Tryptophane in Proteolysis.

BY

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THE last number of the 'Annals of Botany' contains a paper (1) in which I adduce evidence to prove that the proteolytic enzyme of *Nepenthes*, as well as those of the Pine-Apple (bromelin) and of the Papaw (papaïn), are essentially 'tryptic' in their mode of action. The evidence consists in the demonstration, by means of the chlorine-water test, of the presence of a substance, known as tryptophane, among the products of digestion. It is, I believe, generally accepted that the formation of this substance is an indication of the disruption of the proteid molecule into non-proteid substances which is held to be characteristic of 'tryptic' digestion. The correctness of this view of the physiological significance of tryptophane has been confirmed by the recent researches of Hopkins and Cole (2), who find that its formula is $C_{11}H_{12}N_2O_2$, and that it abundantly yields skatol and indol on heating.

I propose, in the present paper, to give a more complete account of my observations on bromelin and papaïn, and to describe further experiments which I have made with the enzymes of the Fig (*Ficus Carica*, L.), of the Coco-Nut (*Cocos nucifera*, L), of germinating seeds of the Bean (*Vicia Faba*, L.) and of the Barley (*Hordeum vulgare*, L.), of Yeast (*Saccharomyces Cerevisiae*, Meyen), and of the Bacteria of putrefaction, as also with animal pepsin. I will so far anticipate as to say at once that in all these cases, under appropriate conditions, I have succeeded in finding trypto-

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phane among the products of digestion of fibrin and of Witte-peptone.

It is already known that the action of certain of these vegetable enzymes is 'tryptic,' since it has been ascertained that they cause the formation of leucin and tyrosin in proteolysis. This is the case with bromelin, papaïn, the enzyme of germinating seeds, and with the enzymes of Yeast and Bacteria. The products of digestion by the enzyme of the Fig (*cradeïn*) have not yet been sufficiently investigated from this point of view.

The only attempts at quantitative estimation of the activity of the vegetable enzymes are those of Chittenden (3) regarding bromelin, and of Martin (4) regarding papaïn. The method employed by these observers was that of supplying a known weight of digestible proteid (albumin) and determining the weight of proteid remaining undigested at the close of the experiment. Whilst the results so obtained are of value, the method is open to the criticism that when HCl or Na_2CO_3 is present in a digestive mixture, the solution of the proteid may be due in part to the action of the acid or of the alkali, and to that extent the numerical result would be vitiated. Nor does this method afford any evidence as to whether or not the proteid that has passed into solution is in the same stage of proteolysis in digestions which may have to be compared. In employing the tryptophane-test for this purpose, I have not had any close quantitative estimation in view. My object has been to ascertain, under various conditions and with various enzymes, the time required to form tryptophane; and to compare the intensity of the reactions as indicated by the depth of colour. In this way some rough idea as to the relative activity of proteolysis has been obtained. It would be, I believe, possible to develop a quantitative method on the basis of the tryptophane-reaction, by ascertaining the volume of chlorine-water necessary to produce the same depth of colour in equal quantities of the various digested mixtures; the coloured chlorine-compound in each being extracted by shaking with equal quantities of amyl-alcohol in which it is readily soluble.

However, the difficulty would still remain that it is impossible at present to prepare equivalent standardized solutions of the enzymes to begin with.

Before proceeding to the description of the experiments, a few general remarks as to the methods employed are necessary. The digestions were carried on in an incubator or thermostat, at a constant temperature of 38°–39° C. The fibrin used had been well-washed in water and in alcohol, and preserved in 50 per cent. glycerin ; it was again well-washed in water immediately before use: it was not previously swollen in dilute HCl. That the results obtained on its digestion were not attributable to an enzyme belonging to the fibrin itself, is proved by the fact that digestion of some of it in 0·2 % HCl, for several days, gave rise to no tryptophane; nor was any found when the fibrin was digested with water for a similar period (see expts. with Bacteria, p. 15). Moreover the results obtained with fibrin were checked by those with Witte-peptone, with which they were generally concordant, though they were produced more slowly and were less marked.

A point of experimental importance arose in connexion with the neutralized or alkaline digestions in which crude vegetable juices were used, such as those of the Pine-Apple, of the Fig, and of germinating seeds. It was observed that the neutral or alkaline reaction of these liquids was maintained for some hours; but on prolonging the digestions over night (20–24 hours) the liquids became more or less strongly acid, showing that a secondary development of acid had taken place. The results recorded in such cases are those that were obtained whilst the liquid was still found to be neutral or alkaline. It is noticeable that when a neutralized or alkaline liquid thus becomes acid again, the tryptophane-reaction that it gives is exceptionally strong, indicating that proteolytic action has been more than usually vigorous. I am unable at present to account for these facts. It seems not improbable that some of the recorded observations as to the activity of certain of these enzymes in prolonged digestions of neutralized or alkaline liquids, may be to some extent vitiated by the failure to recognize this re-acidification.

As a rule the tryptophane-test was applied directly to the digestion-liquid, after ascertaining that the reaction of the liquid was acid. When the liquid was too thick, it was previously filtered. In cases in which a comparison had to be instituted, and where the result appeared to be at all doubtful, measured quantities of the liquids were tested, and measured quantities of chlorine-water added.

In the more prolonged experiments it was advisable to use an antiseptic, and for a time I had recourse to thymol. But I found that this substance seemed to interfere with proteolytic action, especially in the case of pepsin; so I replaced it by hydrocyanic acid (HCN), adding a few drops of a strong (Scheele's = about 4 %) solution; and found that it not only acted as an antiseptic, but in certain cases promoted digestion. When using HCN, it was necessary to keep the liquids during digestion in stoppered bottles to prevent loss of the volatile acid.

Bromelin.

This is the proteolytic enzyme of the Pine-Apple (*Ananas sativus*, Schult.). We owe our knowledge of its properties mainly to the researches of Chittenden (3), who ascertained that it causes the formation of leucin and tyrosin; it is therefore a 'tryptic' ferment, a conclusion confirmed by the fact, to which I have already called attention, that tryptophane is another product of its activity. By means of the weighing method, to which I have alluded, Chittenden found that bromelin is most active in neutral liquids, 'but that the presence of small amounts of acid, especially such as are contained in Pine-Apple juice, and of sodium carbonate (·25 %), interferes with the proteolytic action only slightly.' It is also a very active enzyme, the most active that I have yet met with in plants. Chittenden estimates that, in a neutral liquid, it digested in 2 hours over 20 % per cent. of the albumin supplied, and I have detected the tryptophane-reaction in a bromelin-digestion, whether of fibrin or of Witte-peptone, in 2 hours, in almost all cases.

My results indicate that bromelin is an enzyme adapted

for action in an acid medium; nor is this surprising in view of the fact that, according to Chittenden, the natural acidity of Pine-Apple juice is equal, on the average, to about 0·5 % HCl. The divergence between Chittenden's results and my own is probably due to his having used albumin, and I fibrin and Witte-peptone, as the digestible proteids; he himself found the acid juice to digest fibrin more rapidly than did the neutralized. I did not find that the addition of HCl, up to 0·2 %, to the already acid juice had any marked effect upon the tryptophane-reaction as compared with juice of natural acidity. On the other hand, I found neutralized juice to be distinctly less active than the natural acid juice; and further, that alkalinity, though it did not altogether inhibit, very much diminished the activity of the enzyme.

The following example illustrates the general method and results of the experiments.

The expressed juice is strongly acid, and gives a weak tryptophane-reaction. The amount of liquid in each of the bottles was 50 cc.; the duration of the digestion was 4 hours.

A. Natural acidity:

(1) no proteid added (auto-digestion); faint tryptophane-reaction.

(2) 1 grm. fibrin added: marked reaction.

(3) 1 grm. Witte-peptone added: strong reaction.

B. Neutralized juice:

(4) 1 grm. fibrin added: distinct reaction.

(5) 1 grm. Witte-peptone added: marked reaction.

C. HCl added to 0·2 %:

(6) 1 grm. fibrin added: marked reaction.

(7) 1 grm. Witte-peptone added: strong reaction.

D. Alkaline (0·5 % Na_2CO_3):

(8) 1 grm. fibrin added: faint reaction.

(9) 1 grm. Witte-peptone added: weak reaction.

Next morning, after nearly 24 hours' digestion, Nos. 4, 5, 8, and 9 were distinctly acid, and gave more or less strong tryptophane-reaction. I obtained this result also in other experiments, in which HCN was used as an antiseptic.

Papaïn.

This is the enzyme of the Papaw (*Carica Papaya*, L.), and is obtained from the latex and more especially from the fruit:

for our knowledge of its properties we are chiefly indebted to the researches of Martin, who experimented, not with the enzyme, but with the dried juice. In his first series of observations (4), Martin established the 'tryptic' character of the enzyme by finding leucin, and traces of tyrosin among the products of digestion, coagulated egg-albumin being the proteid supplied when quantitative estimations were made: in the second series of observations (4 *a*), in which the enzyme digested the proteids of its own juice (globulin, albumose, albumin), leucin was found and the presence of tyrosin more definitely ascertained. He concludes that papaïn is active in neutral liquid; more active in the presence of 0·25 % of Na_2CO_3, but less active with larger amounts of the alkaline salt up to 1 %; and that it acts slightly in liquid containing 0·05 % HCl, but not at all with a higher percentage. My own results tend to show that papaïn is more adapted, than Martin found, to an acid medium, and, more especially, that it can act powerfully in a liquid containing ·2 % of HCl: but Martin's results were obtained with albumin, mine with Witte-peptone and fibrin.

The following are some of the experiments upon which my conclusions are based.

Each of four bottles contained 1 grm. of Witte-peptone, and ·5 grm. of 'pure papaïn' (Christy), which gives a neutral watery solution.

Bottle No. 1 contained, in addition, 50 cc. of distilled water.

„ No. 2 „ „ 50 cc. of 0·2 % HCl solution.

„ No. 3 „ „ 50 cc. of 0·5 % citric acid solution.

„ No. 4 „ „ 50 cc. of 0·5 % Na_2Co_3 solution.

To each were added 5 drops of a 4 % solution of HCN.

After $3\frac{1}{2}$ hours in the incubator, the only bottle which showed any tryptophane-reaction was No. 3, where it was weak but distinct.

After 22 hours in the incubator, bottles 2 and 3 gave a strong reaction, whilst bottles 1 and 4 gave a weak reaction.

A similar series of experiments was made with fibrin as the digestible proteid.

Four bottles were taken, as before, each containing 2 grms. of fibrin, and ·5 grm. of pure papaïn.

Bottle No. 1 contained, in addition, 50 cc. of distilled water.
„ No. 2 „ „ 50 cc. of 0·2 % HCl solution.
„ No. 3 „ „ 50 cc. of 0·5 % citric acid solution.
„ No. 4 „ „ 50 cc. of 0·5 % Na_2CO_3 solution.

After 23 hours in the incubator, the strongest reaction was given by No. 3 : next in order came Nos. 1, 2, 4, the reaction in No. 4 being very faint.

I had noticed in experiments with other enzymes, that the presence of a considerable proportion of HCN in acid digestions promoted the enzymotic action to a marked degree. I found this to be the case with papaïn. Comparing digestions, as above, of both Witte-peptone and fibrin (Nos. 2) in 0·2 % HCl alone, with others exactly similar which contained HCN to the extent of 0·2 % in addition, the tryptophane-reaction was much stronger in the latter than in the former. In fact the reactions in the mixtures containing 0·2 % HCN were more marked than any of the others. This is not the case when HCN is the only acid present.

Cradeïn of the Fig (*Ficus Carica*, L.).

The presence of a proteolytic enzyme in the latex of this plant was discovered by Bouchut (5) : but its properties were first investigated by Hansen (6), who found that the latex causes the solution of fibrin, in liquid containing 0·2 % HCl, almost as rapidly as does pepsin ; and also in a liquid containing 2 % of Na_2CO_3, but much less rapidly. The possibility that the enzyme might have 'tryptic' action was present to Hansen ; for he applied the tryptophane-test to the alkaline digestion, and also sought for tyrosin among the products, but with negative results. It is not clear whether or not he similarly investigated the acid digestion. Subsequently the properties of the latex and of the juice of the fruit were studied by Mussi (7). He obtained from the liquid a precipitate, on treatment with alcohol, which was insoluble in water but soluble in the presence of a trace of acid or alkali, the solution having digestive power. He gave the name *cradina* (cradeïn) to the precipitate which consisted, in part at any rate, of the proteolytic enzyme, and confirmed Hansen's statement that it is active in alkaline and in acid (HCl) liquids,

but not in neutral: he does not appear to have investigated the digestive products.

As my experiments have been carried on during the autumn and winter, I have not been able to do much with the latex or with the juice of fresh fruit. I had just time to make one experiment, last October, with a watery extract of the already withering leaves. A mixture, consisting of 50 cc. of the neutral extract, 1 grm. of Witte-peptone, and 0·1 grm. of citric acid, gave a good tryptophane-reaction after 20 hours' digestion. In November, I experimented with the juice of some fresh fruit, and obtained the tryptophane-reaction by the digestion of fibrin: but the material was not satisfactory, and there was the difficulty that the Fig-juice was of a red colour to begin with, so that the tryptophane-reaction could not be readily distinguished. I therefore turned my attention to dried Figs, and obtained satisfactory results with a watery extract. The acid watery extract, digested by itself for 24 hours, gave no tryptophane-reaction; whereas, when either fibrin or Witte-peptone was digested with it, the reaction was marked.

The results that I have obtained with a watery extract of dried Figs do not confirm the conclusions of Hansen and of Mussi that cradeïn is especially active in acid liquids containing HCl, and in alkaline liquids containing Na_2CO_3. On the evidence of the tryptophane-reaction, which I regard as indicating whether or not digestion by the enzyme has taken place, I find that cradeïn is most active in naturally ac id liquids; less active in acid liquids containing HCl; and least active in neutral and alkaline liquids. In some cases I observed that the neutralized and alkaline liquids became strongly acid when the digestion was prolonged to 24 hours; and in such cases the tryptophane-reaction was then more or less strongly marked. It is possible that the results ascribed by Hansen and by Mussi to alkaline liquids may have been due to this secondary development of acid.

The following will serve to illustrate the experiments.

Half a pound of dried figs were extracted with 500 cc. of cold distilled water, the mixture allowed to filter all night in a cold room.

The filtered liquid was slightly acid, and gave no tryptophane-reaction: 50 cc. of it were placed in each of 9 bottles, with various additions, as follows:

1. Extract alone.
2. ,, + 1 grm. fibrin.
3. ,, + 1 grm. Witte-peptone.
4. ,, + 0·1 cc. HCl + 1 grm. fibrin.
5. ,, + 0·1 cc. HCl + 1 grm. Witte-peptone.
6. Neutralized extract + ·25 grm. Na_2CO_3 + 1 grm. fibrin.
7. ,, ,, ,, + 1 grm. Witte-peptone.
8. ,, ,, + 1 grm. fibrin.
9. ,, ,, + 1 grm. Witte-peptone.

To each bottle were added 5 drops of 4 % HCN; the bottles were placed in the incubator at 10 a.m.

After 24 hours' digestion No. 3 gave a marked tryptophane-reaction, Nos. 2 and 5 a weak one; the others gave no reaction; five hours later the result was essentially the same; the experiment was then closed.

The Coco-Nut (*Cocos nucifera*, L.).

The so-called 'milk' is a watery, slightly turbid liquid, which gives weak xanthoproteic and tryptophane-reactions: it is slightly acid, and on the addition of alkali there is a copious precipitate of phosphates. It may be added that the milk gives a strong peroxidase-reaction with H_2O_2 and tincture of guaiacum, and that this property is destroyed by boiling.

I have found the milk to be feebly proteolytic: the enzyme is more active in acid than in alkaline liquids. Its activity would no doubt be greater were the seed germinated.

The following experiment gives a general idea of the proteolytic activity of the milk. In each case 50 cc. of milk were taken and 5 drops of 4 % HCN added: to each, except No. 5, 0·5 grm. of Witte-peptone was added.

1. Nothing further added.
2. Added 0·1 cc. HCl.
3. Neutralized and added 0·25 grm. Na_2CO_3.
4. Neutralized.
5. Milk only.

After 19 hours' digestion in the incubator, the result was—
Marked tryptophane-reaction in 1 and 2.
Distinct „ „ in 3.
Faint „ „ in 4 and 5:
3 and 4 had not become acid.

Germinating Seeds.

There is a considerable literature upon the subject of the proteolytic enzymes of germinating seeds, but it is not necessary to follow it further back than the publication of Green's researches (8) on *Lupinus hirsutus*, L. He obtained from the cotyledons of seedlings four days old, a glycerine-extract which digested fibrin in the presence of 0·2 % HCl, and found leucin and tyrosin to be digestive products. He also obtained (8 *a*) these results with germinating seeds of the Castor-Oil plant (*Ricinus communis*, L.). Neumeister (9) subsequently detected a proteolytic enzyme in the seedlings of barley, poppy, wheat, maize, and rape, and ascertained that it is active only in acid liquids; but the acid present must be organic, not mineral: in this respect his results differ from those of Green, a difference which may perhaps be due to the fact that the plants experimented upon were not the same. Still more recently the matter has been investigated by Butkewitsch (10), the seeds used being those of *Lupinus angustifolius*, L., *Lupinus luteus*, L., *Vicia Faba*, L., and *Ricinus major* (?), with results that on the whole confirm those of Green. He found that, on adding water to the crushed seeds, the naturally acid liquid readily digested the proteids of the seeds; and that this auto-digestion was less rapid when the liquid was rendered alkaline to the extent of 0·1 % NaHO, or acid to the extent of 0·2 % HCl, but more rapid in the presence of 0·1 % HCN.

Experiments with Barley (*Hordeum vulgare*, L.).

Whether or not germinated barley contains a proteolytic enzyme is still a debated question: some of the more recent observations upon it are quoted in Butkewitsch's paper (10). On the whole, the balance of evidence seems to be in favour of

the existence of such an enzyme, and it is generally alluded to in books relating to the chemistry of brewing under the name of 'peptase,' a name which should now be altered.

My own observations enable me to assert that the enzyme is undoubtedly present, and that it is remarkably vigorous. The experiments were conducted with 'green malt'; that is, barley which had germinated on the malting-floor for 11 days, but had not been dried in the kiln.

An extract was prepared by pounding in a mortar about 200 grms. of the barley with 350 cc. of distilled water to which 2 cc. of chloroform had been added; the thick liquid was set to filter all night in a cold room. On the following morning the filtered liquid was found to have a slight acid reaction, and to give distinct evidence of the presence of tryptophane.

About 100 cc. of the liquid were placed in each of three bottles 1, 2, 3: to (1) nothing was added, except 10 drops of 4 % HCN as an antiseptic; to (2), besides 10 drops of HCN, 0·2 cc. HCl was added; (3) was neutralized, and to it were added 10 drops of HCN, and 0·5 grm. of Na_2CO_3. The three bottles were placed in the incubator at 10 a.m. In two hours (1) and (3) gave a somewhat stronger tryptophane-reaction, (2) a weak one; 2 hours later, the results were much the same. 24 hours later (1) gave a strong reaction, (3) a less strong, and (2) still a weak one; it was observed that (3), which had been alkaline on the previous day, was now strongly acid, and that (1) had become more acid.

No proteid was added in any of these experiments, the reserve proteid of the grain providing the necessary material for digestion (auto-digestion).

From these observations it is clear that the enzyme acts strongly in liquid of natural acidity, and that its action is diminished and perhaps inhibited by the presence of 0·2 % HCl. With regard to the effect of an alkaline liquid, the result is less distinct: but it appears that some digestion occurred during the first day when the reaction was alkaline, whilst the stronger reaction on the second day may be attributable to the fact that the liquid had become acid.

I then proceeded to ascertain if tryptophane is present in kiln-dried pale malt; and, as might be expected, I found it.

The malt had been made from barley which had germinated for 11 days, and had been dried at about 90° C. An infusion of the malt with hot water gave a distinct tryptophane-reaction.

Experiments with *Vicia Faba*, L.

These have not yet been carried far. Some that I instituted on the principle of auto-digestion not having proved satisfactory, further observations were made on liquids to which Witte-peptone had been added. The seeds used had been soaked in water for some days in a warm room, and showed signs of germination. As the following record shows, the enzyme seems to act best in neutral or alkaline solutions, and to be inhibited by HCl.

145 grms. of germinated seeds ground to fine paste and extracted with 400 cc. distilled water; the turbid liquid, strained through muslin, is slightly acid, and gives a trace of tryptophane-reaction: 100 cc. placed in each of 4 bottles, with 1 cc. of 4 % HCN:—

1. Without any addition.
2. Neutralized.
3. HCl added to 0·2 %
4. Made alkaline to 1 % Na_2CO_3,

placed in incubator at 4 p.m.: next day at noon—

1. Gives faint tryptophane-reaction.
2. No reaction, is slightly acid.
3. No reaction.
4. No reaction, still alkaline.

As none gave a distinct tryptophane-reaction, I added 1 grm. of Witte-peptone to each; at 3 p.m. the results were—

1. Faint tryptophane-reaction.
2. Marked „
3. Faint reaction.
4. Distinct „

24 hours later, the results were—

1. Distinct reaction.
2. Strong „ (slightly acid).
3. Faint reaction.
4. Marked do. (still alkaline).

It will be observed that my results do not exactly agree with any of those to which I have alluded. The diversity of opinion which exists as to the conditions of proteolysis in germinating seeds is, no doubt, due to the great differences in chemical composition presented by the seeds of the various families of plants. Moreover the experimental difficulties are considerable: it is in many cases almost impossible to obtain clear solutions to test; then there is the re-development of

acid after neutralization to be taken into account ; and finally, seed-extracts are very liable to putrefy. The whole subject requires systematic re-investigation.

YEAST (*Saccharomyces Cerevisiae*, Meyen).

It is a well-known fact that if Yeast be starved by being kept in a liquid which contains no food-materials, the proteids of the cells undergo digestion, and that this auto-digestion is attributable to a proteolytic enzyme. Thus Salkowski (11) has shown that if Yeast be kept in chloroform-water, the liquid eventually contains leucin and tyrosin which can only have been derived from its own proteids. Moreover the formation of these two substances can only be due to the action of an enzyme, since living Yeast-cells are inhibited from enzymotic action by chloroform. More recently Hahn and Geret (12) have detected the formation of leucin and tyrosin in the expressed juice of Yeast. With regard to the proteolytic action of Yeast upon proteid supplied from without, Hahn (13) has ascertained that Yeast decomposes gelatine in the presence of chloroform.

My earlier experiments were merely tentative. In the first instance, I added 5 grms. fresh Yeast, which had been well washed on a filter, to about 100 cc. of three different liquids each containing 1 grm. of Witte-peptone, with the result that, after 24 hours' digestion, I obtained a marked tryptophane-reaction where the liquid was only distilled water, a weaker reaction where the liquid was 0·2 % HCl solution, and no reaction where the liquid was 0·2 % HCN. In a second series of experiments, I ground up fresh Yeast in a mortar with powdered glass and water, and made use of the turbid, faintly acid filtrate, thymol being the antiseptic. In a digestion of 18 hours of a mixture of 20 cc. of the Yeast-extract with 30 cc. distilled water, I obtained a weak tryptophane-reaction when fibrin had been digested, and a strong reaction when 1 grm. Witte-peptone had been digested : in the presence of 0·1 % citric acid, a fibrin-digestion gave no reaction, and a Witte-peptone-digestion, only a weak one. More recently I have used dried Yeast, prepared by drying fresh brewer's-

yeast until it became brittle enough to grind in a mill: 20 grms. of this fine powder were extracted with 400 cc. distilled water for some time, and then thrown on a filter: the whole process of extraction and filtration lasted about 4 hours: the filtered liquid was slightly turbid, distinctly acid, and gave no tryptophane-reaction.

The result of these experiments is to prove the existence in Yeast of a proteolytic enzyme which is active in neutral and in acid liquids, but not in alkaline.

The following mixtures were prepared: each bottle (except No. 1) contained extract of dried yeast diluted with an equal bulk of distilled water, 50 cc. in all; to each 5 drops of 4 % HCN were added.

1. contained 50 cc. undiluted yeast-extract.
2 *a*. added 1 grm. fibrin.
b. „ „ Witte-peptone.
3 *a*. „ „ fibrin + 0·1 cc. HCl (=0·2 %).
b. „ „ Witte-peptone „
4 *a*. „ „ fibrin, neutralized.
b. „ „ Witte-peptone, neutralized.
5 *a*. „ „ fibrin, neutralized, + ·25 grm, Na_2CO_3.
b. „ „ Witte-peptone, neutralized, „

placed in the incubator at 4 p.m.

Next morning at 10 a.m. the results were—

1. weak tryptophane-reaction.

2 *a*. distinct	„	4 *a*. marked reaction.	} now slightly		
b. marked	„	*b*. strong	„	}	acid.
3 *a*. faint	„	5 *a*. none	„	} both	
b. marked	„	*b*. faint	„	}	alkaline.

In a repetition of the experiment with 4 *a* and 4 *b*, I satisfied myself that the striking reaction is given even when the liquid remains quite neutral throughout.

Bacteria of Putrefaction.

The fact that putrefying proteids undergo a decomposition somewhat analogous to that effected by trypsin, but more far-reaching, has long been known. The formation of tryptophane in putrefaction seems to have been first recorded by Claude Bernard (14), as also the fact that continued putrefaction causes the disappearance of this substance.

It is equally well known that the putrefaction of proteid is due to the action of Bacteria: and although it has not yet been isolated, there is evidence that the immediate agent in the process is a proteolytic enzyme.

My observations on putrefaction were undertaken not so much in the expectation of discovering new facts of a fundamental nature, as with the object of ascertaining whether or not tryptophane is formed under all the various conditions of experiment employed in the other investigations, and of determining the time of its appearance in each case: with the object, that is, of instituting a series of control-observations by which the possibility or probability of Bacterial intervention in the other cases might be checked.

The following series afford an idea of the general plan of these experiments and of their results.

Acid liquids: (1) 0·2 % HCl.

2 bottles, each containing 100 cc. of 0·2 % HCl: to the (*a*) one were added 2 grms. of fibrin, to the other (*b*) 1 grm. Witte-peptone.

(*a*) gave no tryptophane-reaction after 10 days in the incubator, nor was there any sign of solution or putrescence:

(*b*) no tryptophane-reaction until the 10th day; on the 5th day Moulds made their appearance (*Penicillium*), and the faint reaction on the 10th day is probably due rather to them than to Bacteria. No offensive odour.

(2) 0·5 % citric acid.

2 bottles each contained 50 cc. of this solution: to the one (*a*) was added 1 grm. of fibrin, to the other (*b*) 1 grm. of Witte-peptone.

(*a*) no tryptophane-reaction after 7 days in the incubator, nor any sign of putresence.

(*b*) no reaction until the 5th day, when Mould had abundantly developed; the faint reaction then given was doubtless due to this cause.

Alkaline liquid: 0·5 % Na_2CO_3.

2 bottles each contained 50 cc. of this solution: to the one (*a*) was added 1 grm. of fibrin, to the other (*b*) 1 grm. of Witte-peptone.

(*a*) distinct tryptophane-reaction on the 7th day; putrescent odour on the 3rd day.

(*b*) distinct reaction and putrescent odour on the 2nd day.

Neutral liquid: tap-water.

2 bottles each contained 50 cc. of water: to the one (*a*) was added 1 grm. of fibrin, to the other (*b*) 1 grm. of Witte-peptone.

(*a*) putrescent odour on 3rd day, distinct tryptophane-reaction on the 8th day.

(*b*) putrescent odour and marked tryptophane-reaction on the 2nd day.

Whilst it is probable that the above results may not be of general application, since other proteids and other organisms might behave differently, yet they serve to illustrate the relation of putrefaction to the other experiments that I was carrying on under generally similar conditions. In the first place, the antiseptic influence of acid, even of such a weak acid as the citric, is clearly demonstrated. It is further shown that the putrefactive enzyme works actively, and about equally, in neutral and in alkaline liquids, and that it decomposes Witte-peptone with much greater facility than it does fibrin. From the experiments with fibrin in neutral and alkaline liquids, it would appear that putrescence, as indicated by the odour, and the formation of tryptophane are not necessarily simultaneous, and that the former may precede the latter. It might have been anticipated from the constitution of tryptophane, as determined by Hopkins and Cole (2), that the indol and skatol, to which the putrid odour is due, would be derivatives of tryptophane, and would only become perceptible when the presence of tryptophane could be detected. As this was not the case, it is probable that a portion only of the indol and skatol formed in putrefaction passes through the tryptophane stage.

In one of the neutral putrefactive digestions of Witte-peptone, a chemical fact presented itself that seems to be worth special mention. After 48 hours, when it gave a strong tryptophane-reaction, the liquid had a marked greenish-blue colouration, turning bright yellow on adding a drop of acetic acid. The colouration was no doubt due to the formation of some indol-derivative.

Pepsin.

In the paper (1) to which I have already alluded, I expressed the conviction that the vegetable enzymes therein referred to, and probably the proteolytic enzymes of all plants, are essentially 'tryptic' in their nature. I arrived at this conviction on the evidence of the tryptophane-reaction, and on the assumption, generally made, that the formation of tryptophane from proteids is characteristic of 'tryptic,' as distinguished from 'peptic,' digestion.

I felt, however, that it was necessary to test the validity of this assumption; to determine experimentally whether or not, as a matter of fact, the formation of tryptophane affords an absolutely reliable distinction between these two modes of proteolysis. I had not been able to find on record any statement to the effect that tryptophane had been found among the products of peptic digestion: but I was aware of the conflict of opinion between the school of Hoppe-Seyler which denied, and the school of Kühne which asserted, that the difference between peptic and tryptic digestion is absolute and fundamental. Lubavin was, I believe, the first to make this suggestion (15), and he did so in consequence of having found leucin and tyrosin among the products of prolonged peptic digestion (9–11 days). More recently Lawrow (16) has stated that he found leucin to be formed in a very prolonged gastric digestion (2 months); and Salaskin (17) and Zunz (18) both assert that crystallizable, nitrogenous, but non-proteid substances are formed under these circumstances [see also Langstein (19)]. There seemed, therefore, to be some probability that tryptophane might be produced in peptic digestion, a probability which has been realized in the investigation that I have made.

It is not necessary, however, that I should give a full account of my experiments; for, when they were complete, I happened to discover that their results had been anticipated; they are, therefore, merely of confirmatory interest. In a paper by Winternitz (20), on the chemistry of the putrefaction

of milk, it is stated in a foot-note, that the author had obtained the tryptophane-reaction in a 7-hours' digestion of fibrin with extract of pig's stomach. It is not surprising that under such conditions of publication, this important discovery should have remained comparatively unnoticed. Malfatti (21) has also obtained tryptophane in neutral, acid, and weak alkaline digestions of Witte-peptone with pepsin: he ascribes the formation of tryptophane in the neutral and alkaline digestions to the presence of trypsin. Further, Glaessner (22) finds that in auto-digestion of gastric mucous membrane (3–4 weeks) in a weak alkaline ($Na_2 Co_3$) liquid, tryptophane is formed. From this he infers that two enzymes are secreted by the stomach; viz. true pepsin, and what he terms 'pseudo-pepsin,' an enzyme which works in alkaline liquids that would destroy pepsin and in acid liquids that would destroy trypsin, and which, unlike true pepsin, forms tryptophane. On this hypothesis he accounts for the many discordant conclusions that have been arrived at as to the nature of the ultimate products of gastric digestion.

Although the position of this question has been thus modified since I began my observations upon pepsin, it is, I think, still worth while to place on record some of the experiments in which I succeeded in detecting tryptophane. The pepsin used was that sold as 'pepsin, pure scales': but I also obtained satisfactory results with glycerin-extract of pig's stomach.

Jan. 24, 50 cc. of solution of 0·2 % HCl, containing 0·5 grm. of pepsin, were placed in each of two bottles (1) and (2):

(1) to this was added 1 grm. of moist fibrin,
(2) to this was added 0·5 grm. of Witte-peptone,
} placed in incubator at 4 p.m.

Jan. 25, 11 a.m., no tryptophane in either: added 0·5 grm. of pepsin to each; also 1 grm. of fibrin to (1), and 1 grm. of Witte-peptone to (2).

4 p.m., faint tryptophane-reaction in both: added more fibrin to (1), and more Witte-peptone to (2).

Jan. 26, 10 a.m., both gave faint tryptophane-reaction: filled up both bottles with 0·2 % HCl solution.

Jan. 27, 11 a.m., tryptophane-reaction marked in (1), strong in (2).

No antiseptic was used; but in view of the results obtained as to the inhibiting action of 0·2 % HCl on Bacteria (p. 15), the result cannot be attributed to putrefaction.

I may mention that in many experiments I used thymol as an antiseptic, but these were generally unsuccessful. It appeared that thymol arrested the action of the pepsin. I therefore had recourse to hydrocyanic acid (HCN). The following experiment shows that this weak acid promotes peptic digestion, when present in sufficient quantity.

Jan. 22. 50 cc. of 0·2 % HCl, containing 0·5 grm. pepsin and 2·5 cc. of 4 % HCN (= 0·2 %), were placed in each of two bottles (1) and (2):

(1) to this were added 2 grms. moist fibrin, } placed in incu-
(2) to this was added 1 grm. Witte-peptone, } bator at noon.

Jan. 23. distinct tryptophane-reaction in both.

Jan. 24. strong reaction in both.

The tryptophane-reaction was obtained more rapidly in this than in any other of the experiments.

I have not had time to repeat my experiments in the light of Glaessner's (22) hypothesis. But if an enzyme having the properties of his 'pseudo-pepsin' were present in the pepsin that I used, it is clear that this pepsin should produce tryptophane in an alkaline liquid: this, however, I have not found to be the case.

Conclusions.

The additional instances that I have now given of the production of tryptophane, selected as they are from various classes and from different parts of plants, bear out my previously expressed opinion that the proteolytic enzymes of plants in general are essentially 'tryptic.' This statement will at any rate hold good until definite evidence is adduced to prove the existence of a 'peptic' enzyme. Moreover, these enzymes, with the exception of that of putrefactive Bacteria, are all active in acid media.

In view of the results obtained as to the action of pepsin, the question at issue has become a broader one. At first it related merely to the nature of the vegetable enzymes, the

object being to ascertain whether they were 'tryptic' or 'peptic.' But, as I have already pointed out, there is a considerable body of accumulated facts tending to show that pepsin itself is capable of effecting 'tryptic' proteolysis. Hence the question now is whether or not such a thing as a 'peptic' enzyme exists at all; an enzyme, that is, which only hydrolyses the higher proteids to peptones, and does not decompose the proteid molecule. The ascertained facts seem to answer this question in the negative: but in view of Glaessner's (22) contention that the apparently 'tryptic' action of pepsin is due, not to pepsin itself, but to another enzyme, his 'pseudo-pepsin,' this answer cannot at present be definitively accepted. The *onus probandi* remains, however, with those who accept Glaessner's interpretation of the facts, and assume the secretion by the stomach of an enzyme which combines in so singular a manner the properties of pepsin with those of trypsin. In the mean time, the terms 'peptic' and 'tryptic' must be used with the reservation that they may refer, not, as hitherto, to what seemed to be fundamentally different processes of digestion, but to different modes of one and the same process. If it should turn out that they are inapplicable in their old sense, they may still be used to indicate the marked differences which exist between pepsin-digestion and trypsin-digestion. Even if pepsin be finally proved to be 'tryptic' in action, it will still be necessary to employ some terms to express the fact that it acts much more slowly than does trypsin, and, as far as it is possible to institute a quantitative estimate, that it produces tryptophane and other ultimate products in relatively much smaller quantity. Moreover the two enzymes differ materially as to the medium in which they respectively act: the one acts, whilst the other does not, in a HCl solution; and conversely, the one acts in an alkaline liquid whilst the other does not.

In respect of the reaction of the medium, the vegetable enzymes may be taken to show affinity with pepsin on the one hand and trypsin on the other. The results described in the foregoing pages may be conveniently summarized in the

form of a provisional arrangement of the enzymes from this point of view, based upon the tryptophane-reaction.

I. *Active in Acid liquid*:
  1. active *only* in acid liquid:
    (*a*) most active with HCl: Pepsin.
    (*b*) active with HCl or natural acid: Nepenthin.
  2. more active in acid than in neutral or alkaline liquid:
    (*a*) equally active with HCl or natural acid: Bromelin: Coco.
    (*b*) more active with natural or organic acid: Papaïn; Cradeïn: 'Peptase' of Barley.

II. *Active in Neutral or Acid liquid*:
  enzyme of Yeast.

III. *Active in Neutral or Alkaline liquid*:
  (*a*) active in either: enzyme of Bean (?): putrefactive Bacteria.
  (*b*) more active in alkaline: Trypsin.

In conclusion, I would add a few words regarding the observation that hydrocyanic acid promotes proteolysis in certain cases. I regard this as a matter of some importance, as it may, if followed up, throw light upon the physiological significance of this acid in plants. Its general occurrence in certain families has long been known; and it was assumed, by a too facile oecology, that its importance lay in the protection which it was assumed to afford, by reason of its poisonous properties, against the depredations of animals. It is only recently that the matter has been seriously investigated. As the result of experiments, extending over several years, made upon *Pangium edule*, Reinw., a tropical tree which contains it in all its parts, Treub (23) has come to the conclusion that hydrocyanic acid is an early product in the nitrogenous anabolism of the plant. The presence of the acid in many germinating seeds, such as the Almond, the Peach, &c., is quite consistent with this view, for it may serve as nitrogenous plastic material for the growing embryo. At the same time the observations that I have made suggest that the acid may also be of importance in facilitating the proteolysis of the reserve-materials of the seed, a suggestion which I propose to test experimentally.

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