The Incapacity of the Date Endosperm for Self-Digestion.

BY

RAYMOND H. POND,

North-Western University, Chicago.

INTRODUCTION.

THAT the digestion of nourishment stored in the endosperm is accomplished by the influence of enzymes which are secreted, or at least become active under conditions of germination, is universally accepted. The results and conclusions of various investigators are by no means uniform, as to whether or not the enzymes are developed in the embryo only or also in the endosperm independently of the embryo. The prevailing opinion, as recognized by Pfeffer, is that the endosperms of many grasses, palms, &c., possess a capacity for auto-digestion; that is, that such endosperms from which the embryos have been removed will, under optimum conditions of germination, provided digestion products may easily escape, digest themselves and become depleted of their storage substance. This view has become so widely accepted as no longer to be questioned.

When one examines the evidence upon which this conclusion rests he is surprised at its weakness. Moreover, associated with this view is a conception which is wholly out of date. The various authors have regarded as an almost self-evident fact that digestion by enzymes is evidence of vitality. The idea that a given tissue which happens to contain something capable of doing enzyme work is to be regarded as living matter, is no longer modern.

This paper is intended to show, first: that the literature as it stands does not furnish convincing proof of auto-digestion by endosperms, and second, that the date endosperm which Puriewitsch found to be capable of auto-depletion, and which he regards as living matter, does not possess such capacity.

The weakness of the evidence as presented by various authors is in nearly every case to be explained by unreliable data. For instance, the seeds have been allowed to germinate before removing the embryo when

it is well known that the latter contains enzyme. Some have based conclusions on the fact that the 'culture fluid' reduced Fehling's solution. The latter is of no significance unless it be known that reducing substances are not already present in the endosperm.

It is of course essential to understand what constitutes auto-depletion. The mere diffusion of soluble cell-substance from the tissue cannot be regarded as such. There must be evidence of chemical change, such as corrosion of starch-grains or dissolution of cell-wall. That the storage organs in some seeds are the cotyledons must not be overlooked, and the distinction must always be made between the behaviour of fleshy embryos and endosperms.

HISTORICAL.

Gris ('64, p. 90), in a notable contribution to the anatomy and physiology of germination, mentions some changes which were observed in the cell contents (aleurone especially) during germination, but Van Tieghem was the first to attack the problem directly. Van Tieghem ('77, p. 582) found that isolated endosperms of *Ricinus communis* under germination respire and are self-digesting, since the aleurone and oil are consumed, while the endosperms of *Canna* (amylaceous) and date (horny) are 'passive' and remain unchanged. He clearly implies that endosperms rich in oil and aleurone are self-digesting and possess vitality, while starchy endosperms and those containing reserve cellulose are not self-digesting and do not possess vitality. This conclusion rests purely on microscopical observations, and while the changes observed in *Ricinus* do indicate self-digestion, the possibility of a feeble digestion by the date is not excluded by such observations. So far as I am aware, this conclusion of Van Tieghem's regarding *Ricinus* has never been questioned.

Green ('90, p. 146) investigated the germination of *Ricinus communis* and found a 'ferment to exist as zymogen in the resting seed which is readily developed by warmth and weak acids into an active condition.' As to the relative influence of the endosperm and embryo he found 'the changes to be initiated in the endosperm, for they take place, although more slowly when the embryo is carefully removed.' The changes referred to consist (Green, '90, p. 147) in the saponification of fat into glycerine and ricinoleic acid especially, also the formation of sugar. Unfortunately the details of this work are not sufficient to permit any judgement as to the degree of demonstration attained.

Green ('86, p. 57) made glycerine extracts of the cotyledons of date-seedlings and of the endosperm-residue. Both extracts were applied to ground endosperms of resting date-seeds, but neither gave evidence of hydrolytic activity. We do not know what evidence was searched for, as the author merely states that, 'after prolonged action neither of them

(extracts) seemed to have had any effect, at least the quantity of ground cellulose had not apparently diminished.' However, another portion of the cotyledon extract was found by Fehling's test to have formed reducing sugar by its action on manufactured cotton fibre, while a similar portion boiled to destroy the enzyme was found to have no action on cotton fibre. From these results, and an examination of the cotyledon-structure, Green concludes that the ferment exists only in the epidermal cells of the cotyledon, and since the endosperm extract seemed to yield no sugar after contact with the ground resting endosperms, he finally says regarding the endosperm, 'there was no evidence that the extract of the endosperm had any action on cellulose at all. Evidently in it there is no ferment and the change is due to agencies quite external to its cells.'

Brown and Morris, after an extended study of the barley endosperm, agreed with Van Tieghem that the amylaceous endosperm of the grasses is a 'dead store house' of reserve material. In the same paper ('90, p. 480) the possibility is left open that the aleurone layer may contain some residual vitality, but the starch-bearing cells do not, and the conclusion including the 'dead store house' applies only to these starch-grain-cells. They further found ('90, p. 525) that the diastatic capacity of scutellum-cells is destroyed by treatment with vapour of chloroform. But when 'portions of the integuments of germinating barley, with their adherent aleurone layer, are treated in a similar way with chloroform vapour, we have found that their diastatic power *is not in the least impaired*, the starch under such sections being as quickly and completely attacked as under sections which have not been treated. Moreover, we have observed the same thing even after the fragments have been immersed for some time in absolute alcohol.' These authors assume that such treatment must have destroyed vitality, yet they acknowledge that the diastatic capacity remains undiminished. If then diastatic activity can proceed in dead tissue, how did these authors conclude that the amylaceous tissue of the endosperm constitutes a 'dead store house' because it does not possess diastatic activity? The inference of course is, that if such cells had been found able to do enzyme work they would have been regarded as living. As a matter of fact, their conclusion is that the aleurone cells are living because they show diastatic activity, and yet this activity was manifested under conditions which must have prohibited vitality. This affords an excellent example of the trouble which arises when vitality and enzyme activity are confused. It must be said here that these authors probably do not hold such views at the present time, but in a review of this kind it is necessary to deal with the literature as it stands. They further find ('90, p. 509) that translocation diastase is probably produced in the endosperm-cells by the embryo during its ante-resting development and that residual translocation diastase constitutes the diastase of the resting

grain. It may be said in this connexion that no one has proved that the diastase of the resting grain is in the endosperm as well as in the embryo. They do show ('90, p. 507) that during formation of the seed the diastase increases. The proportion of diastase was estimated at three stages, namely: endosperm about one half developed, endosperm about two-thirds developed, endosperm fully developed but not ripe. This does not show, however, as stated by Green ('01, p. 18), that the increase of enzyme takes place in the endosperm unless we assume that during the early development the embryo is too small to be considered. Brown and Morris do not state that they estimated the diastase present in the endosperm, but that they estimated the diastase in the grain when the endosperm was in varying stages of maturity. In this connexion they also state that a similar quantity of mature ungerminated grains yielded a similar quantity of cupric oxide. In this later test, however, the ungerminated seeds were allowed to steep, just how long is not stated, nor at what temperature, but if as long as in a preceding experiment (p. 496) the steeping lasted 24 hours and the temperature was not high enough to kill the embryo. Even if the steeping were only twenty-four hours the possibility of enzyme passing from the embryo to the endosperm is open¹. On p. 507, they show that the major part of the soluble enzyme in the resting seed (after steeping) is located in the proximal half (embryo half), and that some also exists in the distal half. If it were not for the steeping a demonstration of enzyme in this distal half would be conceded. This paper of Brown and Morris may then be summarized as attributing diastatic capacity and vitality to the aleurone cells but as denying both to the amylaceous endosperm.

Grüss ('93, p. 288) found evidence of enzyme activity in the endosperm of maize from seedlings whose hypocotyls had elongated to six or seven centimeters. Since the aleurone layer and scutellum contain a much greater quantity of enzyme, he concluded that the diastase is secreted by these tissues and by diffusion from them penetrates the endosperm. Later, Grüss ('96, p. 439) found that isolated endosperms of maize under sterile conditions of germination for twelve days gave a very positive reaction with the guaiac-hydrogen-peroxide test. A similar result was obtained with the endosperm of barley. If this guaiac test is to be accepted as reliable we must regard the capacity of the endosperms of maize and barley to generate enzyme independently of the embryo as demonstrated. To support this by further evidence the direct sugar test was made with Fehling's solution. In this case, the barley endosperms were in moist sand four days, but no statement is made that sterile conditions were maintained. The glycerine extract from these endosperms after digestion for eighteen

¹ Brown and Escombe ('98, p. 11) concede the possibility of enzyme from the embryo penetrating the endosperm during steeping, and say that the embryo should be removed while the seed is still dry.

hours with 1 per cent. starch-paste and diluted gave a mixed solution 5 cc. of which reduced 1.8 cc. of Fehling's solution. A similar solution from normally germinated seeds reduced 1.9 cc., while such a solution from ungerminated seeds reduced 1.1 cc. This experiment is not conclusive, for the following reasons:—It is not positively stated or implied that the endosperms remained sterile; the starch-paste may have contained a little sugar; the digestion during eighteen hours may not have been free from the influence of organisms, as no statement of such precaution is made; ten hours is the limit of safety without antiseptics at 37°C.; the end reaction with Fehling's solution is not accurate to even 1 cc. with mixed solutions containing proteid and starch in varying stages of alteration; the small differences of .1 cc. between the reducing power of the solution from isolated endosperms and of the solution from normally germinated seeds is suspicious. Moreover, if the endosperm contains reducing substance an extract from material soaked four days would probably contain more of such reducing substance than a similar extract from dry resting endosperms even though the time of extraction in both cases were equal.

In the case of the date-seed, Grüss ('96, p. 421) found the resting endosperm to be practically free from enzyme by the guaiac-hydrogen-peroxide test. In the endosperm of germinating seedlings ('96, p. 422) he found by test isolated blue spots indicating the local generation of enzyme independently of the embryo. But concerning this it may be said that as long as the cotyledon was present during germination such a test cannot be regarded as demonstrating independent activity by the endosperm. In summarizing the work of Grüss we may say that the autolysis of the endosperm in the cases of barley and maize is proven only in so far as the guaiac-hydrogen-peroxide test is specified for the presence of hydrolyzing enzymes. In the case of the date there is little approach to convincing evidence.

Hansteen ('94, p. 426) regards Van Tieghem's conclusion, concerning self-digestion as occurring in oily seeds but not in amylaceous seeds, as untenable. First, because Van Tieghem made no provision for the escape of the products of digestion. The accumulation of such products in starchy seeds would soon inhibit further digestion. Such inhibition in the case of oily seeds would, however, occur much later, because the oil is first changed to transitory starch and considerable disappearance of oil might be noted before the accumulation of the products of starch transformation would inhibit further digestion. Second, because Hansteen considers himself to have demonstrated that isolated endosperms of barley and corn under sterile conditions of germination favourable to the escape of digestion-products are self-digesting to a considerable extent, and that actual depletion and corrosion of the grains may be noted. The surrounding water into which the digestion-products diffuse becomes reducing to Fehling's

solution. The only objection to Hansteen's tests are, first : that he allowed the grains to soak for two days before removing the embryo, so that the possibility of the diffusion of enzyme from embryo to endosperm is open. That this is a valid objection is recognized by Brown and Escombe ('98, p. 11). On the other hand, Pfeffer ('97, p. 612) regards Hansteen's experiments as demonstrating the capacity of corn and barley endosperms for auto-depletion. Second, he does not show that the endosperm does not already contain some reducing sugar, which, if present, could easily diffuse into the surrounding water.

Linz ('96, p. 311) has shown that in isolated endosperms of maize under conditions of germination a marked increase in sugar content occurs during a period of twelve days. But since he allowed the grains to soak for two days before removing the embryos it is impossible to attribute this increase of sugar exclusively to the activity of enzymes in the endosperm. He draws the unqualified conclusion that the endosperm is living matter. A similar objection may be taken to Linz's ('96, p. 318) unwarranted conclusion that in such endosperms it is not the aleurone layer which furnishes the enzyme. He merely found that after two days' soaking the aleurone layer contains less enzyme than the endosperm, which may mean that during the two days' soaking enough enzyme diffused from the aleurone layer to establish the relative distribution he found. Brown and Escombe found that the aleurone layer (barley) only contains enzyme, and that the corrosion of starch is more notable in the tissue contiguous to it. Moreover, this observation disagrees with that of Grüss ('93, p. 288) who found the aleurone layer and scutellum to contain a much greater quantity of enzyme than the endosperm in the case of seedlings whose hypocotyls had elongated six or seven centimeters.

Leclerc du Sablon ('97, p. 395) made analyses of the date-seed before and after germination. He found oil and sugar to be present in the resting endosperm, together with some little aleurone intermixed with the protoplasm. The sugar is present in only about 1 per cent., and is to be regarded as a saccharose, since it reduces Fehling's solution with precision only after boiling with acid. The carbohydrates insoluble in 90 per cent. alcohol, but soluble in water, are present in only about 2 per cent. Determinations of the amount of sugar present in the resting endosperm were made, and also in the endosperm during germination, when the radicle had elongated to various lengths. His results show a constant increase in the sugar content of the endosperm, but he regards the increase as insignificant, and concludes that the date endosperm is incapable of self-digestion. Examination of his data reveals that he found 0.019 grms. of sugar in a quantity of endosperm tissue when the radicle measured 0.50 cms., and when the radicle measured 4.0 cms. he found 0.024 grms. which is an increase of 26 per cent. Such an increase would be significant

if occurring in the endosperm under conditions which would permit us to say that the embryo had no influence. Sablon notes the interesting fact that during germination the relative quantity of oil in the undigested portion of the endosperm remains about constant, 7 to 9 per cent. He also finds that the sugar and dextrin also remain about constant, but that the resting grain contains a little more because it has not lost by exosmosis. He concludes that the enzyme from the embryo does not penetrate the endosperm.

Puriewitsch ('97, p. 13) studied the depletion of the date endosperm, and concluded that it is capable of auto-digestion. How such a conclusion could be drawn from the data he has recorded cannot be understood. In the first place he allowed the seeds to germinate in sand until the radicle was from 3-7 cms. long. This would require, according to temperature, from two to four weeks, and enzyme from the embryo would certainly pass into the endosperm. Newcombe ('99, p. 68) found an enzyme in the endosperm during later germination, and if my own conclusion is correct, that an enzyme cannot be detected in the resting endosperm, it is evident that such diffusion must occur during germination¹. Moreover, Puriewitsch does not record actual evidence of digestion. He found merely that the 'culture fluid' was reducing to Fehling's solution. This is not significant, because the resting endosperm contains reducing substance, and further the tannin present would give the same reaction. As a matter of fact his record furnishes more evidence for a negative conclusion, because in microscopic sections of his material he did not find any notable difference between test objects and controls. How could auto-digestion be attributed to a tissue which, after thirty-six days at 27° C., showed no evidence of dissolution as compared with controls? Further he regards the endosperm as living matter capable of initiating and continuing vital processes ('97, p. 16).

In '98, after the researches of Hansteen and Puriewitsch, which are regarded by Pfeffer ('97, p. 613) as demonstrating that the endosperms of barley, maize, and date are capable of self-digestion, Brown and Escombe ('98, pp. 3-24) undertook a reinvestigation of the work of Brown and Morris ('90, p. 458). After quoting the general conclusions of the latter as follows: 'Although the peripheral layer of the endosperm, the so-called aleurone-layer, undoubtedly consists of living cells, no evidence could be obtained of the existence of any residual vitality in the amyliiferous cells which constitute by far the greater portion of the endosperm,' they state that there is a general agreement in the work of Hansteen, Pfeffer, Grüss,

¹ Puriewitsch makes a brief statement which shows that this source of error was not overlooked, but he does not show that the error was actually avoided. His statement seems to refer to the cereal endosperms especially, and cannot be applied to the date, because in the text descriptive of manipulation in the date experiments he states that the seeds were allowed to germinate.

and Puriewitsch that the amyliferous cells of the endosperm of the grasses have a definite power of digesting their reserve materials, this power being entirely independent of any influence of the embryo, the only necessary condition for its exhibition being that the products of metabolism shall not be allowed to accumulate within the endosperm.

As a result of this reinvestigation Brown and Escombe ('98, p. 14) have demonstrated a hydrolytic capacity of the aleurone cells of barley. Degermed endosperms steeped in saturated aqueous chloroform twenty-four hours show no depletion for several days, until invaded by organisms, while others not chloroformed show marked sub-aleuronic depletion and dissolution of starch grains. Cytological study of aleurone cells shows a 'well defined nucleus and all the usual cytological evidence of activity,' and these cells are commonly regarded as living units. They also find ('98, p. 18), from a cytological study of the amyliferous cells (barley), that the latter are in a very different condition from that of the aleurone cells. The nucleus is deformed, and the general structure indicates senescence. Actual trial showed the amyliferous cells to be without digestive capacity. Endosperms without embryos or aleurone, under favourable conditions for germination and removal of metabolic products, showed no difference as compared with similar ones treated with saturated aqueous chloroform for twenty-four hours. No visible changes occurred in either case until appearance of organisms, when visible changes did occur, so that it is possible, though rather improbable, that changes might have occurred later if the organisms had been kept out. This work of Brown and Escombe may, then, be regarded as demonstrating the capacity of the aleurone layer of barley for endosperm depletion and as rendering such capacity on the part of the amyliferous cells highly improbable.

The literature thus examined shows that auto-digestion by the amylaceous endosperms of grasses or by the horny endosperms of palms has not been successfully demonstrated, although such a claim has been made by different authors, and this claim has been accepted hitherto. On the other hand, the presence of an enzyme in the aleurone cells of barley has been demonstrated, and Puriewitsch has made reasonably certain a similar statement for other cereals. The question of the vitality of either aleurone cells or of endosperm cells is quite open, for the simple reason that actual vitality tests have not been applied. The work of Brown and Escombe above cited indicates, but does not demonstrate, that the aleurone cells are living, and that the amyliferous cells are not.

INVESTIGATION.

Material and its preparation.—By applying to a large wholesale candy house I was able to secure a large quantity of seeds without buying the fruit. There is a further advantage in that such firms get the best and

freshest, so that seeds saved from such fruit are large and nearly all capable of germination. The seeds so obtained are partially cleaned, but it is always necessary to wash them thoroughly without soaking, after which they may be stored in a dry place for use as needed. The fleshy mesocarp contains reducing sugar.

In all the material used embryo substance was absolutely excluded. This was accomplished by rotating the point of a pocket-knife in the embryo cavity of each and every dry seed until, by examination, all traces of embryo substance were removed. In all the material used this task was done by myself. Since the membranaceous endocarp which still clings to the seeds as so far cleaned contains tannin, it is necessary to remove this in some way. This may be accomplished either by sifting over an air blast after each grinding, and before the powder becomes at all fine, or by washing and drying rapidly after each time through the mill. By either method so little tannin remains that digestion in water at ordinary temperature for six hours gives a liquid which does not react with ferric chloride. Both methods were used: the former before it was known that the contained proteids are insoluble in water, and the latter after.

Estimation of the hydrolytic product.—Having found Fehling's solution unsuitable for my purpose, I decided to find a method which would give me more reliable data. Since starch, dextrine, and enzyme are insoluble in 95 per cent. alcohol, while the reducing sugars arising from enzyme activity are soluble, this reagent was employed for separation. The following test shows the efficiency of such a method. A solution of soluble starch¹ was made, and its content of anhydrous starch per cubic centimeter determined by evaporating over steam triplicate volumes, drying the residue at 110° C. for one hour, cooling in the desiccator and weighing. The anhydrous content of a filtered solution of amylopsin was similarly determined. It was thus possible to titrate a given weight of starch with a given weight of enzyme, and then to determine the weight of the hydrolytic product as follows: After digestion the solutions were made 95 per cent. alcoholic and then allowed to stand until the precipitate settled. The alcoholic solution was decanted, filtered, the precipitate washed with alcohol and the filtrate then evaporated to dryness over steam in tared beakers. The residue was air-dried at 110° C. to constant weight. This residue is brown, with a sweetish, toast-like odour, very hygroscopic, easily soluble in water, and reduces Fehling's solution, but the reaction is not precise, and much of the precipitate remains in suspension for some time, but finally settles. From the following tables it is evident that the hydrolytic product soluble in alcohol is very constant for a given ratio of enzyme to starch.

¹ Dry potato starch was extracted with glycerine (180°–190° C.) for thirty minutes and precipitated with alcohol. Purification was accomplished by reprecipitation with alcohol from aqueous solution.

All the figures represent anhydrous weight in grams. Three ratios of enzyme to starch were tried in duplicate. The starch solution contained in 1 cc. 0.01625. The enzyme solution contained in 1 cc. 0.00160. Titration closer than .1 cc. not attempted.

	Enzyme.	Starch.	Hydrolytic Product.
1:100	No. 1—0.004960	0.500	0.4700
	No. 2—0.004960	0.500	0.4700
1:200	No. 1—0.002560	0.500	0.4870
	No. 2—0.002560	0.500	0.4950
1:300	No. 1—0.00160	0.500	0.4180
	No. 2—0.00160	0.500	0.4150

These figures show that a reasonably accurate determination of hydrolytic product can be made with varying dilutions of enzyme. Such a determination is far more satisfactory than the usual colour method with iodine. The enzyme used was advertised to convert into dextrine and maltose 250 times its own weight of starch in three hours at about 40° C. The fact that the hydrolytic product for the ratio 1:200 is greater than that for the ratio 1:100 is to be attributed to difference in digestion conditions, since no attempt at comparison was made except for the duplicates of a given ratio. As dextrine is known to be one of the products of enzyme activity, since dextrine is insoluble in 95 per cent. alcohol, it is evidently excluded in this method. Since the ratio of the hydrolytic product thus obtained is so constant for a given ratio of enzyme to starch, it must be regarded as a very convenient and accurate method for testing the hydrolytic capacity of a given enzyme.

In applying this method to the study of the Date it was found necessary to evaporate to dryness and then extract with alcohol instead of depending upon the alcohol to precipitate all unhydrolyzed matter. This evaporation must continue until the residue is hard. If to the viscous residue alcohol be added, some of the carbohydrates, though insoluble in alcohol, mix with it and when heated an apparently clear solution is obtained. The alcoholic extract should cool to room temperature before filtering. Failure to observe these precautions gave me results from which a conclusion contrary to the one here given was published¹.

During extraction with alcohol the beakers were covered with watch crystals thus making a sort of reflux condenser.

Properties of the aqueous extract.—The powdered endosperm (400 grms.) prepared as described above yields, after about six hours' digestion with distilled water (1,200 cc.), a liquid which when filtered has the following properties. It is neutral to litmus and remains neutral until by putrefaction it becomes acid. It does not contain tannin, as the test with ferric chloride

¹ Pond, '04, p. 181.

was negative. It does not contain coagulable proteid, for on boiling with acetic acid in several dilutions no flocculation occurred. Care was taken to acidify after boiling and not before. On standing twenty-four hours after boiling there was so sediment. Prompt disappearance of turbidity on acidification suggested the presence of phosphates (tribasic calcium phosphate). On testing with molybdic solution a fine yellow precipitate fell promptly, and on standing twenty-four hours an abundant yellow sediment settled (ammonic-phospho-molybdate). The extract, in contact with concentrated nitric acid, failed to give even the slightest turbidity at junction or anywhere, which suggested the absence of all forms of proteid matter except perhaps secondary proteoses and peptones. The xanthroproteic reaction is doubtful, because the extract has a faintly yellow colour and a slight opalescence. The colour, slight as it is, turns to orange with alkali (NaOH and NH_4OH), and similar change occurs with concentrated nitric acid, the colour in the latter case deepening on addition of alkali. Reversing the order of addition reverses the effects, or if heat is generated other colours, such as brown or reddish-brown, are produced. The colouring matter does not react sharply however. The same tests with commercial tannin did not give identical results, although various colourations were obtained. Dilute alkali causes the change of colour but dilute acid does not. The opalescence is, however, destroyed by both reagents. Glacial acetic acid does not produce visible change. Mineral acids, however, if concentrated, seem to have decided positive effect.

Negative results were obtained in the following tests for proteids: Mayer's reagent, Millon's reagent, acetic acid and potassium ferrocyanide, and boiling in an acidified, saturated solution of ammonium sulphate. The latter solution, after filtering, was negative in the Biuret test. The latter reaction and the xanthroproteic test are not reliable with the extract because the colour produced by the alkali or strong acid obscures any evidence of change.

The extract reduces Fehling's solution if not too much reagent is used. If some freshly prepared reagent is diluted and boiled, and then the extract be added slowly, a prompt reduction occurs very soon. If the reagent is present in excess the reduction may not occur. In some cases I noted a heavy flocculation without reduction. This flocculation was found to be produced by the copper sulphate alone. On removing the precipitate the filtrate was promptly reducing. That this reduction was not due to tannin is certain because the test with ferric chloride was negative.

The reaction with phenylhydrazin is positive. Carbohydrates insoluble in alcohol are present, and they may be fractionally precipitated with different strengths of alcohol, but very little precipitation occurs after a concentration of 80 per cent. is reached. The substance precipitated by alcohol when dried is of a dirty white colour and partially soluble

in water. It does not respond to any of the tests for proteids. This behaviour excludes albumins and globulins, and adds emphasis to the negative tests with the extract itself. These observations do not agree with those of Puriewitsch ('97, p. 66) who obtained flocculation on boiling his 'culture fluid' with acetic acid, and a white precipitate with acetic acid and potassium ferrocyanide. Either his observations were faulty or else some coagulable proteid passed from the embryo to the endosperm during germination.

Extraction with sodium chloride. The same powder, after the preceding extraction with water, was digested with 5 per cent. sodium chloride for sixty hours (toluol, 5 per cent.). This extract was neutral to litmus. On heating, a milky turbidity appeared which was more opaque than was that of the aqueous extract. The heating was continued to boiling for a minute, when a flocculent precipitate appeared which redissolved on cooling and reappeared on heating. The turbidity was destroyed by acetic acid and did not reappear until the acidity was neutralized by ammonium hydroxide. Other reactions agreed with those of the aqueous extract, except that a white precipitate formed with acetic acid and potassium ferrocyanide. This latter result was found to be due to impurities in the toluol, as the acetic acid alone with the toluol gave the white precipitate. The flocculation on boiling was found to be due to impurities in the sodium chloride. This leaves the sodium chloride extract negative in all tests for proteids, just as was the aqueous extract, and positive for phosphates. The sodium chloride itself did not react for phosphates with molybdic solution.

Extraction with sodium hydroxide, .1 per cent. The residue remaining after the extraction with sodium chloride was digested sixty hours with sodium hydroxide, .1 per cent. and toluol, 5 per cent. The filtered extract was found to be only slightly alkaline so that the alkalinity had evidently decreased during the digestion. This extract does not have the opalescent hue of the other extracts but instead is of a deep amber colour. This colour is undoubtedly due to the alkali, as it appeared almost instantly when the latter was added. As the neutral point was slowly approached with acetic acid the amber gradually disappeared, being replaced slowly by a dirty opalescence. As the reaction became faintly acid a cloud was noticed, which gradually spread through the liquid as the reaction became distinctly acid. On standing a few minutes this turbidity developed to a flocculation and a curdy precipitate finally settled.

This precipitate changed to brick red on boiling with Millon's reagent. The filtrate did not react with any reagents for proteids nor with ammonium sulphate. The extract itself did not respond to any of the proteid tests except with ammonium sulphate. The latter test was positive because the small amount of proteid present was concentrated into a precipitate. Coagulation did not occur when the extract was boiled at the neutral point.

Another extract was made from fresh dry powder. After two hours digestion the brownish red extract gave a precipitate at the neutral point. Continuing the extraction over night (sixteen hours), the extract did not give a precipitate at the neutral point, and boiling at the neutral point was without apparent effect. When slightly but distinctly acid the precipitate settled.

Extraction with sodium hydroxide, 2 per cent. The residue remaining after the extraction with sodium hydroxide, .1 per cent., was exhausted sixty hours with a 2 per cent. solution of the same reagent. The powder immediately turned a dark blood red with a brownish tinge. The filtered extract was of course alkaline. On close neutralization with acetic acid no change occurred, not even after boiling. Continuing the acidification slowly, no change was apparent when a decided acid reaction was attained, but continuing still farther the brownish tinge gradually disappeared, and when strongly acid a change to cherry red occurred, and a copious flocculent precipitate settled. This result made it clear that a fairly large amount of proteid can be extracted with a proper strength of alkali. The .1 per cent. alkali is evidently too weak to extract all the proteid. Since the 2 per cent. alkali, however, might have effected some decomposition of the proteid, it seemed best to try a moderate strength.

Extraction with sodium hydroxide, .5 per cent. On adding 500 cc. of .5 per cent. NaOH solution to 500 grms. of freshly prepared powder the usual change to brownish red occurred. After forty-eight hours it was noticed that this colour had disappeared, and the reaction was found to be acid to litmus¹. The same volume of alkali solution was then added and the brownish red colour returned and remained for sixty hours. On acidification with acetic acid the filtered extract yielded a copious flocculent precipitate. The latter, after fusing with caustic potash, gave an unusually abundant yellow precipitate of ammonic-phospho-molybdate. This result, in connexion with the data already recorded, may be accepted as evidence of the presence of considerable nucleoproteid. The wet precipitate resembles the extract in colour, being perhaps more brown than red. No colour was imparted to any of the following reagents on stirring the still wet precipitate into them: acetone, methyl alcohol, ethyl alcohol, benzol, petroleum ether, turpentine and carbon bisulphide. Some of the matter which colours the precipitate is soluble in hot water, and this solution gave a weak reaction for tannin with ferric chloride².

Why this extract became acid after forty-eight hours while the .1 per cent. extract was still alkaline after sixty hours is not known. The two cases are not comparable, however, because one was with fresh powder and the other with material which had been successively exhausted with water and sodium chloride.

² The remainder of the precipitate was studied further by Dr. Gies, as far as the quantity would permit, and the following observations were made by him. After drying at room temperature and being reduced to a powder, which was dark red, the precipitate was redissolved in dilute alkali

Demonstration of tannin in the endocarp. The thin membranous endocarp may be obtained in quantity as a chaff by sifting the powder, especially after the first time through the mill. On boiling some of this endocarp chaff in water, a light brown solution was obtained, which gave with ferric chloride a brown precipitate and a brownish green filtrate. By using a more dilute solution of the reagent a typical blue green colour without a precipitate was obtained. On adding lead acetate a grey precipitate fell leaving a water clear filtrate. Potassium ferrocyanide caused a grey precipitate to fall. Iodine gave a reddish brown precipitate.

A cold aqueous extract of the endocarp chaff gave a typical reduction of Fehling's solution, probably due to the reducing sugar held by the membranes. Another portion of chaff, after being washed until an hour's aqueous extract at ordinary temperature did not reduce Fehling's solution, was extracted with boiling water. This extract reduced Fehling's solution, presumably because of the tannin rather than because of sugar. That tannin was present was made certain by the test with ferric chloride. Further, this extract gave the same colour reaction with alkali as the powdered endosperm from which the tannin was not obtained after long digestion in cold water. The endosperm substance entirely freed from endocarp substance by scraping the dry seed does not develop this colour with alkali. Thus it seems probable that the endosperm powder prepared as previously described above contains so little tannin or associated substances that not enough goes into solution in cold water to react with ferric chloride, but that enough is present to produce the change noted when alkali is added.

Recapitulation of preceding data. The facts of special interest now revealed are: the endosperm contains reducing sugar, the endocarp contains tannin, the only proteid present in significant amount is the nucleoproteid.

Experiment No. 1. Is the aqueous extract of the resting endosperm auto-hydrolytic? For this test 400 grms. of powdered endosperm were

(KOH, 2 per cent.) and reprecipitated by slight acidification with dilute acetic acid. This process was conducted three times. The filtrates were amber coloured. The final precipitate seemed as deeply coloured as the first. The filtrates were free from phosphate. The precipitate contained phosphorus. On digestion in pepsin-hydrochloric-acid (2 per cent. each), a slight residue resembling the original precipitate remained, containing phosphorus. It was soluble in dilute alkali and appeared to be a nuclein or a mixture containing it. Hydrated with 2 per cent. HCL a deep red solution was formed, and a brownish black, flocculent material collected. The latter was slowly soluble in dilute alkali and, after being reprecipitated with acid, the colour remained the same and no effort was made to determine the nature of the latter. The filtrate was made strongly alkaline with NH_4OH . Treatment with an excess of ammoniacal silver solution had no effect. There was no precipitate of silver-purin compounds after standing twenty-four hours. These observations indicate that the original precipitate is not a pure substance. It contains not only nucleoproteid but apparently the colouring matter already noted. It is possible also that other substances or compounds of nucleoproteids are present, and further study with more material would be necessary to establish definite conclusions.

used. This quantity of powder was digested fifteen hours in the refrigerator ($12^{\circ}\text{C}.$) in 1500 cc. of tap-water which had been filtered through a Berkefeld filter. No antiseptics were used, the low temperature sufficing to inhibit putrefaction, as was shown by the neutral reaction of the filtered extract. The test for tannin in the filtered extract was negative, so 75 cc. of it were delivered from pipette into each of four beakers. Two of these beakers, group 1, were immediately evaporated to dryness over steam. The remaining pair, group 2, were digested eight days at $35^{\circ}\text{C}.$ During this period a strength of 5 per cent. toluol was maintained. The beakers were covered with a watch crystal and stirred daily. After evaporating to dryness the duplicates of group 2, all four beakers were then extracted with 95 per cent. alcohol by boiling for three hours. After cooling and decanting over night, the alcoholic extracts were filtered into four tared beakers and evaporated to dryness over steam. The residues thus obtained were dried to constant weight at $120^{\circ}\text{C}.$ and being very hygroscopic were cooled in the desiccator and weighed as rapidly as possible. The duplicates of group 1 were respectively .5765 gm. and .5805 gm., while those of group 2 were .5465 gm. and .5610 gm. Evidently the quantity of monoses in solution did not increase and hydrolysis could not have occurred. Since the aqueous extract contains non-reducing carbohydrates, which are converted into reducing sugars by the action of dilute acids, no reason is apparent why hydrolysis should not have taken place, if any enzyme were present, unless it might be that the non-reducing carbohydrates were present in sufficient amount to inhibit any enzyme action. That this was not the cause of the negative result seems clear from the results of subsequent experiments. That the temperature of digestion was favourable must be assumed because the seed germinates much more rapidly at this temperature than at lower ones.

Experiment No. 2. Is the endosperm powder capable of auto-digestion? Into each of four beakers 25 grms. of powdered endosperm were placed. The weighing itself was accurate, but no attempt to get the actual weight of the powder was made, the moisture remaining after three days' exposure to room temperature after grinding being included. Into each of two of these beakers 75 cc. of 1 per cent. CuSO_4 were delivered from pipette, and into each of the remaining two were placed 75 cc. of 1 per cent toluol. All four beakers were incubated ten days at $35^{\circ}\text{C}.$, being stirred daily and the toluol renewed. On the tenth day the liquor from each beaker was filtered, and into four tared beakers 50 cc. of each filtrate were respectively placed. The anhydrous alcohol soluble residues were: toluol, .4326 gm. and .4110 gm.; copper sulphate, .4400 gm. and .4647 gm. The latter residues were coloured by the copper sulphate and no doubt a small amount of the latter was

dissolved by the alcohol, but this would not affect more than the second decimal place. The figures certainly indicate a negative result, and the only possible objection is that mentioned in the preceding experiment, namely, the inhibition of enzyme activity by the presence of substances in solution.

Experiment No. 3. Does an enzyme develop in the endosperm during germination? In experiment number 1, the aqueous extract was from the resting endosperm. In experiment number 2, the endosperm substance was under conditions comparable to those of germination, except that aerobic respiration was impossible and the escape of any hydrolytic products could not proceed normally. So it seemed best to test the behaviour of endosperms which had been under conditions favourable for germination. For this purpose a quantity of degermed but otherwise whole endosperms was divided into two groups. The individuals of group 1 were located on a perforated, floating board so that each rested in a thin film of water. The volume of water which floated the board was about twenty litres and this was renewed daily. The endosperms were kept sterile by washing them for three minutes daily in a one per cent. solution of copper sulphate. The germination of a few seeds whose embryos were intentionally left undisturbed was not prevented by this treatment. That is, of course, the radicles protruded. The individuals of group 2, intended as controls, were placed in crystallizing dishes and enough one per cent. copper sulphate solution added to about one half submerge them. This solution was renewed every three days, so as to make the loss by diffusion comparable with that occurring in group 1. After twenty-eight days at room temperature, the endosperms were ground and the tannin removed as usual. With the powder thus obtained a repetition of experiment number 2 was made, except that the residue from 25 cc. of filtrate was determined instead of from 50 cc. The residues of group 1 were .1310 and .1360 grm. Those from group 2 were .1835 and .1883 grm. Evidently there is no more manifestation of enzyme in this experiment than in the preceding ones. The possibility of the same disturbing factor was not excluded, however, although its potency would necessarily be reduced by the loss of substance from the endosperm during the period under conditions of germination.

The residues are less than one half those found in the preceding experiment. This would be expected, as sugar originally present was lost by diffusion during the twenty-eight days under germination conditions. In both cases the copper sulphate residues are larger, and since by calculation from the solubility of copper sulphate in alcohol, it may easily be seen that not all of the difference can be attributed to this, it seems quite probable that the escape of sugar from the endosperm

proceeds more slowly in the copper sulphate solution. This granted, the result is just what would be expected as a consequence of simple exomose in the absence of enzyme.

Experiment No. 4. Do degermed but otherwise whole endosperms show any internal corrosion or evidence of auto-digestion after a long period under optimum conditions of germination? While preceding tests render a negative answer highly probable, the following experiment was made as a conclusive test. Twenty degermed and carefully cleaned endosperms were placed in a vertical position behind a rubber band which encircled a small crystallizing dish. The preparation was then placed in a dish containing sufficient 1 per cent. toluol to leave the endosperms standing about one-fourth submerged. Diffusion and escape of hydrolytic products were thus well provided for and respiration was not interfered with. The preparation was then kept in the incubator at 35° C. for fifty-six days. A control preparation in 1 per cent. copper sulphate was carried. The solutions of these preparations were, with few exceptions renewed daily. The toluol of this strength is such a weak antiseptic that a daily renewal is necessary as also the bathing of the unsubmerged portions of the endosperms. Seeds will commence germination under these conditions.

At the conclusion of the test period the endosperms were sectioned, stained in Bismarck brown and mounted in glycerine. After clearing over night microscopic examination failed to reveal the slightest evidence of auto-digestion. The walls were intact. No such corrosion markings as figured by Grüss ('94 a, taf. 14 and 15) could be found. Comparison was made with dry resting endosperms but no positive difference could be detected. The middle lamella showed very plainly in all three sets of sections, but no difference either in cell wall or cell contents could be noted.

Conclusion :—The endosperm of *Phoenix dactylifera* is incapable of self-digestion.

While this investigation has been continued intermittently for some years, the major part of the results here recorded have been obtained during my incumbency of a research scholarship, June, July, and August, 1905, in the New York Botanical Garden. I am especially indebted to Professor Wm. J. Gies, consulting chemist of the Garden, and to Dr. D. T. MacDougal, assistant director, for advice and facilities in carrying out the work.

LITERATURE.

- BROWN AND ESCOMBE ('98): On the Depletion of the Endosperm of *Hordeum vulgare* during Germination. *Pro. Roy. Soc., Lond.*, lxxiii, pp. 3-24.
- BROWN AND MORRIS ('90): Researches on the Germination of some of the Gramineae. *Journ. Chem. Soc.*, lvii, p. 458.
- BUNGENER AND FRIES ('86): Ueber das diastatische Vermögen der Gerste. *Zeitsch. Ges. Brauwesen*, N. F., ix, p. 261.
- GREEN ('86): III. On the changes in the Proteids in the Seed which accompany Germination. *Phil. Trans. Roy. Soc., Lond.*, clxxviii, B, p. 39.
- ('90): On the Germination of the Seed of the Castor-oil Plant (*Ricinus communis*). *Pro. Roy. Soc. Lond.*, xlvii, p. 147.
- ('01): The Soluble Ferments and Fermentation. Revised Edition of 1899.
- GRIS, ('64): Recherches anatomiques et physiologiques sur la germination. *Ann. Sci. Nat. Bot.*, 5^e sér., ii, p. 90.
- GRÜSS ('93): Ueber den Eintritt von Diastase in das Endosperm. *Ber. Deutsch. Bot. Gesell.*, xi, p. 288.
- ('94): Ueber das Verhalten des diastatischen Enzyms in der Keimpflanze. *Jahrb. wiss. Bot.*, xxvi, p. 412.
- ('94a): Ueber die Einwirkung der Diastase-Fermente auf Reservecellulose. *Ber. Deutsch. Bot. Gesell.*, xii, p. 60.
- ('95): Die Diastase im Pflanzenkörper. (Vorl. Mitt.) *Ber. Deutsch. Bot. Gesell.*, xiii, p. 2.
- ('96): Beiträge zur Physiologie der Keimung. *Landw. Jahrb.*, xxv, p. 385.
- HABERLANDT ('90): Die Kleberschicht des Gras-Endosperms als Diastase ausscheidendes Drüsengewebe. *Ber. Deutsch. Bot. Gesell.*, viii, p. 41.
- ('04): Physiologische Pflanzenanatomie. 3. Aufl., pp. 444, 476.
- HANSTEEN ('94): Ueber die Ursachen der Entleerung der Reservestoffe als Samen. *Flora*, lxxix, p. 419.
- HÉRISSEY ('03): Recherches chimiques et physiologiques sur la digestion des mannanes et des galactanes par la séminase chez les végétaux. *Rev. Gén. Bot.*, xv, pp. 345-392, 406-417, 444-464.
- LINTNER ('88): Ueber das diastatische Ferment des ungekeimten Weizens. *Zeitsch. Ges. Brauwesen*, N. F., xi, p. 497.
- LINTNER AND ECKHARDT ('90): Studien über Diastase, III. *Journ. prakt. Chem.*, N. F., xli, pp. 91-96.
- LINZ ('96): Beiträge zur Physiologie der Keimung von *Zea Mais*. *Jahrb. wiss. Bot.*, xxix, p. 267.
- NEWCOMBE ('99): Cellulose-Enzymes. *Ann. Bot.*, xiii, p. 49.
- PFEFFER ('93): Ueber die Ursachen der Entleerung der Reservestoffe aus Samen. *Ber. math.-phys. Klasse königl. sächs. Gesell. Wiss. Leipzig*, xlv, p. 421.
- ('97): Pflanzenphysiologie. I, pp. 612-613; Eng. ed. ('00), I, pp. 598-599.
- POND ('04): The Endosperm Enzyme of *Phoenix dactylifera*. Preliminary report. *Science*, xx, p. 181.
- PURIEWITSCH ('96): Ueber die selbstthätige Entleerung der Reservestoffbehälter. (Vorl. Mitt.) *Ber. Deutsch. Bot. Gesell.*, xiv, p. 208.
- ('97): Physiologische Untersuchungen über die Entleerung der Reservestoffbehälter. *Jahrb. wiss. Bot.*, xxxi, p. 13.
- SABLON ('97): Sur la digestion de l'albumen du Dattier. *Rev. Gén. Bot.*, ix, p. 395.
- VAN TIEGHEM ('77): Sur la digestion de l'albumen. *Compt. rend.*, lxxxiv, p. 582.

Pond, Raymond H . 1906. "The incapacity of the date endosperm for self-digestion." *Annals of botany* 20, 61–78.

<https://doi.org/10.1093/oxfordjournals.aob.a089083>.

View This Item Online: <https://www.biodiversitylibrary.org/item/234848>

DOI: <https://doi.org/10.1093/oxfordjournals.aob.a089083>

Permalink: <https://www.biodiversitylibrary.org/partpdf/318822>

Holding Institution

Smithsonian Libraries

Sponsored by

Biodiversity Heritage Library

Copyright & Reuse

Copyright Status: Not in copyright. The BHL knows of no copyright restrictions on this item.

This document was created from content at the **Biodiversity Heritage Library**, the world's largest open access digital library for biodiversity literature and archives. Visit BHL at <https://www.biodiversitylibrary.org>.