The Proteases of Plants (IV).

BY

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IN the course of the year 1905 an interesting contribution to this subject was made by Dean, consisting of two papers (1 and 2) on proteolytic enzymes, the principal results of which may be briefly given as follows.

The objects of the investigations detailed in the first paper, as stated by the author, were two : 'in the first place, . . . to test a number of different plant-tissues in order to confirm, if possible, the results of Vines ;' whilst 'the second and more important part of the investigation was to make a careful study of the enzymes in some one or two plants.'

With regard to the first object, it is somewhat singular that the author expresses no opinion as to whether or not he succeeded in confirming my results ; but it may be inferred from the account of the experiments that he was successful. For instance, in the case of the leaves of the Spinach (*Spinacia oleracea*), his conclusion is that they 'contain an enzyme capable of attacking Witte-peptone, but not acting on the proteids of the tissue, or if it does, these proteids do not yield tryptophan on hydrolysis ;' a conclusion which is, on the whole, in accordance with my earlier observation (3) that these leaves act upon Witte-peptone, but are unable to digest fibrin. A similar parallelism exists between his observations and mine on the leaves of the Cabbage (*Brassica oleracea*). Other experiments, made with material that I had not used, the blossoms of the Wild Carrot (*Daucus Carota*), the leaves of the Chestnut (*Castanea sativa americana*), etiolated seedlings of a Bean (*Phaseolus Mungo*), seedlings of *Cucurbita maxima*, gave results supporting the view, which I have expressed more than once, of the widespread, if not universal, occurrence of ereptase in the tissues of plants.

With regard to the second object of Dean's paper, it was pursued by the investigation of the seeds of *Phaseolus vulgaris*, which seems to include the White Haricot Bean and the Scarlet Runner. The result was remarkable. It was found that the cotyledons, whether the seeds were ungerminated or

in different stages of germination, contained an ereptase, inasmuch as the material actively digested Witte-peptone as shown by the tryptophane-reaction; but 'at no time could any evidence of the presence of an enzyme capable of attacking the proteids of the seed be obtained.' 'The enzyme acts on the proto-proteose, the hetero-proteose, and the deuterio-proteoses, separated from Witte-peptone; it is quite inactive on phaseolin of the bean, excelsin of the Brazil nut, edestin of the hempseed and boiled fibrin.' The author concludes as follows:—'There can be no doubt but that the large proteid store in bean-seeds is utilized in germination, and in all probability this utilization is preceded by cleavage to the amido-acids, hexon bases, &c. What effects this cleavage, and what part, if any, the ereptase plays, are unsolved problems.' It should be noted that the times of digestion mentioned in the various experiments were 41, 19, and 18 hours.

Dean's second paper gives an account of more minute investigations into the chemistry of the seeds of *Phaseolus vulgaris*, with results that confirm the main conclusions of the first paper. He says: 'The results of this study have shown that, as was to be expected, the proteids of the seed undergo proteolysis during germination as a preliminary to the transportation of the nitrogen and its utilization in the formation of new organs. There are three ways in which this process might be carried out: by the action of a tryptic enzyme; by the combined action of the protoplasm and of an enzyme which is, by itself, incapable of carrying out the whole process; or by the action of the protoplasm alone. The results of a study of the proteolytic enzymes of the resting and germinating bean show that there is no enzyme present which is able to digest the proteids of the seed. There is present, however, an enzyme of the ereptase group which is capable of digesting the proteoses resulting from the partial hydrolysis of the seed proteids. It may be, therefore, that the protoplasm of the cells starts the process of proteid decomposition, and carries it to some stage at which the ereptase takes up the work and completes the process.'

EXPERIMENTS WITH SEEDS.

I turn now to my own observations. Until recently, I had not specially investigated seeds, having only made a few incidental experiments in connexion with the determination of the general occurrence of ereptase in plant-tissues. The first experiments, described in my paper on 'Tryptophane' (4), were made with Malt and with *Vicia Faba*, and showed that extract of Malt readily undergoes autolysis, whereas, in the case of *Vicia*, no autolysis could be detected in 20 hours, though Witte-peptone was more or less peptolyzed in 24 hours. I took occasion to point out at the time that 'the whole subject requires systematic reinvestigation;' and the experiments described in this paper are a first attempt to satisfy that requirement.

In a subsequent paper (3) it was shown that extract of Green Peas (*Pisum sativum*) acts strongly on Witte-peptone; and that the 'germ' of Wheat not only acts upon Witte-peptone, but also digests fibrin. My last paper on proteases (5) contains an account of more detailed investigation of the proteolytic properties of Malt, carried on with the object of elucidating the nature of the enzymes which effect not only peptolysis but also peptonization.

I begin with a general description of the methods adopted. The seeds, as a rule without their integuments, were ground in a hand-mill: in nearly every case 10 grms. of the flour were triturated with 100 c.c. distilled water (10 % mixture): the mixture was generally used as such, but in some cases it was allowed to settle and the supernatant liquid decanted off: the antiseptic was HCN 0.1 %: the ox-fibrin had been preserved in a mixture of dilute glycerin (50 %) and alcohol, and was well washed before an experiment: the mixtures for digestion were put into stoppered bottles to prevent loss by evaporation, more especially of the HCN, a few drops of which were added from time to time when the experiments were protracted: the temperature of the incubator was about 40° C. The fibrin was suspended in the digesting mixture by a thread, so that it could be readily examined from time to time. The fibrin used was of the same stock throughout the whole of the experiments; the fact that in many instances the fibrin was not digested, suffices to remove any suspicion of self-digestion.

The seeds employed were those of the following plants:—*Vicia Faba* (Broad Bean), *Pisum sativum* (Pea), *Phaseolus multiflorus* (Scarlet Runner), *Phaseolus vulgaris* (White Haricot Bean), *Lupinus hirsutus* (Blue Lupin), and *Zea Mais* (Maize). The idea was to compare a number of more or less closely allied seeds, in this case Leguminous, introducing an altogether different one by way of contrast. In every case it was ascertained that the sample of seed used was capable of germinating. The seeds were germinated in a hot-house.

The experiments were designed to determine whether or not (1) auto-lysis, or (2) fibrin-digestion, took place in the various mixtures.

(1) *Germinated Seeds.*

Vicia Faba. 19 days after sowing: 30 grms. of ground cotyledons extracted for 48 hours with 100 c.c. distilled water, at room-temperature: the extract gave marked tryptophane-reaction: 40 c.c. of the extract decanted off digested 0.2 gm. fibrin within 24 hours in the incubator.

Phaseolus multiflorus. 7 days after sowing: 10 % mixture gave distinct tryptophane-reaction, which became strong after 24 hours in the incubator: the fibrin (0.3 gm.) had begun to break up after 72 hours, and had entirely disappeared in 120 hours.

50 grms. of the same cotyledons were triturated with 200 c.c. distilled water and

left to stand at room-temperature for 24 hours: 150 c.c. liquid were then decanted off, and gave distinct tryptophane-reaction: the fibrin (0.3 gm.) remained unaltered until the seventh day in the incubator, when it was seen to be breaking up, the tryptophane-reaction being strong: the fibrin had disappeared on the ninth day.

Same crop, 13 days after sowing: the 10% mixture of the cotyledons gave marked tryptophane-reaction: after 24 hours in the incubator, the fibrin (0.3 gm.) was partly digested, and the tryptophane-reaction had become strong: 21 hours later, the fibrin had completely disappeared.

Pisum sativum. 6 days after sowing: epicotyls about 1 inch above the soil: the 10% watery mixture of the ground cotyledons gave marked tryptophane-reaction: after 24 hours in the incubator, the tryptophane-reaction had become strong: the fibrin (0.3 gm.) remained apparently unaltered until 72–96 hours in the incubator, when it was found to be attacked: 48 hours later it had disappeared.

Some seedlings of the same crop were left to grow for 5 days more: a 20% watery mixture of the cotyledons was then made, which gave strong tryptophane-reaction, and digested 0.3 gm. fibrin within 24 hours.

Lupinus hirsutus. 4 days after sowing: radicles 1–2 inches long: 10% mixture gave faint tryptophane-reaction: after 24 hours in the incubator, the tryptophane-reaction had become strong: after 120 hours, the fibrin (0.3 gm.) remained apparently unaltered, but had quite disappeared at the end of 168 hours.

Same crop, 7 days after sowing: 10% mixture gave distinct tryptophane-reaction, which had become strong after 24 hours in the incubator: the fibrin was found to be breaking up after 72 hours, and had disappeared by the end of 120 hours.

Same crop, 11 days after sowing: cotyledons green: the 10% mixture gave faint tryptophane-reaction: after 48 hours in the incubator the tryptophane-reaction was found to have become strong and the fibrin to be breaking up: by the end of 96 hours, the fibrin had disappeared.

Phaseolus vulgaris. 8 days after sowing: the epigeal cotyledons were green: 10% mixture gave faint tryptophane-reaction: after 48 hours in the incubator the tryptophane-reaction had become strong, and the fibrin (0.3 gm.) was breaking up: within 72 hours the fibrin had disappeared.

Zea Mais. 4 days after sowing: radicles well developed: ground up entire grains and seedlings: the 10% mixture gave distinct tryptophane-reaction: after 24 hours in the incubator both the tryptophane-reaction and the fibrin (0.3 gm.) were unaltered: 24 hours later the tryptophane-reaction was marked, and most of the fibrin had gone: 24 hours later the fibrin had disappeared.

The results of these experiments are altogether harmonious. In all cases it was found that, after germination,

- (1) the cotyledons, or the entire grain (Maize), contained tryptophane;
- (2) the amount of tryptophane rapidly increased during the experiment; and,
- (3) fibrin was more or less rapidly digested.

The conclusions to be drawn are obviously these. (1) There was present in these germinated seeds an enzyme that produced tryptophane

from the reserve proteids. The fact that, in the leguminous seeds, the original watery mixture of the cotyledons gave relatively weak tryptophane-reaction, which soon became strong under experimental conditions, indicates, no doubt, that tryptophane, like the other amido-acids, does not accumulate in the cotyledons of the seedling, but is distributed to the growing parts. (2) There was present also an enzyme capable of digesting fibrin. It may be fairly inferred that an enzyme that can digest fibrin can also digest the reserve proteids of the seed.

I am quite unable to reconcile my results and conclusions with those of Dean. Far from being unable to obtain evidence 'of the presence of an enzyme capable of attacking the proteids of the seed,' I have never failed to obtain such evidence whenever the seeds were actively germinating and the duration of the experiment was sufficiently prolonged. In endeavouring to account for the contradiction between us, I am inclined to attribute it, in part at any rate, to the relatively short duration of Dean's experiments: this is, I feel sure, the explanation of my own failure to detect autolysis in my first experiments with *Vicia Faba* (see *ante*, p. 114). But I also attribute it, and more particularly, to what seems to me to be a questionable proceeding in his method of testing. I notice that, before applying any test, whether biuret or tryptophane, the digestive mixtures of *Phaseolus vulgaris* were boiled, then acidified with acetic acid and filtered: in no case was the biuret- or the tryptophane-reaction given by a liquid so treated. The facts, so far as I know them, are these. A watery extract of seed of *P. vulgaris* gives good biuret-reaction: if the extract be boiled and filtered, the filtrate still gives a good reaction, indicating the presence (as I pointed out more than twenty-five years ago with regard to seeds generally) of a proteose or phytalbumose: if, however, the extract be boiled and also acidified with acetic acid and filtered, the reaction is hardly perceptible, owing, as I think, to the precipitation of the proteose by the acid. If now a small quantity of tryptophane be present in such a solution, it is not improbable that it might be carried down wholly or in part by the precipitates formed by boiling and by acidification. It is highly probable, that had the tests been applied to the mixtures in question previously to the treatment indicated, both the biuret- and the tryptophane-reactions would have been obtained. Moreover, I found that some of these leguminous seed-extracts did not give the tryptophane-reaction very readily, so that it was necessary to add the chlorine-water gradually and to the extent of as much as 2-3 vols. before it could be decided whether or not tryptophane were present: possibly this detail may have had something to do with the observed results. In any case it is obviously desirable to exhaust all possible modifications in the methods of such a research, before attempting to explain its results by so speculative an hypothesis as that of the direct intervention of the protoplasm in the proteolysis of the seed.

To return to the interpretation of my own results. Peptolysis and fibrin-digestion both took place: but the question remains, were they effected by a single protease of the nature of tryptase; or were they effected by two distinct proteases, the one an ereptase, the other possibly a peptase?

Dean recognizes the presence of ereptase in the seeds of *Phaseolus vulgaris*, and the facts that I have recorded seem to extend this conclusion to the various seeds with which I have experimented. The important point is that, whereas the production of tryptophane was immediate, the digestion of fibrin was delayed, the less so the longer the seeds had been germinating before the experiment. This difference in point of time suggests that two distinct proteases are concerned in the proteolysis of the seed: were both tryptophane-production and fibrin-digestion effected by one and the same enzyme, the two processes should be carried on more nearly *pari passu* than was observed to be the case. However, it is not urged that the results are absolutely conclusive.

With the object of obtaining some more definite answer to this important question, I instituted a series of experiments with ungerminated seeds of the same plants and from the same parcels. I must explain that in some cases the seeds were used *with* their integuments, though I did not observe that this made any material difference. In other respects the methods employed were the same as those already described.

(2) Ungerminated Seeds.

The experiments themselves require no special description, and were exactly the same in all cases: so the results may be at once considered.

The 10 % watery mixtures of the six species of seed investigated gave no tryptophane-reaction, to begin with, except in the case of *Lupinus hirsutus*, where a faint reaction was detected. This being so, the development of a tryptophane-reaction, whilst in the incubator, would be evidence of autolysis; and, as a matter of fact, tryptophane was produced in every instance, with various degrees of rapidity, but rather slowly and gradually as a rule, as indicated in the following table compiled from a number of experiments:—

	24 hrs.	48.	72.	96.	120.	144.
<i>Vicia Faba</i>	—	distinct	marked	—	—	strong
<i>Phaseolus multiflorus</i> .	faint	—	marked	strong		
<i>Pisum sativum</i> . . .	faint	distinct	—	marked	strong	
<i>Lupinus hirsutus</i> . .	strong					
<i>Phaseolus vulgaris</i> .	faint	marked	strong			
<i>Zea Mais</i>	faint	distinct	marked	strong		

In every experiment, then, evidence of autolysis was obtained: clearly a protease was present in the ungerminated seeds that could act upon

some constituent of the reserve proteid. But the differences in the rapidity of action remain to be accounted for: they may be due (1) to variation in the amount of enzyme present in the seeds; or (2) to differences in the amount of the digestible proteid stored in the seeds: or perhaps to a combination of these conditions. I have not attempted to go minutely into the matter; but I have made an observation, based upon my earlier experiments with *Vicia Faba*, that throws some light upon it. I have found that 10 % mixtures of the six species of seeds investigated gave in every case a more or less strong tryptophane-reaction within 24 hours when Witte-peptone had been added to the extent of 0.5 %. Undoubtedly these ungerminated seeds all contained sufficient protease: hence the results of the autolysis-experiments indicate an absence, not of the enzyme, but of the particular proteids upon which the enzyme could act, except in the case of the Lupin, where the tryptophane-reaction was developed as rapidly without as with the addition of Witte-peptone. The slow development of the tryptophane-reaction in the other cases suggests a gradual conversion of less tractable reserve proteids, probably forms of proteoses, into others more readily digestible, probably deuterio-albumoses and peptones. However, it is impossible to account fully for the differences observed in the proteolysis of these seeds without an adequate investigation of the nature of their reserve proteids; and this I have not attempted. But I may record my impression that the differences in the constitution of the proteid reserves are not so much dependent upon the kind of seed—that is, are not altogether *specific* differences—as upon the degree of ripeness of the seed. It would appear that the more completely ripe the seed, the more intractable are the reserve proteids. This point, again, can only be settled by further investigation: by comparative experiments with seeds at different stages in the process of ripening.

Having thus dealt with the autolysis of ungerminated seeds, the possibility of fibrin-digestion remains to be considered. The experiments on this question were carried on in the same way as those already described for germinated seeds: in every case 0.3 grm. fibrin was suspended in a mixture consisting of 10 grms. seed-flour and 100 c.c. distilled water, with 0.1 % HCN as the antiseptic.

The results were these: in the case of *Vicia Faba*, *Phaseolus multiflorus*, and *Phaseolus vulgaris*, the fibrin showed no appreciable diminution even when, as in one instance, the experiment was continued for 14 days: in the case of *Pisum sativum*, *Lupinus hirsutus*, and *Zea Mais*, the fibrin completely disappeared in the course of several days. Thus, with *Pisum*, the fibrin showed signs of diminution on the ninth day, and had disappeared on the tenth: with *Lupinus*, in one experiment, the fibrin began to break up on the fourth day and disappeared on the fifth; in another experiment, it disappeared on the sixth day: with the Maize, the fibrin was seen to be

breaking up on the seventh day, and had disappeared on the eighth. Thus it was found that the mixtures of the ungerminated seeds of the Pea, the Lupin, and the Maize, gradually acquired, under experimental conditions, the capacity for digesting fibrin. I cannot explain why this capacity was not equally developed in *Vicia Faba*, *Phaseolus multiflorus*, and *Phaseolus vulgaris*: I can only suggest that in these cases the process is slower, and that possibly fibrin-digestion might have been observed had the experiments been more prolonged.

The probability of the suggestion made in connexion with the experiments on germinated seeds, that two proteases are active during germination, is materially increased by these experiments on ungerminated seeds. It is not reasonable to attribute the digestion of the fibrin, which took place five to ten days after the experiment had begun, to the protease in the resting seed that was found to digest Witte-peptone within a few hours: for if this protease were capable of digesting fibrin, why was its action so long delayed? It is more in accordance with the observed facts to conclude that the ungerminated seeds contained, to begin with, an ereptase but no protease capable of digesting fibrin; and that, in certain cases, such a protease was gradually developed in the course of the experiment.

It occurred to me that it might be possible to accelerate the fibrin-digestion by chemical means; and, with this object in view, I made some experiments in which the distilled water was replaced in the mixtures by NaCl-solutions. The attempt did not prove successful, but the experiments are nevertheless worth mentioning. In the first set, 5 % NaCl-solution was used: the result was that in most cases (*Vicia Faba*, *Phaseolus vulgaris*, Pea, Lupin) the fibrin completely disappeared within 72 hours. This seemed to be satisfactory, but there was the possibility that it might be due, wholly or in part, to solvent action of the salt; and, as a matter of fact, it was found, in a control-experiment, that 0.3 gm. fibrin was completely disintegrated, though not altogether dissolved, when digested for three days in 100 c.c. 5 % NaCl-solution alone, with 0.1 % HCN. In a second set of experiments, a 2.5 % NaCl-solution was used, with the result that the fibrin disappeared in about ten days in the case of the Pea and of the Lupin, but not in any of the Beans (the Maize was not tried), a result which is much the same as that obtained when distilled water was used. It may be added that digestion of 0.3 gm. fibrin with distilled water only, containing 0.1 % HCN, produced no effect though continued for more than a fortnight.

Summary.

The facts established by the foregoing experiments are briefly these:—

(1) The ungerminated seeds were found to contain a protease which acted (a) immediately on Witte-peptone, and (b) more or less slowly on the reserve proteids of the seeds:

(2) The germinated seeds were found to contain a protease that digested fibrin: this protease was also found in certain cases (Pea, Lupin, Maize) to be slowly developed in the substance of the ungerminated seed in the course of the experiment.

The object of this research was to obtain, from the study of seeds, evidence bearing upon the conclusion at which I had arrived (5) as the result of experiments with Yeast, Malt, Mushroom, and other vegetable substances, that, in those cases in which fibrin-digestion takes place, two proteases are present: the one, essentially peptolysing, ereptase, the other essentially peptonizing, peptase as I believe. The conclusion was based mainly on observations showing that by appropriate chemical means, such as variations in the reaction of the liquid, it is possible to induce differential effects, to retard or inhibit the one process without similarly (if at all) interfering with the other. It is, I consider, definitely supported by the results stated above, which afford evidence of a new kind—the separation of the proteases in point of time. Thus, in the ungerminated seed, one protease only, ereptase, was found to be present, to begin with: no evidence of the presence of the fibrin-digesting protease could be obtained until several days after the experiment had proceeded or germination had been going on.

It must, however, be admitted 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.

The general course of proteolysis in the germinating seed can now be intelligibly followed. At first it is confined to the action of the ereptase upon the more digestible reserve proteids such as the proteoses. Within a few days after germination has begun, the fibrin-digesting enzyme is developed: this hydrolyses the higher proteids of the seed, and so produces material upon which the ereptase can continue to act. There does not seem to be any reason whatever for the assumption of the direct intervention of the protoplasm in the process.

LIST OF PAPERS REFERRED TO.

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2. — : On Proteolytic Enzymes, II; *ibid.*, vol. xl, Aug., 1905, p. 121.
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