Proteolytic Enzymes in Plants (II).

BY

S. H. VINES,

Sherardian Professor of Botany in the University of Oxford.

IN the January number of the present volume (1) I published some observations tending to show that proteolytic enzymes are of very general occurrence in plants. Whilst it had previously been implicitly assumed by physiologists that this was probably the case, the first experimental demonstration of the fact was, I believed, that contained in my paper. It turns out, however, that I was mistaken. My attention has since been directed to a paper by Buscalioni and Fermi (2), published in 1898, which somewhat anticipates my results: but though our conclusions are concordant on the whole, our methods were widely different. The method of Buscalioni and Fermi is an adaptation of the gelatine-culture of Bacteria. A layer of gelatine, with carbolic acid (·5–1 %) as the antiseptic, covers the floor of a Petri-dish, and upon this are placed the objects (seeds, portions of leaves, &c.) whose proteolytic action is to be determined; the test being, of course, the liquefaction of the gelatine. By this simple method the authors were able to detect more or less marked proteolytic activity in many Fungi, but by no means in all those tried; in some Algae (*Codium tomentosum*, *Padina Pavonia*, *Chara* sp., *Dictyota dichotoma*, *Ceramium* sp.); and in some Lichens: but the experiments with a Moss (*Funaria hygrometrica*), a Liverwort (*Lunularia vul-*

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garis), with *Equisetum* sp. (rhizome), and with the leaves and rhizomes of various Ferns, all gave negative results.

Turning to the Phanerogams, the authors give first their results with laticiferous and resinous plants, and these are rather conflicting. They found no digestive action in the liquids of the various Fumariaceae (*Corydalis lutea*), Papaveraceae (*Papaver somniferum*, *P. Rhoeas*, *Argemone mexicana*), and Compositae (*Crepis setosa*, *Sonchus tenerrimus*, *Taraxacum officinale*, *Lactuca sativa*) that they investigated. On the other hand, positive results were obtained with all the Urticales tested, viz. *Ficus Carica* and *F. elastica*, *Morus alba* and *tatarica*, *Broussonetia papyrifera*, and *Maclura aurantiaca*. In the remaining orders, some species were, whilst others were not, found capable of liquefying the gelatine ; for instance, in the Euphorbiaceae, *Euphorbia Lathyris*, *E. Tirucalli*, *E. canariensis*, *E. balsamifera*, *E. coerulescens*, *E. grandidens*, and *Poinsettia pulcherrima* were active, whilst *Euphorbia tigridis* and *dulcis*, *Homalanthus populifolius*, and species of *Croton* were not: similarly in the Convolvulaceae, *Convolvulus sylvaticus* and *Calonyction* (*Ipomoea*) *macrantholeucum* were active, but not *Convolvulus arvensis* and species of *Ipomoea*: in the Asclepiadaceae, *Tweedia neerifolia* and *Asclepias curassavica* were found to be active, but not *Hoya carnosa*: in the Apocynaceae, *Tanghinia venenifera* and *Plumeria alba* were active, but not *Vinca minor*, *Acokanthera spectabilis*, nor *Echites flavescens*. It is interesting to note that the resin of *Pinus halepensis* showed some activity, if only weak, as did also the secretion of *Nelumbium speciosum*.

Of juices expressed from the plant, but few were active, namely those obtained from the young leaves of *Agave mexicana* and *A. americana*, from the young stems of *Phytolacca dioica*, from the stems of *Anagallis arvensis*, from the apices of the shoots of *Glycine sinensis*. Amongst the considerable number of inactive juices were those obtained from old leaves of *Agave mexicana*, and from adult branches of *Phytolacca dioica*; hence it would appear that young tissues are more likely than old ones to contain the protease.

The action of sections of stems and leaves was next investigated: and here again the number of positive results is much smaller than that of negative. Out of a list including about fifty species, only a few were markedly active; namely, sections of the leaf of *Dyckia princeps*, of young shoots of *Phytolacca dioica*, of *P. abyssinica*, and of *Portulaca oleracea*. In the case of *Phytolacca abyssinica*, it is specially noted that sections of young tissues acted much more powerfully than those of older parts.

In the case of roots, those which proved to be active were about equal in number (28) to those that failed to act, though in many cases the activity was slight. The most active roots were those of *Amorphophallus Rivieri*, of *Aspidistra elatior*, and of an undetermined Bromeliad. The authors contrast the more general distribution of the enzyme in the roots with its more restricted distribution in the green parts, where its presence seems to be especially associated with rapid growth. At the same time they find reason for doubting if the penetration of the tissues of the parent member by endogenously developing roots is due in any degree to the action of the proteolytic enzyme; in fact they assert that the 'poche digestive' of van Tieghem has no significance so far as the solution of proteids is concerned. In this connexion they mention that their researches were carried on in the month of July, when conditions were most favourable for ferment action.

The number of bulbs, tubers, and tuberous roots examined was but small, only twelve, and the positive and negative results were equally divided. The tubers of *Tamus communis* and of *Dioscorea bulbifera*, as also the tubercular roots (containing *Anabaena*) of *Cycas revoluta* were found to be most active, whilst the root-tubercles of the Leguminosae acted but feebly. The bulbs examined were those of *Allium sativum*, *Cepa*, and *Porrum*, but they were not found to be active; nor were the tuberous roots of *Beta* and of *Dahlia*. From these facts the conclusion, which seems to me to be hardly justified by the facts, is drawn that of these organs those that are

modified roots are more generally active than those that are modified shoots: for of the three cases in which vigorous liquefaction was observed, one only (*Cycas*) is a true root, whilst the organs of the other two are of cauline origin; this is certainly true of the tuber of *Tamus communis*, and I believe it is also true of *Dioscorea bulbifera*.

Next come the experiments with flowers and fruits, of which about sixty are recorded. Of the various parts of the flower, the stamens and the pollen proved to be by far the most active. Only three experiments were made with pollen (*Hedychium maximum*, *Hibiscus speciosus*, *Cucurbita maxima*), and in all three liquefaction was marked. A considerable number of fruits was tested, among others the Grape, the Orange (epicarp), the Lemon (unripe), the Red Currant, the Peach, the Apricot, the Cherry, and the Strawberry, but with invariably negative result.

These are followed by the experiments with seeds. A few unripe seeds were investigated, and of these only a small number gave positive results, one of them being *Phaseolus multiflorus*. Among a number (22) of ripe seeds, those of *Sorghum cernuum*, *Cannabis sativa*, *Linum usitatissimum* (especially the seed-coat), and *Anagallis arvensis*, were found to be active. Contrary to what might have been anticipated, the authors found that many germinating seeds, whether albuminous or exalbuminous, were quite inactive.

Some observations were also made on parasitic Phanerogams. The presence of a protease was indicated only in the haustoria of *Orobanche Hederae* and of *Cuscuta*, the other parts of these plants apparently containing none of it: nor was any trace of it detected in *Viscum album* or in *Loranthus europaeus*.

Finally, attention was directed to 'insectivorous plants.' The results obtained with *Drosera* were sometimes positive, sometimes negative. The investigation of *Nepenthes* was effected by placing pieces of the pitcher upon the gelatine, some having the inner and some the outer surface in contact with it: in the former case the gelatine was liquefied, but not

in the latter. The liquefying action of both *Utricularia* and *Aldrovanda* was but slight, as was also that of *Sarracenia purpurea.*

The memoir concludes with some general considerations as to the action of heat and light upon the enzyme, and as to the influence of the reaction of the medium upon its activity. With regard to the latter point, the general conclusion arrived at is that in the large majority of cases the presence of acid increases the activity of the enzyme, the presence of alkali diminishes it. The acids employed were chiefly organic, the citric, tartaric, and oxalic, in 1 % solutions: in a few instances 1 % HCl was used, and was found to promote liquefaction by *Ficus* and *Phytolacca abyssinica*, but more frequently its effect was unfavourable. In only one case, that of *Tuber aestivum*, was liquefaction limited to an alkaline medium (3 % Na_2CO_3). In certain others, however, such as the style and stigma of *Hibiscus speciosus*, the latex of *Ficus Carica*, and the unripe seeds of *Phaseolus multiflorus*, experiments of 24 hours' duration in the alkaline medium showed vigorous liquefaction. It is not impossible that these results may have been due to Bacteria; the authors themselves do not seem to attach importance to them.

I have thought it necessary to give this rather full account of the researches of Buscalioni and Fermi, because their work is not, I believe, as well known as it deserves to be, at least among English botanists; and also because a certain amount of detail is necessary for the discussion of the relation of their results to those that I have obtained by an altogether different method. I am glad to find that our conclusions are in agreement so far as general principles are concerned. The demonstration of the wide distribution of a proteolytic enzyme in the plant-body, is the outcome of their experiments as of my own. There is, not unnaturally, some divergence in matters of detail. For instance, they found such laticiferous Composites as the Lettuce and the Dandelion to be inactive, whereas I found them to be active, and I have since found the leaves of the Endive to be active. The same divergence

exists with regard to the Beet-root, and to the epicarp of the Orange. These divergences probably depend to some extent upon seasonal differences in the material examined, upon the higher temperature which I employed, and perhaps to an even greater extent upon the antiseptics used in the different experiments. Buscalioni and Fermi used exclusively carbolic acid; whereas I have never done so, but have used chiefly hydrocyanic acid or chloroform-water. In subsequent pages of this paper, I propose to consider the relation of various antiseptics to proteid-digestion in some detail.

Further, we are in agreement in the general conclusion that the vegetable proteases are most active in an acid medium. But as regards the products formed in digestion we necessarily part company: for it was not possible by the gelatine-method to obtain the information afforded by the tryptophane-method. Buscalioni and Fermi mention, indeed, that in certain cases they obtained the biuret-reaction, indicating the presence of albumoses or peptones, in the liquefied gelatine, but they did not attempt to pursue the subject further. The advantage of the tryptophane-method adopted by me, is that it throws light upon this fundamental question, and that any kind of proteid matter can be subjected to experiment. Whilst the gelatine-experiments of Buscalioni and Fermi were the first to indicate the wide distribution of proteases in plants, my tryptophane-experiments demonstrated that these widely-diffused substances are completely proteolytic in action, so far as they have been investigated.

Proteases and Antiseptics.

I have already suggested that such divergences as exist between the observations of Buscalioni and Fermi and my own are probably due, at any rate to some extent, to the fact that we respectively made use of different antiseptics. This suggestion is based on results that I have obtained tending to show that the same protease is affected differently by different antiseptics, as also that different proteases are diversely affected by the same antiseptic. In the following paragraphs

I give an account of my experiments in this direction, so far as they have gone.

In March, 1902, I published in this periodical a paper (3) which dealt, among other topics, with the digestive properties of papaïn. I there adduced evidence to prove that this protease proteolyses fibrin and Witte-peptone, and is more active in acid than in neutral or alkaline liquids, as indicated by the tryptophane-reaction. As regards its proteolytic action, my results confirmed those of Martin (4), who had found leucin and tyrosin among the products of digestion. After my MS. had left my hands, I received a paper on the subject by Mendel and Underhill (5), which contains observations apparently disproving the proteolytic activity of papaïn, and suggesting that it can only peptonize the higher proteids. This was followed, after an interval of several months, by a second paper (6), which, without adducing fresh experimental evidence, restates the conclusions of the previous paper, criticizing also the view, to which I have more than once given expression, that all known vegetable proteases decompose the proteid molecule into leucin, tyrosin, tryptophane, &c., that is, are completely proteolytic.

The facts upon which Mendel and Underhill rely, are that in over sixty trials made with four different samples of papaïn, and with casein, fibrin, coagulated egg-albumin, and boiled muscle-tissue as the material to be digested, they failed to detect leucin, tyrosin, or tryptophane. Only with fresh, unboiled muscle were these products obtained, a result that these authors attribute to the self-digestion (autolysis) of the tissue. In all the experiments, sodium fluoride (NaF 1 %) was the antiseptic employed. On this evidence they conclude that papaïn is an enzyme differing from both pepsin and trypsin. 'While the products of the papaïn digestion of proteids resemble quite closely those of pepsin, . . . the enzyme differs from animal pepsin in that it acts readily in both neutral and alkaline media. On the other hand, although papaïn is comparable with trypsin in exerting a solvent action in fluids of various reactions, the failure to form leucin, tyrosin,

or tryptophane in appreciable quantities—at least under conditions in which they are readily formed in large quantities by the other tryptic enzymes—places it in a class of its own for the present.'

In endeavouring to account for the wide divergence between their conclusions and my own, I was at first inclined to question the activity of the papaïn employed by Mendel and Underhill; but the numerical results which they give show conclusively that a considerable amount of the proteid supplied (as much sometimes as 70 %) was dissolved, and the peptonization of casein was definitely proved. Hence there is evidence that the papaïn was active. This being so, the only remaining difference in the material of the two sets of experiments lay in the antiseptics employed, sodium fluoride in theirs, hydrocyanic acid in mine. I had already drawn attention to the fact that papaïn-digestion is promoted by HCN, and I thought it not improbable that this might prove to be an important factor in the problem. I accordingly instituted the following comparative experiments, in which NaF and HCN were the respective antiseptics, with results that fully realized my anticipation.

In the first instance I made use of Witte-peptone as the digestible material, and sodium fluoride (NaF), hydrocyanic acid (HCN), and chloroform-water as the antiseptics, the solutions being neutral, acid, or alkaline. The result proved that the proteolysis, as indicated by the tryptophane-reaction, was much more marked in the acid liquid containing HCN than in any of the others: it was less marked in the chloroform-water liquids, and scarcely perceptible in those containing NaF.

The details of the experiment were as follows: 5 grms. of papaïn ('purified papaïn,' Christy) were extracted for 3 hours with 250 cc. distilled water; the liquid was then filtered: the filtrate was a clear brownish liquid, distinctly acid, giving good biuret-reaction but no tryptophane-reaction. 10 grms. of Witte-peptone were similarly extracted with 250 cc. dist. water: on filtration a yellowish, neutral solution was obtained giving no tryptophane-reaction.

25 cc. of each of these solutions were then placed in each of 10 stoppered bottles: the contents of 3 of these were acidified by the addition to each of ·25 grm. of citric acid (= 0·5 %), the contents of other 3 made alkaline by the addition to each of 0·12 grm. of Na_2Co_3 (= ·25 %), whilst to the remaining 3 neither acid nor alkali was added: the contents of the last 3 were slightly acid, but they are distinguished below as 'neutral.'

To an acid, an alkaline, and a neutral bottle, 0·5 grm. of NaF was added (= 1 %): to a similar set, HCN was added to 0·2 %: to a third set of 3, chloroform was added to 0·5 %.

A control bottle contained 25 cc. of boiled papaïn-solution, with 25 cc. of Witte-peptone solution.

After 18 hours' digestion at 40° C., the tryptophane-reactions were as follows:—

	HCN.	*Chlorof.*	*NaF.*
Acid	very strong	marked	distinct
Alkaline	distinct	faint	faint
Neutral	distinct	faint	faint.

The control bottle gave a scarcely perceptible reaction.

These results indicate to how great an extent the activity of papaïn is affected by the antiseptic employed; and more especially that sodium fluoride exerts a strong inhibitory influence. Moreover, the advantage of an acid over an alkaline or a neutral medium is apparent.

In order to more definitely establish these conclusions, the experiment was repeated with fibrin instead of Witte-peptone: each bottle contained 1 grm. of fibrin, otherwise the contents were the same as in the preceding experiment, except that the percentage of Na_2CO_3 in the alkaline bottles was increased to 0·5 %. After eighteen hours' digestion, at 40° C., the results were:—

	HCN.	*Chlorof.*	*NaF.*
Acid	fibrin quite disintegrated marked tryptophane	scarcely attacked faint trypt.	distinctly attacked faint trypt.
Alkaline	fibrin quite disintegrated faint tryptophane	scarcely attacked doubtful trypt.	scarcely attacked doubtful trypt.
Neutral	fibrin nearly all gone distinct tryptophane	distinctly attacked faint trypt.	mostly disintegrated faint trypt.

24 hours later, the results were :—

		HCN.	*Chlorof.*	*NaF.*
Acid	{	fibrin as before	as before	mostly disintegrated
	{	strong tryptophane	faint trypt.	faint trypt.
Alkaline	{	fibrin as before	as before	as before
	{	faint tryptophane	doubtful trypt.	doubtful trypt.
Neutral	{	fibrin quite disintegrated	about half gone	mostly disintegrated
	{	marked tryptophane	distinct trypt.	faint trypt.

The contents of all the bottles gave a good biuret-reaction at the close of the experiment. The alkaline bottles retained their reaction throughout.

The results with fibrin not only serve to confirm those with Witte-peptone, but they give valuable information as to the nature of the action not only of the antiseptics but also of the reaction of the medium. With regard to the first point, it appears that neither chloroform nor NaF inhibits the peptonizing action of papaïn, but that they both (especially NaF) impede further proteolysis with the formation of tryptophane. With regard to the second point, it is clear that the presence of acid is altogether favourable, whilst the presence of alkali is as distinctly unfavourable, impeding even peptonization in the bottles containing either chloroform or NaF.

These results suffice to make clear the reason of the failure to obtain the tryptophane-reaction in the experiments of Mendel and Underhill, and they establish the accuracy of my previous observations. They strikingly demonstrate the remarkably favourable effect of the presence of HCN upon the proteolytic activity of papaïn, as also the inhibitory effect of NaF.

In view of these results, I thought it worth while to make comparative experiments with a number of the antiseptics in general use for these purposes. In the first series, Witte-peptone was the digestible material; in the second, fibrin.

Experiment with Witte-peptone.

A clear solution (2 grms. in 200 cc. dist. water) of papaïn, and a clear solution of Witte-peptone (2 grms. in 200 cc. dist. water), were prepared: in the latter .1 grm. of citric acid was dissolved. 25 cc. of

each solution were placed in each of 8 bottles, and to each (except one, the control) one of the following antiseptics was added: NaF, 1 %; Salicylic acid, 1 %; thymol, ·5 %; chloroform, ·5 %; toluol, ·5 %; formalin, ·8 %.

After 7 hours' digestion at 40° C., the tryptophane-reactions were:—

Distinct; HCN bottle.
Faint; thymol, toluol, control, chloroform.
None; Salicylic acid, NaF, formalin.

17 hours later, the reactions were:

Strong; HCN.
Distinct; thymol, toluol, control.
Faint; Salicylic acid, chloroform.
None; NaF, formalin.

A similar series of experiments, in which fibrin (2 grms.) replaced the Witte-peptone, and in which a stronger solution of papaïn (4 grms. extracted with 200 cc. dist. water) was used, gave confirmatory results.

Experiment with Fibrin.

The same antiseptics in the same strength as before. After 7 hours' digestion, the results were:—

HCN; fibrin completely disintegrated; distinct tryptophane reaction.
Salicylic acid; fibrin unaffected; no tryptophane.
Thymol; fibrin slightly attacked; no tryptophane.
NaF; fibrin partly disintegrated; no tryptophane.
Chloroform; fibrin unaffected; no tryptophane.
Toluol; fibrin gelatinous; no tryptophane.
Formalin; fibrin unaffected; no tryptophane.
Control; fibrin largely disintegrated; distinct tryptophane.

19 hours later, the results were essentially similar: the fibrin was rather more attacked in one or two cases, but it had not been completely disintegrated in any but the HCN and the control bottles, and these were still the only bottles the contents of which gave any tryptophane-reaction, strong in the HCN bottle, distinct in the control. The contents of all the bottles gave good biuret-reaction.

In these experiments the influence of HCN in promoting proteolysis by papaïn is very evident. In order to determine

that HCN does not exercise any direct proteolytic action, the following experiment was made:—

30 cc. of a papaïn solution like the above were placed in each of 2 bottles, that in one of the bottles having been previously boiled: to each bottle were added 1 grm. Witte-peptone, ·25 grm. citric acid, and 20 cc. dist. water containing HCN so that the percentage of HCN in the mixture was 0·2. After 18 hours' digestion, the unboiled contents of the one bottle gave strong tryptophane-reaction, whilst the boiled contents of the other gave none.

I then proceeded, for purposes of comparison, to make a similar experiment with the juice of the Pine-apple.

50 cc. of expressed juice were placed in each of 7 bottles, with 1 grm. of moist fibrin: in 5 of the bottles the juice was of natural acidity, and to each of these antiseptics were added respectively as follows: 0·2 % HCN, 1 % NaF, 0·5 % thymol, 0·5 % toluol, 0·5 % chloroform: the juice in the sixth bottle was neutralized and then made distinctly alkaline by the gradual addition of 1·7 grm. Na_2Co_3, when 0·2 % HCN was added: no antiseptic was added to the seventh bottle.

After 24 hours' digestion at 40° C., the results were as follows. The fibrin had been quite or almost completely dissolved in all the bottles: least in the NaF and chloroform bottles. The tryptophane-reactions were:—very strong in NaF, thymol, toluol, and chloroform bottles; marked in the toluol bottle and in the alkaline HCN bottle; less marked in the acid HCN bottle and in the bottle without antiseptic.

These results are altogether contradictory to those obtained with papaïn: for in this case proteolysis was most active in the presence of NaF, and least active in the presence of HCN. It seems natural to infer that the difference in the behaviour of the two proteases with the two antiseptics indicates a fundamental diversity in their properties. It is generally agreed that bromelin is a more active protease than papaïn, though no digestion-experiments have been made with equivalent weights of the pure substances; and until that has been done, there is no real basis for comparison. There can, however, be little

doubt that the undiluted juice used in this series of experiments contained a much higher percentage of protease than did the extracts of papaïn in the previous series; and it seemed possible that the diverse results might be due rather to the relative amount of the proteases in the solutions than to a difference in their properties. With this possibility in view, I instituted the following experiments with papaïn-extracts of different strengths, and with diluted and undiluted Pine-apple juice, NaF and HCN being the antiseptics employed.

Papaïn.

50 cc. of 4 % watery extract were placed in each of two bottles, together with 0·2 grm. citric acid and 1 grm. of moist fibrin: to one bottle 0·5 grm. NaF (= 1%) was added, to the other 2·5 cc. of 4 % HCN (= 0·2 %).

Two exactly similar bottles were prepared in which, however, the strength of the papaïn-extract was 2 %.

After 18 hours' digestion at 40° C., the fibrin was completely dissolved in both the HCN bottles; only partially dissolved in the NaF bottles. The tryptophane-reaction was strong in the bottle containing the 4 % extract and HCN; distinct in the bottle containing the 2 % extract and HCN; faint in both the NaF bottles.

30 hours later, the fibrin was completely dissolved, except for a small residue, in all the bottles. The tryptophane-reaction was strong in both the HCN bottles; faint in both the NaF bottles.

Bromelin.

50 cc. of undiluted Pine-apple juice were placed in each of 2 bottles with 1 grm. moist fibrin: to the one 0·5 grm. NaF (= 1 %) was added, to the other 2·5 cc. 4 % HCN (0·2 %).

Two exactly similar bottles were prepared in which the juice had been diluted with an equal volume of distilled water.

After 18 hours' digestion, the fibrin was mainly dissolved in all the bottles. The tryptophane-reaction was strong in the bottle containing undiluted juice and NaF; marked in the bottle containing diluted juice and NaF; distinct in that containing undiluted juice and HCN; faint in that containing diluted juice and HCN.

30 hours later, the fibrin was dissolved in all the bottles. The tryptophane-reaction was very strong in the NaF bottle with undiluted

juice, strong in the NaF bottle with diluted juice, marked in both the HCN bottles.

From these experiments it is clear that the influence of such antiseptics as NaF and HCN on proteolysis depends, not upon the amount of the protease present, but upon the nature of the protease, probably upon its chemical constitution.

The general conclusion to be drawn from all these experiments with various antiseptics is that these substances exert a considerable influence, greater than is usually supposed, upon proteolytic processes. It is, I think, made clear that in investigating the action of any protease, it is necessary that experiments should be conducted with more than one antiseptic before any conclusion as to the properties of the enzyme is arrived at. I am also justified in reasserting that all vegetable proteases, so far as they have been investigated, are essentially proteolytic; and that no merely peptonizing protease has yet been discovered.

I may incidentally mention here an experiment upon the action of Pine-apple juice at the ordinary temperature of the laboratory instead of in the incubator: that is, at about 17° C. instead of 40° C. The results show that proteolysis is effected under these conditions, but more slowly than at the higher temperature.

50 cc. of Pine-apple juice were placed in each of two bottles, with 1 grm. moist fibrin; to the one 0·5 grm. of NaF (= 1 %) was added, to the other 2·5 cc. of 4 % HCN (= 0·2 %).

After 19 hours' digestion the fibrin was quite disintegrated in both: the NaF bottle gave distinct tryptophane-reaction, the HCN bottle gave no reaction.

29 hours later, the NaF bottle gave strong tryptophane-reaction, the HCN bottle a distinct reaction.

Dahlia variabilis.

The tuberous roots of the Dahlia have long been the subject of investigation on account of their peculiar chemical contents. They have largely provided the material for the

study of inulin, though the discovery of the enzyme *inulase* was made by Green (1887) in the tubers of the Jerusalem Artichoke (*Helianthus tuberosus*). But the fact of more immediate interest in connexion with the subject of the present paper is that Leitgeb (7) found the roots, in a state of rest, to contain considerable quantities of asparagin and tyrosin as nitrogenous reserve-material. On this ground the Dahlia-roots seemed likely to be promising material for digestion-experiments, which I have accordingly made, together with some incidental observations on the presence of tyrosin.

The expressed juice of the tuberous roots immediately assumes a dark colour owing to the action of the oxidase which Bertrand (8) found to be present, and which he termed *tyrosinase*, upon the tyrosin in solution. On filtration, a brown, opalescent, distinctly acid liquid is obtained, which gives strong xanthoproteic reaction, strong Hofmann's reaction with Millon's reagent, and oxidase-reaction with guaiacum, but no biuret-reaction: it gives a tryptophane-reaction which is not easy to perceive on account of the brown colour of the liquid. On boiling the juice there is a considerable precipitate: the clear filtrate gives the same Hofmann's and xanthoproteic reactions as the unboiled liquid.

These reactions, especially the Hofmann's reaction with Millon's reagent, in which a brilliant pink colouration appears on heating, followed by the formation of a similarly coloured precipitate, indicate the presence of tyrosin in considerable quantity. More definite evidence is afforded by the application of Mörner's (9) test. As this reagent is not yet well known, I give its preparation. It is a mixture of 1 vol. of formalin (40 %) with 45 vols. of distilled water, and 55 of concentrated sulphuric acid. Heated with tyrosin, a striking green colour is produced. I found that on adding some of this reagent to Dahlia-juice, the green colour was developed without heating; the effect of heating was to give rise to a brown colour, due probably to the action of the H_2SO_4 on the inulin present.

Turning now to the question of the proteolytic activity of the juice, I may mention that Buscalioni and Fermi found the tuberous roots of the Dahlia to be proteolytically inactive, but this is not in accordance with my results. I made experiments (*a*) with the juice alone (autolysis); (*b*) with Witte-peptone added; (*c*) with fibrin added; in all cases there was distinct evidence of proteolysis. The material used was the root in the resting condition, and the experiments were carried on at intervals from January to March.

Autolysis.

40 cc. of slightly diluted expressed juice were placed in each of 4 bottles: to (1) nothing was added; to (2) HCN 0·2 %; to (3) citric acid 0·5 %; to (4) HCN 0·2 %, and citric acid 0·5 %.

After 21 hours' digestion at 40° C., the contents of (1) and (2) gave a distinct tryptophane-reaction, those of (3) and (4) a marked reaction. 30 hours later, (1) and (2) still gave the same reaction, whilst that of (3) was marked, and that of (4) had become strong.

Proteolysis of Witte-peptone.

In each of four bottles were placed 50 cc. of expressed juice diluted with equal vol. of dist. water, and 0·5 grm. Witte-peptone: the further additions to the bottles were precisely as in the preceding experiment.

After 4 hours' digestion at 40°C., the tryptophane-reaction was strong in the bottle containing citric acid, and in that to which neither citric acid nor HCN had been added; marked in the citric acid and HCN bottle; distinct in that to which HCN but no citric acid had been added.

19 hours later, the reactions were essentially the same.

Fibrin.

4 bottles were prepared precisely as those in the preceding experiment, except that 1 grm. moist fibrin was substituted for the Witte-peptone.

After 19 hours' digestion at 40° C., the fibrin had become shrivelled and stringy in all the bottles, and did not appear to have been at all dissolved. The tryptophane-reactions were:—distinct in the citric acid and HCN bottle, as also in the bottle with HCN but no citric

acid, and in the bottle to which neither citric acid nor HCN had been added; marked in the bottle with citric acid but without HCN.

48 hours later, the fibrin presented the same appearance, and the tryptophane-reactions were:—marked in all the bottles except the one containing HCN but no citric acid, where it remained distinct.

I have further succeeded in preparing a proteolytically active glycerin-extract from the roots. 100 grms. of the root, cut into small pieces, were macerated in strong alcohol for twenty-one hours, and then dried at room-temperature: the dried material, which weighed only 13 grms., was well triturated with 50 cc. glycerin, and left to stand for three days. The mass was then strained through muslin, yielding a turbid brownish extract, the activity of which was tested as follows:—

30 cc. of the glycerin-extract were mixed with 130 cc. chloroform-water, and 40 cc. of the mixture were placed in each of 4 bottles. To the liquid in No. 1, which was slightly acid, 0·2 grm. Witte-peptone was added: to No. 2, 0·2 grm. Witte-peptone, and 0·2 grm. citric acid (= 0·5 %): to No. 3, 0·2 grm. Witte-peptone and 0·2 grm. Na_2CO_3 (= 0·5 %) so that the reaction was distinctly alkaline: to No. 4 nothing was added.

After 4 hours' digestion at 40° C., the contents of Nos. 1, 3, and 4 gave a faint tryptophane-reaction; those of No. 2, a distinct reaction. 19 hours later the tryptophane-reaction was distinct in No. 1, marked in No. 2, faint in Nos. 3 and 4.

From these experiments it is clear that the tuberous root of the Dahlia contains an enzyme which proteolyses the proteids of the root; that it also proteolyses Witte-peptone is shown by the rapid development of a more or less strong tryptophane-reaction when this material is presented to it. There is, however, no evidence that the protease attacks fibrin, for in no case did there appear to be any definite solution of it; the tryptophane-reactions given by the contents of the bottles in the fibrin-experiments were so nearly the same as those given by the bottles in the autolysis-experiments that they do not appear to have been to any extent due to the presence of the fibrin.

Helianthus tuberosus.

I have already stated (1) that the tissue of the tuber of this plant proteolyses Witte-peptone. I have since ascertained that the expressed juice of the tuber proteolyses Witte-peptone, as also its own proteids.

In view of the presence of inulin in this tuber, I thought it worth while to determine whether or not the inulin were accompanied by tyrosin, as is the case in the tuberous root of the Dahlia. I found that there was no such storage of tyrosin in this plant.

The expressed juice is a brown, turbid, slightly acid liquid; it gives the oxidase-reaction with guaiacum, and strong xanthoproteic reaction. On boiling there is a dense precipitate; the clear filtrate gives faint tryptophane-reaction, no biuret, only a faint Millon's reaction, and none with Mörner's reagent for tyrosin, in striking contrast to the juice of the Dahlia-root.

Crambe maritima.

The etiolated shoots of the Sea-kale occurred to me as probably interesting material for investigation. The expressed juice is a yellow acid liquid, giving good peroxidase but no oxidase-reaction with guaiacum; it also gives weak xanthoproteic and Millon's reactions. A precipitate is formed on boiling; the filtrate gives no biuret, but faint tryptophane-reaction. Digestion-experiments showed that autolysis is feeble, but the proteolysis of Witte-peptone is active.

50 cc. of expressed juice, diluted with an equal vol. of dist. water, were placed in each of 4 bottles: to (1) only a little thymol was added; to (2) a little thymol and 0·5 grm. Witte-peptone; to (3) thymol, 0·5 grm. of Witte-peptone, and 0·25 grm. citric acid (= 0·5 %); to (4) 0·5 grm. Witte-peptone, 0·25 grm. citric acid, and HCN to 0·2 %.

After 19 hours' digestion at 40° C., (1) gave faint tryptophane-reaction; (2) a strong reaction; (3) and (4) a marked reaction.

The action of the juice upon fibrin was then investigated. Inasmuch as in the previous experiment the activity of the juice had been found to be greatest in the bottle to which no acid had been added, no acid was added in this case: but the contents of the bottles were very distinctly acid at the close of the experiment. The result indicates that the juice does not act upon fibrin.

40 cc. of expressed juice, diluted with an equal vol. of dist. water, were placed in each of 3 bottles, with some thymol: to (1) nothing further was added; to (2) 0·5 grm. of Witte-peptone; to (3) 1 grm. of moist fibrin.

After 29 hours' digestion at 40° C., (1) and (3) gave distinct tryptophane-reaction, (2) a strong reaction. The fibrin in (3) did not appear to have been attacked to any extent, so that the tryptophane-reaction was due to autolysis.

Inasmuch as neither the enzyme of the Sea-kale, nor that of the Dahlia acts upon fibrin, they are to be referred, like those of many other plants (see my previous paper, 1), to the erepsin-group of proteases.

Betula alba.

I happened to have the opportunity of investigating the sap poured out by a 'bleeding' Birch-tree.

The sap is a clear, yellowish, neutral liquid: it gives the peroxidase- but not the oxidase-reaction with guaiacum, also faint xanthoproteic and Millon's reaction, no tryptophane or biuret-reaction, but strong sugar-reaction with Fehling's solution.

Digestion-experiments were made with and without added proteid (Witte-peptone and fibrin), the sap being acidified with citric acid or made alkaline with Na_2CO_3, also with or without the addition of a few drops of HCN solution as an antiseptic, but in no case was any tryptophane-reaction observed, even when digestion was prolonged to forty-eight hours. The sap apparently contains no protease.

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