solution; all my experiments, so far, have been made with extracts containing other substances in solution, the presence of which materially affects the action of acids or alkalies upon the enzyme. For instance, I have found (17) the reaction-range of peptolysis in a 5 per cent. Yeast extract to be from 0.6 per cent. HCl to 2 per cent. Na_2CO_3 , a very wide range which could certainly be very much reduced were the experiments to be repeated with a liquid containing only the ereptase in solution.

I have not yet come across any facts to indicate that the ereptases of various plants are materially different from each other.

The Peptases are proteases of which the digestive activity is limited to the peptonization of the more complex proteins.

There is some ground for thinking that there are at any rate two kinds of peptases, which differ from each other (*a*) in the mode of their occurrence, and (*b*) in the relation between their respective digestive activities and the reaction of the medium. The one kind exists in the tissues of plants, fruits, seeds, latex, &c., and may therefore be designated *endopeptase*; the other kind is to be found in the excretions of plants, for example, in the pitcher-liquid of *Nepenthes*, and may be distinguished as *ectopeptase*.

(*a*) *Endopeptase* is an enzyme which can be readily extracted from the tissues, &c., in which it occurs by NaCl-solutions; and also, to a less extent, by water, and by 50 per cent. alcohol. The extraction by means of water is due to the presence of salts and other substances which are extracted with the peptase; for, when obtained as free as possible from foreign bodies, it is not soluble in distilled water (*vide* Yeast, p. 9), but is still soluble in 2 per cent. NaCl-solution. Its digestive activity, like that of ereptase, is greatest at the natural reaction, generally somewhat acid, of the plant-extract which contains it. Very slight addition of mineral acid (0.05 HCl) or of a rather larger quantity of organic acid (.3 per cent. citric acid) arrests the action of a pure solution of the enzyme, which digests fibrin actively when neutral or slightly alkaline; an increase of alkalinity retards and finally stops digestion.

(*b*) *Ectopeptase* is an enzyme which is in solution in the excretion of at

any rate one carnivorous plant, *Nepenthes*. This is the only one of the carnivorous plants which has been adequately investigated from the point of view of digestive activity; but it is not improbable that the facts ascertained with regard to this plant will be found again when the other carnivorous plants are examined.

The pitcher-liquid of *Nepenthes* is, under normal conditions, a clear colourless or yellowish liquid, either neutral or acid in reaction, containing very little organic matter and not more than 1 per cent. of mineral matter. It is, therefore, a fairly pure aqueous solution of the protease. The characteristic feature of its digestive activity upon fibrin and other complex proteins is that acid reaction is absolutely essential: neutral liquid has no action; digestion is rapid at natural acidity, and also in the presence of added acid, whether organic (citric acid) or mineral (HCl 0.3 per cent.). It is especially in its relation to HCl that ectopeptase differs essentially from endopeptase: for the activity of the latter, when in pure solution (thus corresponding nearly to the pitcher-liquid) is arrested by the presence of as little as 0.05 per cent. HCl.

In considering the relation of the peptases to the acidity of the medium, it may be pointed out that there is some evidence (observations of Weis and others on Malt, quoted in No. 17, p. 291, of the list of references) to prove that the acid reaction which is always given by extracts of parts of plants, is due, at any rate in Malt, and probably also in other seeds, to the presence, not of free acid, but of acid phosphates. It is in an acid medium of this kind that endopeptase seems to be most active. Ectopeptase, on the contrary, is most active in the presence of free acid; this is well established in the case of *Nepenthes*.

It is impossible to conclude this discussion of the nature of the proteolytic enzymes of plants without some reference to those of animals. Certain analogies are obvious. The enzyme that I have termed 'ectopeptase' agrees in all essential properties with animal pepsin; this agreement is particularly interesting because it justifies the original conclusion (see p. 10) that the excretions of carnivorous plants contain pepsin. The only modification of that original view that is necessary is that these excretions (at least that of *Nepenthes*) contain ereptase in addition. Then, again, the ereptase of plants differs from that of animals only in that its reaction-range is more extensive in the direction of acidity, and is, perhaps, less extensive in the direction of alkalinity.

But it is not so easy to find an animal analogue for 'endopeptase'. It does not correspond to trypsin, because that substance is held to be an enzyme that both peptonizes and peptolyzes; it would, however, correspond fairly well with the peptonizing factor in trypsin, if that were regarded as separable; and it may prove to be separable after all.

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