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Researches on the Germination of the Pollen Grain and the Nutrition of the Pollen Tube

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X. *Researches on the Germination of the Pollen Grain and the Nutrition of the Pollen Tube.*

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That the deposition of the pollen grain upon the stigma is followed by a process of true germination was established by Van Tieghem as long ago as 1871,* who pointed out the similarity of its behaviour to that of the spores of Lycopodiaceæ and of many ferns, and indicated that the pollen tube may be compared to the prothallium proceeding therefrom, especially in the cases of those prothalli which contain no chlorophyll. Its growth is prepared for by the deposition in the grain of several forms of reserve material, chiefly of carbohydrate nature. During the process of germination these reserve materials have been found to change, indicating an active metabolism, while at the same time active respiration goes on, as shown later by Mangin.† Not only do we find reserve materials deposited in the pollen grain, but further, we can identify a similar store in the tissues of the style, especially when that organ is a long one, and the pollen tube has consequently some distance to travel before reaching the ovules.

In most cases of the storage of reserve materials in plants we find evidence of the utilization of such stores by the presence and activity of enzymes. Thus in seeds, Gorup-Besanez,‡ Guignard,§ Brown and Morris,‖ and others, have proved their presence; in tubers, Baranetzki¶ found them in the potato; in leaves, Brown and

* Van Tieghem, "Recherches physiologiques sur la régétation libre du pollen, &c.," 'Ann. des Sc. Nat., Bot.,' 5e série, vol. 12, 1871.

† Mangin, "Recherches sur le pollen," 'Bull. Soc. Bot. de France,' vol. 33, 1886.

‡ Gorup-Besanez, 'Deutsch. Chem. Gesell. Ber.,' 1874.

§ Guignard, 'Journal de Botanique,' 1890, p. 385, *et seq.*

‖ Brown and Morris, "Researches on the Germination of some of the Gramineæ," 'Journ. Chem. Soc.,' 57, 1890.

¶ Baranetzki, 'Die stärkeumbildenden Fermente,' 1878.

Morris,* and Vines† have demonstrated their existence, and their relation to the temporary deposits of starch in the chlorophyll grains. The writer‡ has shown various enzymes to exist in seeds and tubers, transforming for the nutrition of the plant, proteids, fats, and carbohydrates.

Pollen being the seat of a germinative process, physiologically comparable to those already mentioned, the probability of the activity of enzymes therein, is at once apparent. Nor is evidence wanting that such bodies exist there; Van Tieghem§ has found that when the pollen of Narcissus, Crocus, and some other species, is cultivated in 10 per cent. solution of cane sugar, in no very long time after the sowing some of the sugar becomes inverted. The result is brought about when the grains are allowed to grow freely, or when such growth is prevented by the presence of chloroform in the culture fluid.

Strasburger‖ also points out that when pollen grains are allowed to grow in the presence of a very thin starch paste, a transformation of the starch into sugar can gradually be noted, indicating the presence of diastase.

The probability of certain pollen grains containing a cytolytic enzyme has been noted by other observers. Thus it has been shown that in the progress of the pollen tube of grasses through the tissue of the style it often passes between the cells, instead of through them, burrowing thus through the middle lamella. Strasburger¶ notes similar behaviour of the tube in several genera of Dicotyledons, especially certain of the Caryophyllaceæ and the Malvaceæ.

In all these cases, however, the action of the living pollen grain only has been observed. The question of an enzyme capable of being extracted by appropriate solvents, and of acting while in such solution, still remains open to investigation. In a paper which the writer communicated to the British Association at Cardiff, in 1891,** the existence of diastase in such a condition and the possibility of extracting it, were dealt with. Since that date more extended experiments have been carried out, which form the subject of the present paper.

From the work already quoted above, the enzymes which were to be expected in pollen grains appeared to be diastase, invertase, and a cytolyst. The presumption in favour of the latter was not so strong as that in favour of the two first named,

* Brown and Morris, "A Contribution to the Chemistry and Physiology of Foliage Leaves," 'Journ. Chem. Soc.,' May, 1893.

† Vines, 'Brit. Assoc. Reports,' Cardiff, 1891.

‡ Green, "On the Changes in the Proteids in the Seed, &c.," 'Phil. Trans.,' vol. 178, 1887, B. *Ibid.*, "On the Germination of the Seed of the Castor-oil Plant," 'Proc. Roy. Soc.,' vol. 48, p. 370. *Ibid.*, "On the Germination of the Tubers of the Jerusalem Artichoke," 'Ann. of Botany,' February, 1888.

§ Van Tieghem, "Inversion du Sucre de Canne par le pollen," 'Bull. Soc. Bot. de France,' vol. 33, 1886.

‖ Strasburger, "Ueber fremdartige Bestäubung," 'Jahrb. f. wiss. Bot.,' vol. 17, p. 94.

¶ "Neue Untersuchungen über den Befruchtungsvorgang bei den Phanerogamen," 1884.

** 'Brit. Assoc. Reports,' Cardiff, 1891, p. 696.

though it was supported by the discovery of such an enzyme by MARSHALL WARD in the hyphæ of a species of Botrytis examined by him in 1888.* These hyphæ were found to perforate cell walls in much the same way as pollen tubes make their way through the tissue of the style and ovary, and they yielded on appropriate treatment a fluid which softened and dissolved cellulose, just as the extracts of the scutellum of Barley grains prepared by BROWN and MORRIS.† The appearance and mode of growth of the pollen tube simulate very closely the phenomena observed by MARSHALL WARD in the development of the mycelium of the Botrytis.

The method adopted in investigating the existence of isolable enzymes in pollen was the following. Flowers were selected whose stamens were just beginning to dehisce and the pollen extracted from them by careful dissection. When a large quantity of pollen was required, such stamens were taken out and allowed to dry at the ordinary laboratory temperature in watch glasses; they slowly opened in drying and were then well shaken and sifted through fine muslin. The pollen so collected was ground up in a glass or agate mortar, till microscopic examination showed most of the grains disintegrated. In some cases the powder was then mixed with thin starch paste (1 per cent.), and the effect observed. In others, the powder was suspended in either glycerine or 5 per cent. solution of NaCl, to which ·2 per cent. of KCy was added as an antiseptic. In yet other cases, a few drops of either chloroform or oil of cinnamon were used to keep away bacteria, the latter essence having been shown by CADEAC and MEUNIER‡ to be a strong bactericide. The extracts were allowed to stand for a few hours and then filtered, and the resulting filtrate used to act on starch paste, or solution of cane sugar. Sections of delicate cellular tissue were used to examine for the cytolyst, being put in a small quantity of the extract in watch-glasses. The digestions were carried out either at the ordinary laboratory temperature, or at 38° C. in an incubator, controls, in which the extract had been boiled for several minutes, being carefully kept in each experiment. In many cases it was found that the pollen extracts themselves had the power of reducing FEHLING's fluid. When this was the case, a further control was prepared, consisting of the extract diluted with water instead of either cane sugar solution or starch paste, the proportions being carefully adjusted in each set, so that they might be strictly comparable. After a sufficient time had been given for action, the quantity of reducing sugar formed in each digestion tube was determined by the method of boiling with excess of FEHLING's fluid, filtering, combustion, and weighing the resulting CuO. When chloroform or oil of cinnamon were used as antiseptics, they were removed by boiling the solution till they had evaporated, before titrating with the FEHLING's fluid.

* 'Annals of Botany,' II., 319.

† "Researches on the Germination of some of the Gramineæ," 'Journal of the Chemical Society, June, 1890, 458.

‡ 'Ann. de l'Inst. Pasteur,' vol. 3, 1889, p. 317.

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In the case of diastatic action, the effect of adding iodine from time to time to a portion of the digestion was also noted.

Details of some of the experiments in each case are appended.

I. *Diastase.*

Lilium candidum.—1. Ground pollen used without extraction—A quantity of the ground pollen, the contents of two anthers, was mixed with 10 cub. centims. of thin starch paste of 1 per cent. strength, 5 cub. centims. boiled and cooled, 5 cub. centims. left as prepared.

After 46 hours the unboiled tube gave a purple colour with iodine, the boiled one a blue. After 24 hours longer action the unboiled tube gave no colour with iodine, while the control became blue. Testing now with FEHLING's fluid, the unboiled tube gave a copious reduction, the control gave none.

2. Glycerine extract prepared from pollen from several stamens, as described above. When filtered, 5 cub. centims. were boiled, and 5 cub. centims. taken as prepared. Each was mixed with 5 cub. centims. starch paste and exposed at the temperature of the laboratory. At intervals, the two were tested with iodine, usually ten drops being taken from each. The results are expressed in the following table.

Time of digestion.	Unboiled extract.	Boiled extract.
26 hours	Purple-blue	Blue
2 days	Purple	,,
3 ,,	Red-brown	,,
6 ,,	Colourless	,,

The contents of the tubes were then boiled with FEHLING's fluid, when the unboiled gave a copious reduction and the boiled one a faint trace.

Though the action was carried on for several days, no bacteria appeared in either tube. The filtrate, as prepared was quite clear and free from any *débris* of pollen.

Helianthus.—Pollen very small. 1. Mixed at once with 1 per cent. starch paste, and half the mixture boiled. Digested both at 18°C. and at intervals tested a few drops with iodine.

Time.	Unboiled.	Boiled.
2 days.	Red-purple	Blue
3 ,,	Colourless	,,

Boiling both these digestions separately with FEHLING's fluid, the unboiled gave a copious reduction, the boiled one scarcely a trace.

2. Glycerine extract of ground grains prepared as in case of Lily.

Time of action.	Unboiled.	Boiled.
1 day	Reddish-purple	Blue
2 days	Red-brown	,,

The action was stopped at this point, and each tube boiled with FEHLING'S fluid. The unboiled reduced it copiously, the boiled one gave hardly a trace of Cu_2O.

Corylus avellana.—Extract made by steeping ground pollen in 5 per cent. NaCl + ·2 per cent. KCy solution for twenty-three hours and filtering. In this case further controls were prepared, the tubes being mixed as follows :—

A. 5 cub. centim. extract + 5 cub. centims. 1 per cent. starch paste.
B. 5 ,, ,, boiled + 5 cub. centims. 1 per cent. starch paste.
C. 5 ,, extracting fluid + 5 cub. centims. 1 per cent. starch paste.
D. 5 ,, water + 5 cub. centims. 1 per cent. starch paste.

These were left to digest at 20° C. in the laboratory, with the following results when samples were treated with iodine.

Time of digestion.	A.	B.	C.	D.
19 hours	Red-purple	Blue	Blue	Blue
44 ,,	Red-brown	,,	,,	,,

On boiling the remainder of each with FEHLING'S fluid, at the end of the 44 hours, A reduced it copiously, the others not at all.

Comparing this experiment with those previously narrated, the advantage of the salt solution over glycerine as a solvent is evident.

Lilium pardalinum.—A more careful quantitative experiment made with the pollen of this species may be quoted here, though it was made with another object which will be referred to later. ·3 grm. of pollen was weighed out, carefully ground up and mixed with 60 cub. centims. of starch paste (1 per cent.). After agitation to ensure an equal diffusion of the *débris* with the starch, it was rapidly divided into two, and half of it heated for fifteen minutes in a water bath to 98° C. The two, labelled P and P*b*, were then put for three days in an incubator at 38° C. At the end of that time P was perfectly limpid and clear, while P*b* was apparently unchanged. No bacteria had developed. They were both then passed through filters to remove the *débris* of the pollen, which had settled to the bottom of the beakers, and the filtrates boiled with excess of FEHLING'S fluid. Both gave a reduction, that of the control being less than the other, and no doubt due to a reducing sugar which other experiments

had shown to be present in the pollen of this species, as will be described later. The resulting precipitate was thrown in each case on to a filter, washed, dried, and after combustion in a crucible, weighed as CuO. The results were as under :—

	P.	Pb.
Gross weight of crucible, ash of filter, and CuO	1·357 grm.	1·282 grm.
Weight of crucible and ash	1·246 „	1·246 „
Weight of CuO	·111 „	·036 „

There was a quantity of reducing sugar in the grains of pollen represented by ·036 grm. CuO. Deducting this from the CuO found in P., we have ·075 grm. of CuO, due to the sugar formed by the diastase in half the ·3 grm. pollen used.

These experiments show that diastase exists in pollen, and that it can be extracted as readily from the grains as from the cells of other parts of the plant.

Besides the species already mentioned, experiments showed its presence in the pollen of Gladiolus, Anemone, Antirrhinum, Tropæolum, Pelargonium, Crocus, Brownea, Helleborus, Alnus, Tulipa, and Clivia; also in that of Zamia after germination begins. The action on starch grains suggests that it belongs to the translocation variety of Brown and Morris,* as it dissolves the starch grains without corrosion. More on this point will, however, be described in connection with experiments made on the pollen tube and its contents.

II. *Invertase.*

The first experiments made with a view to the identification of this ferment were qualitative only.

Eucharis grandiflora.—Pollen was collected from several anthers just commencing to dehisce, and was ground up in an agate mortar, and mixed with 10 cub. centims. of solution of 5 per cent. NaCl and ·2 per cent. KCy. After standing 20 hours it was filtered till clear; 2 cub. centims. of this extract were then mixed with 10 cub. centims. of a weak solution of cane sugar, and a similar control was prepared with 2 cub. centims. of the extract boiled.

The two were tested at intervals by boiling 1 cub. centim. of each with 1 cub. centim. of Fehling's fluid diluted with 4 cub. centims. of water. Results were as under :—

* *Op. cit.* Also Green on "Vegetable Ferments," 'Ann. of Bot.,' vol. 7, p. 86.—March, 1893.

Time.	Unboiled.	Boiled.
After 19 hours . .	Trace of reduction	No reduction
„ 43 „ . .	Liquid turned yellow, but gave no precipitate	Faint greenish tint replaced the pure blue
„ 6 days . .	Copious reduction and precipitate of Cu_2O	No further reduction

The pollen used in this case was only the contents of three or four small anthers, but the result shows that a workable quantity of invertase can be extracted from even so little.

Narcissus papyraceus albus.—The pollen, after grinding up, was extracted in this case by 10 cub. centims. dilute glycerine, to which ·5 per cent. of asparagin had been added, according to the suggestion of EFFRONT,* the amide having the property of accelerating the action of any enzyme present.

After extraction the residue was filtered off and suspended in another 10 cub. centims. of the same mixture, with a view to ascertaining whether all the enzyme could be extracted by one exposure to the fluid. The extracts were labelled A and B respectively.

5 cub. centims. of each was then boiled, and digestion tubes were prepared as under :—

A. 2 cub. centims. extract A + 2 cub. centims. solution of cane sugar ;
B. Boiled control ;
C. 2 cub. centims. extract B + 2 cub. centims. solution of cane sugar ;
D. Boiled control ;
E. 2 cub. centims. glycerine mixture + 2 cub. centims. solution of cane sugar, without either extract.

All were placed in the incubator at a temperature of 38° C.

After 24 hours a small sample of each was boiled with FEHLING'S solution. A gave a strong red reduction ; C a yellowish-red one ; while the controls all alike showed a faint greenish tinge, presumably due to a trace of inverted sugar in the cane sugar used.

After a further 24 hours the differences noted were intensified, showing a progressing inversion in A and C. The controls remained as before, all showing a very slight trace of reduction.

The pollen was thus shown to contain invertase, and to yield it up only incompletely to extraction.

Some quantitative experiments were subsequently made upon two other species.

Narcissus pseudo Narcissus.—A quantity of pollen, weighing ·4 grm., was ground

* EFFRONT. "Sur les Conditions Chimiques de l'Action des Diastases," 'Comptes Rendus,' vol. 115, p. 1324.—December 26, 1892.

up in an agate mortar, and mixed with 100 cub. centims. of salt mixture (5 per cent. NaCl + ·2 per cent. KCy), and allowed to extract for several days. The mixture was then filtered, and the filtrate examined microscopically, and found to be free from bacteria. As invertase had been demonstrated to exist in an allied species, a boiled control was not employed in this experiment.

Two tubes were prepared, one containing the extract + 100 cub. centims. of cane-sugar solution, and the other 100 cub. centims. of the same salt mixture as that with which the pollen had been extracted, added to the same quantity of sugar solution. After three days' action, the two were boiled with excess of FEHLING'S solution, and the resulting precipitate was filtered off, washed, and subjected to combustion in a platinum crucible. The quantity of CuO from the one was ·038 grm., and from the other ·021 grm., giving ·017 grm. CuO due to invert sugar, produced by the action of the invertase on the sucrose.

Narcissus poeticus.—·1 grm. of pollen was taken and extracted in 15 cub. centims. chloroform water, without bruising, for two days. Then it was filtered, and the filtrate added to 25 cub. centims. of 10 per cent. solution of cane sugar. A control was prepared, consisting of 15 cub. centims. chloroform water and 25 cub. centims. of the same sugar solution. A few drops of oil of cinnamon were added to each as a further antiseptic,* and the two were digested at the ordinary laboratory temperature for four days. Then, after boiling for some time till the chloroform and oil of cinnamon were removed, they were again boiled with excess of FEHLING'S fluid, and the oxide filtered off, washed, heated in platinum crucible to redness till the weight was constant, and weighed. The control gave ·0098 grm., the pollen extract gave ·0978, or nearly ten times as much.

Besides these pollens, invertase was found in that of *Helleborus*, of *Richardia* (the so-called Arum-lily), of *Lilium pardalinum*, and of *Zamia skinneri*.

Of other pollens examined for the two enzymes, diastase was found to be absent from Lupinus, Lathyrus, Eucharis, Richardia, and Narcissus; invertase was not found in Alnus and Clivia.

A few experiments were made with a view to determining the existence of a cytolyst and a proteolyst, but in no case could either be found.

The enzymes present in resting pollen grains are, therefore, chiefly diastase and invertase, but their distribution is irregular, some containing one, some the other, and some both. Where diastase occurs, it is the form described by BROWN and MORRIS as the "translocation" variety. This is apparently the form indicated in STRASBURGER'S experiments already referred to.

The enzymes are with difficulty completely extracted by solvents, even several days' action of the various extracting fluids leaving some behind in the residue. Of the solvents used, 5 per cent. NaCl solution is the most effectual.

* CADEAC, *op. cit.*

Changes in the Quantity of Enzyme during the Germination of the Pollen Grain and the Growth of the Pollen Tube.

Many experiments were made with various pollens to ascertain which species would germinate most freely, and in what culture fluids they could most easily be made to put out pollen tubes. Eventually, various species of *Narcissus* and of *Lilium* were selected as yielding invertase and diastase respectively. The pollen of these genera was found to germinate in water, and in various strengths of cane-sugar solution. Some experiments made with the pollen of *Zamia skinneri* also yielded instructive results.

In making these experiments, a quantity of pollen was collected from several hundreds of anthers, and equal weighed quantities were cultivated on glass plates under bell jars over water. As it was impossible for them to grow in the presence of antiseptics, the cultures were carefully watched and examined at short intervals to guard, as far as possible, against the danger of ruining the experiment by the introduction of micro-organisms. In many cases the tubes attained a good degree of development in a few hours, some species of Lily putting them out in two hours or less. In other cases, the cultures proceeded for one or two days in safety. After the culture was made, the germinating grains and their tubes were digested under various conditions with either cane sugar or 1 per cent. starch paste, further germination being inhibited by addition of antiseptics, usually ·2 per cent. of potassic cyanide. Controls with ungerminated pollen, or extracts of it, were kept side by side with the others. In some cases, the cultures were dried on the plates at a low temperature (40° C.), and the dried residue collected and ground up in the agate mortar, the controls in each experiment being treated in the same manner as the cultures.

The most striking experiment with invertase was made on the pollen of *Narcissus poeticus*. The pollen was collected from 906 anthers, a quantity weighing ·3 grm. being yielded by this number. This was divided into three parcels of ·1 grm. each. One parcel (A) was steeped at once in 10 cub. centims. chloroform water; another (B) was set to germinate in water on a glass plate; and the third (C) similarly in cane-sugar solution (15 per cent.). When germination was well advanced the cultures were carefully washed from the plates, and all made up to 15 cub. centims. with chloroform water. They were then all filtered, mixed with 25 cub. centims. of 10 per cent. cane-sugar solution, and allowed to digest for 93 hours at the ordinary laboratory temperature.

At the expiration of this time, after removal of the chloroform by boiling for some time, 20 cub. centims. of each were again boiled with excess of FEHLING'S solution, and the copper oxide filtered off, washed and weighed, after combustion, in a platinum crucible.

A yielded ·1 grm. CuO; B, ·24 grm.; and C, ·65 grm.; a blank experiment showing ·01 grm.

Calculating the sugar produced in each case in 100 cub. centims. of the digestion

mixture for purpose of comparison, we find that 100 cub. centims. containing the extract of the ungerminated pollen would yield an increased production of CuO of ·45 grm.; that germinated in water an increase of 1·159 grms.; and that germinated in cane sugar solution one of 3·187 grms. As 1 grm. of cane sugar inverted produces 2·321 grms. of CuO, we find that in 100 cub. centims. of the three mixtures respectively ·19 grm., ·5 grm., and 1·37 grm. of cane sugar were inverted, showing a marked increase of invertase as the tubes developed. The difference between the two latter is not so great as the figures quoted seem to show, as no doubt a good deal of inversion took place during the culture in cane-sugar solution before the extracts were made. In allowing for the difference in time it will be remembered that in the case of C during extraction and digestion alike, the pollen grains and their enzymes were in the presence of considerable excess of cane sugar, so that the progress of the action may be taken to have been uninterrupted and regular. The culture was continued for 45 hours, and the subsequent digestion for 93 hours. In the cases of A and B sugar was present only during the latter period. Dividing the total sugar produced by the number of days during which the enzyme was acting in each case, we have as a day's inversion ·049 grm., ·13 grm., and ·24 grm. of cane sugar severally, which may be taken as a very fairly accurate measurement of the invertase present in the pollen grains and tubes under the conditions set forth.

Commenting upon this and similar experiments, it appears probable that a thin-walled pollen grain yields its enzymes fairly readily to extracting solutions without any preliminary bruising, and that the pollen tube with its still thinner membrane is especially likely to do so. In cultures made under the conditions described, inversion of the cane sugar began very soon after the experiment was set going, progressing continuously all the while the pollen tube was growing, the experiments so confirming those of Van Tieghem,* while carrying them further.

Comparing the thickness of the coat of the pollen grain with that of the wall of the pollen tube, it could be objected to the experiment detailed above that the difference observed might be partly explained on the ground of difference in the completeness of extraction. Though this objection does not seem a very strong one in face of the duration of the experiment, an effort to ascertain whether better extraction would explain the difference was made in the following manner.

Two parcels of ·025 grm. pollen each were weighed out and exposed under similar conditions to contact with solution of cane sugar, germination being allowed to proceed in one case, and in the other being inhibited by the addition of chloroform. As is well known, this reagent does not inhibit the action of invertase, though it prevents the pollen grain from putting out its tube. As soon as the tubes were just beginning to make their appearance germination was stopped. The tubes then were not more than half the length of the diameter of the grain. It may be assumed that the extraction of the enzyme from the grains had been practically the same in the two

* Van Tieghem, *loc. cit.*

cases. The cultures were then separated from the fluids, ground up separately in an agate mortar and extracted with the usual salt mixture, with a trace of chloroform as an extra precaution, the culture fluids being later added to the extracts. After 24 hours the two were digested with considerable excess of 10 per cent. cane-sugar solution for 22 hours, then, after removal of the chloroform as in other cases, boiled with excess of FEHLING'S fluid and the CuO estimated as before. The result was that the germinated grains inverted nearly 25 per cent. more cane sugar than the ungerminated ones. Though this increase is slight, as compared with that found in the last experiment quoted, it must be remembered that germination was stopped very soon after it had started, in order that there might be no possibility of extraction from a long thin-walled tube. It is still sufficiently marked to support the conclusion that the enzyme is actually increased when germination of the grain commences. For the present it will be best to defer discussing how this increase is brought about, and examine the phenomena of germination in pollen containing diastase.

The alterations of diastatic power evinced during germination were studied in the pollens of various species of *Lilium* between June and September. The first experiment, commenced on June 29, gave results that may be quoted in detail.

The pollen was collected from *Lilium croceum*, and parcels weighing ·0215 grm. each were used for comparison. One was cultivated in water for 24 hours, till the tubes were of a good length, say twenty times the diameter of the grain. In consequence of the possibility of loss in filtration, and the difficulty of grinding up the culture while wet, the grains with their tubes were mixed at once with thin starch paste, containing about 1 per cent. of starch, and the quantity made up to 100 cub. centims. The other parcel of pollen was ground in an agate mortar, and mixed with an equal quantity of the same starch paste. The 100 cub. centims. were in each case divided into two equal volumes, those containing the germinated pollen labelled A and B, and those with the ungerminated grains labelled C and D. A and C were put into the incubator to digest at 38° C.; B and D were allowed to remain at the temperature of the laboratory, then 18° C. After 24 hours, a few drops from the A and C set were tested with iodine, when A gave a red and C a purple colour. With FEHLING'S solution, a measured sample from A gave a more copious reduction than a similar quantity from C. A little later both digestions were boiled with excess of FEHLING'S solution, and the resulting precipitate treated as in other cases. The reduction in A was found to be nearly five times that in C, a very considerable increase. The total quantities were small, as would be expected from the preliminary experiments already quoted, which show that the pollen does not contain much ferment, 24 hours being usually hardly long enough to show decided action. The B and D set were allowed to digest at 18° C. for a week, when the diastatic power was found to be increased in about the same proportion, the quantities of maltose obtained being, of course, much greater.

Similar results were obtained with the pollen of *Lilium pardalinum*. The pollen

of the Lily has a very strongly thickened coat, impregnated with a considerable quantity of a resinous colouring matter, rendering extraction a matter of some difficulty. An experiment was consequently made to ascertain whether extraction could be complete by the use of a solvent, or whether, as in so many cases, particularly of leaves,* the residue of the grains after extraction retained much of the diastase. Two parcels of pollen of *L. pardalinum* were taken, each weighing ·2 grm. One was put to germinate in water, and the other steeped at once in water containing ·2 per cent. of potassic cyanide to inhibit this process. After the tubes had obtained a fair length, the culture was filtered and the filter washed with water, the washings being added to the filtrate. The grains, with their tubes, were then extracted with the usual salt mixture for 48 hours, when the extract was filtered off. The residue was then suspended in a further quantity of the extracting fluid. The other parcel was treated similarly, so that there were prepared from each a filtrate from the culture, a salt extract of the grains, and a residue suspended in fluid. Each of the six was made up to 25 cub. centims., and the KCy adjusted that each should contain ·2 per cent. of the antiseptic. The two sets were labelled G and H respectively, and each 25 cub. centims. was mixed with 30 cub. centims. of ·1 per cent. starch paste. Digestion was carried out for three days at 18° C., its progress being noted by testing a few drops with iodine at regular intervals, and finally titrating with Fehling's fluid. The first outcome of the experiment was that the total diastase was increased by about 50 per cent. in the germinated pollen. There was a good deal of difference in the distribution of the diastase in the several digestions of the two sets. Of the total amount found in the G set, 45 per cent. was in the filtrate, 29 per cent. in the extract, and only 26 per cent. in the residue; while in the H set, with a smaller total quantity, 23 per cent. was in the filtrate, 13 per cent. in the extract, and as much as 64 per cent. in the residue. This shows how difficult it is to extract the enzyme from the thick-walled pollen grain, and how relatively easy to obtain it from the thinner-walled pollen tube. The total result, however, shows that the increase observed in the whole experiment is a real one, and not a question of incomplete extraction.

One experiment made upon this pollen appears to indicate that the increase noted above is not an immediate one, but that it is preceded by a diminution during the early stages of the growth of the tube. This is a different result from that arrived at in the case of the invertase, and shows that the process is not exactly alike in all cases. Too much stress should not be laid upon it, though the results are rather striking. It was made with the last sample of the pollen of *L. pardalinum* which would germinate, and for want of fresh material it could not be repeated. The probable explanation of the result will be dealt with later, when discussing the general question of the formation of the pollen tube. In conducting the experiment two parcels of the pollen, each weighing ·1 grm., were taken; one was ground up at once

* *Cf.* Brown and Morris, "On the Chemistry and Physiology of Foliage Leaves," 'Journal of the Chem. Soc.,' May, 1893, p. 634. Also Vines, 'Annals of Bot.,' 1891, p. 409.

and steeped in water, the other was germinated for seven hours, till fair tubes had made their appearance. The culture was then removed from the plate, and the grains and tubes ground up as in the case of the other parcel. Without filtering, each was mixed with 20 cub. centims. of 1 per cent. starch paste, and put in the incubator at 38° C. At the end of 18½ hours both were filtered rapidly, the filtrates boiled with excess of FEHLING'S solution, and the resulting precipitate treated as usual. The final weights of CuO were—

From the digestion with germinated pollen ·036 grm., corresponding to ·027 grm. maltose.

From the digestion with ungerminated pollen ·0595 grm., corresponding to 044 ·grm. maltose.

(1 grm. of maltose reduces 1·345 grm. of CuO.)

The course of action in the pollen of *Lilium pardalinum* appears, therefore, to be that during the first few hours of germination, there is a diminution of the quantity of diastase, followed by a recovery and subsequent increase.

Some further experiments with reference to the existence of diastase were carried out on the pollen of *Zamia skinneri*, one of the Cycads. The pollen grains of this plant differ from those of the Lily in not containing starch as a reserve material, though when their tubes are growing in a suitable environment, starch soon makes its appearance in them. The pollen grains of *Zamia* are roundish to oval in shape, with a crease-like mark down their longest diameter. They will not germinate in water, but will do so fairly readily when sown upon pieces of boiled or raw pear or apple pulp. Less freely they may be cultivated in the expressed and filtered juice of either of these fruits. As said above, they contain no starch. Examination was made by mounting them in a strong solution of chloral hydrate, to which a little alcoholic tincture of iodine had been added. This reagent slightly swells the grains, and at the same time renders them extremely transparent, while the iodine colours any starch that may be there.

Experiments failed to show any sufficient evidence of diastase in the resting grain, though it was sought for carefully, as starch soon appeared when the germination began.

Several experiments made with apple juice and its various constituents taken separately, soon showed that the question of the growth of the tube mainly turned on the question of the absorption of carbo-hydrate material, and that the vegetable acid of the juice is not essential, though it is possibly advantageous. The process of germination was very slow, so slow indeed that usually the cultures were spoiled by the growth of moulds, the mycelia of which could be seen to be infesting the cone. In all cases, however, the output of a pollen tube was preceded by the appearance of starch in the grains, and it was soon possible to detect the commencement of germination by this occurrence, which generally was noticeable about twenty-four hours after sowing the pollen.

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To determine whether germination was accompanied by a development of diastase, which would probably mean the same thing as an increase of the original quantity in the case of the Lily, three equal parcels of pollen were taken. One was steeped in water, and one in apple juice, while the third was used dry. After two days the grains soaked in juice were swollen and contained numbers of starch granules; those steeped in water were swollen like the others, but contained no starch: their protoplasm was somewhat more granular than before. The two were then dried in the incubator at 38° C., and all three ground up separately, and extracted with ·2 per cent. KCy solution for twenty-four hours, the acidity of the residue of the juice being carefully neutralized before extracting. The extracts were filtered, and mixed with a little 1 per cent. starch paste. Care was taken especially to see that all three were exactly alike in the quantity of starch, of KCy, and of water, also that the reaction was alike in all three.

Each was then divided into two, and half of it boiled for fifteen minutes on a water bath. Action was allowed to proceed in the incubator at 38° C. for three days, when it was stopped, and the several digestions boiled with excess of FEHLING's fluid. The resulting precipitates were collected on filters of known ash, and washed with hot water. The filters were then dried and subjected to combustion in a platinum crucible till the weight was constant.

The results were as under:—

Juice culture.	Unboiled tube.	Boiled tube.
	grms.	grms.
Weight of crucible, ash, and CuO	1·304	1·298
Weight of crucible, 1·244 grm. ash ·001 „	1·245	1·245
Weight of CuO	·059	·053

giving ·006 grm. reduced by the maltose produced by the diastase in the germinating grains.

Neither the resting grains, nor those that had been steeped in water gave any evidence of diastatic action, there being only the merest suggestion of reduction on boiling the digestions with FEHLING's fluid.

In *Zamia*, then, as starch is produced after the absorption of sugar by the pollen grains, and before visible germination commences, there is a simultaneous formation of diastase to provide for its digestion. No such formation takes place, unless the sugar is absorbed. Either cane sugar or grape sugar will give rise to this appearance of starch. What the antecedent condition of the diastase may be, or whether it is secreted by the protoplasm when required, is a point that will be referred to later.

One more curious fact with regard to the diastase of the pollen of *L. pardalinum*

may be narrated here. Attempting to confirm the initial diminution of diastase on the commencement of germination, as described above, the pollen was found to have lost its power of putting out tubes. Out of a large quantity sown, very few grains even commenced growing. It seemed desirable to investigate the diastatic power of the pollen in this condition, and to see whether any connection could be traced between the power of germinating and the activity of the enzyme. The pollen remaining weighed ·3 grm.; it was ground up in an agate mortar and mixed with 60 cub. centims. of starch paste (1 per cent.). Half of it was then boiled for fifteen minutes, and the two were set side by side in the incubator at 38° C., labelled P and P*b* respectively.

The digestion was continued for 22·75 hours, when both were boiled with excess of FEHLING'S fluid, and the resulting precipitates collected, treated as usual, and weighed as CuO. Deducting the small amount yielded by P*b*, which was due to a little sugar in the pollen, P had formed sugar corresponding to ·075 grm. CuO, which, estimated as maltose, equals ·057 grm.

While this pollen still retained the power of germination, the experiment described on pp. 396–397 had been carried out with it, and as the diastase in the grains before germination was then determined, the two experiments may now be compared. In the first, ·05 grm. of pollen yielded diastase which, working in the presence of excess of starch, formed ·044 grm. maltose in 18·5 hours. In the second, ·15 grm. pollen, working under the same conditions exactly, formed ·057 grm. maltose in 22·75 hours. Reducing these two to ·05 grm. pollen, working for one hour, we get, in the first case, a formation of ·0024 grm., and, in the second, of only ·0008 grm. of maltose, showing that with the failure of power to germinate, the amount of diastase was reduced to one-third the original quantity.

Growth and Nutrition of the Pollen Tube.

The variations in the amount of enzyme obtainable from the grains at different periods of their life, taken in connection with the different contents of the grains of various species of plants, suggested that the growth and development of the pollen tube is not a uniformly simple process, but one showing a very definite relation to the environment in which each finds itself, and to the various nutritive materials occurring in the grains themselves, and in the styles of the plants to which they belong.

To examine this in some detail was the object of many experiments, of which the most important were made upon the pollen of *Narcissus*, *Lilium*, and *Zamia*. All these can be made to germinate with fair success, the last named being the most refractory, and its cultivation, for the reasons already stated, being attended with most difficulty.

The grains were sown in various media, in hanging drops in closed glass chambers which could be transferred to the stage of the microscope. The most convenient form

of chamber was that first used by Professor MARSHALL WARD. It consisted of a glass tube, in the centre of which an oval bulb was blown. This was broken above and below, and the two apertures of the fracture ground smooth. One aperture was then cemented to a glass slide, while a coverslip, on which was placed the hanging drop, was laid upon the other, the chamber being kept full of moist air by loosely plugging the ends of the tube with wetted cotton wool. The chamber and the coverslip were luted by a little olive oil. In these tubes the cultivation proceeded satisfactorily for several days.

The grains of Narcissus grew fairly well in drops of water, but were best developed in solution of cane sugar, 15 per cent. being found to be the most favourable degree of concentration. After two or three days, the tubes attained a length of 20 or 30 times the diameter of the pollen grain. They were long narrow tubes with clear transparent walls, and had usually somewhat dilated ends, in some cases forming globular swellings, which were often larger than the grain itself. These globular ends had softer and thinner walls than the rest of the pollen tube. When, as was not infrequently the case, the end did not dilate, the walls of the tip were thicker than those of the rest of the tube. The mode of growth suggested a good deal of internal tension, accompanied usually by a softening of the tip, much like that of the hyphæ of Botrytis, as described by Professor MARSHALL WARD.* In most cultures the globular swellings did not appear, and it is probable that they are abnormal appearances produced by mal-nutrition. In the Lily pollen tubes they never occurred, the wall there not being particularly different at the ends and along its length. The contents were always vacuolated, with an accumulation of granular matter, particularly towards the tip. The granules were very large and refringent. In external contact with the tip of the pollen tube generally a large number of these refringent granules appeared, looking as if extruded from the tube. That this was the case seems probable, for, in many instances, the two masses of granules within and without the tube seemed almost continuous, the thin wall, however, being usually visible between them. In one case an appearance was presented supporting strongly the idea of excretion from the tube; this was in a tube of *Narcissus poeticus*, where a well-defined aperture, with regular and even edges, was visible on one side of the tip just behind the apex, and the granules could be seen streaming from it. This aperture had not the appearance of an irregular rupture, which was observed many times in swollen tubes.†

The protoplasm surrounding the vacuolation all along the tubes was extremely granular, and the more so the more quickly the tubes had been developed. Very marked movements or currents of circulation were exhibited by the protoplasm,

* *Loc cit.*

† VAN TIEGHEM, in his paper on pollen already referred to, describes similar appearances. He says the tubes are often pierced at the end of the terminal swelling, sometimes at a single point, when the greater part of the plasma escapes in the form of a large drop or tear, and sometimes at many points, each then exuding a droplet. *Loc. cit.*, 1871.

particularly in the tubes of the Lily. The tubes of *Zamia* developed very slowly, and the culture was always ruined by the appearance of mould before they had attained a length of more than five or six times the diameter of the pollen grain. They were chiefly noteworthy for certain peculiarities in their reserve materials.

In most of the cultures, besides the appearances described leading to the hypothesis of extrusion of granules without rupture of the tube, a large number of the tubes ruptured irregularly, often suddenly, with a violent expulsion of the finely granular protoplasmic contents. Sometimes a piece of the tube was broken off, sometimes the split was lateral. This appearance is no doubt abnormal, and due to excessive absorption of water from the liquid culture medium, which is not the environment naturally furnished. The distension of the tip alluded to is possibly due to the same cause. The granular matter extruded from these ruptures was very different to the refringent granules leaving the tube at the apex and remaining for a time in contact with it.

During this process of development and growth it is evident that the tube must receive nutriment in some form. The appearances described and the evidence already given of the excretion of ferment into the culture fluids point to a source of this nutriment in the tissue of the style. At the same time, it has been shown by De Planta, Mangin, and others, that the grain itself is a storehouse on a small scale, various grains differing in the nature of their contents, the latter, however, being almost always starch or some form of sugar.

The presence of starch in those pollen grains in which it exists can be readily demonstrated by mounting the mature grains in strong solution of chloral hydrate to which a little alcoholic tincture of iodine has been added. After a few hours the chloral hydrate renders the grains nearly transparent, while the iodine stains the starch. When treated in this way the starch grains appear usually as very minute specks embedded in the brown-stained protoplasm. Their number varies very much, some grains staining almost black from the amount present; others showing the isolated specks with great distinctness. Examining pollen of *L. pardalinum* in different stages of development it became evident that the starch begins to be deposited there early in the maturing of the anthers, and that the quantity gradually increases till the pollen grains are mature. In ripe grains some granules were seen to give not a blue, but a purplish-red colour with the iodine.

In many pollen grains the quantity of starch increased at the onset of germination when the latter took place in a nutrient fluid.* In *Zamia*, as already mentioned, no starch is present in the resting grain, but germination is always preceded by its appearance. As this secondary storage is not observable in water cultures, the inference is clear that a larger supply of nutritive material than serves for immediate requirements leads to the reinforcement of the reserves of the resting grain.

A second reserve material, not uniformly present, is dextrin, to which the purple-red

* This was noted by Mangin in his experiments on respiration of pollen, *loc. cit.*, p. 517.

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colour of certain of the granules is due. This is probably not a constant constituent, but due to enzyme action within the grain, as will appear later.

The presence of various sugars has been pointed out by many writers, particularly by De Planta, who found 14 per cent. of cane sugar in the pollen of *Corylus avellana*,* and 11 per cent. in that of *Pinus*. In experiments on this group of constituents, the pollen of *Lilium pardalinum* was especially examined for the presence of cane sugar. A quantity of pollen was taken, which weighed 2·346 grms. It was carefully washed with ether till all colouring matter was removed, when its weight was 2·061 grms., showing a loss of ·285 grm., or 12·15 per cent. due to resin and colouring matter. It was then extracted with boiling absolute alcohol on a water bath, an inverted condenser being fitted to the flask. After an hour it was set aside to cool, and allowed to remain under the alcohol for some days. The latter was then decanted off, and the pollen again extracted as before with a fresh quantity of the spirit. The second extraction was followed by a third, conducted similarly. The alcoholic extracts were mixed together and evaporated to dryness on a water bath, when a sticky residue was left. This was dissolved in 36 cub. centims. of water and the solution divided into two equal quantities. Half was mixed with an appropriate quantity of pure invertase† and digested in the incubator at 36° C. for several hours, to invert any cane sugar present.

After this digestion was complete, both quantities were boiled with excess of Fehling's solution, the resulting precipitates collected on filters of known ash, washed, dried, and incinerated in a platinum capsule till the weights were constant. These were found to be as under :—

	Inverted half.	Uninverted half.
Crucible + ash + CuO . .	1·413 grms.	1·3065 grms.
Crucible + ash	1·251 ,,	1·251 ,,
CuO	0·162 ,,	·055 ,,

The increase in reducing power was due to the inversion of cane sugar by the invertase. In the 36 cub. centims. of extract there was therefore cane sugar corresponding to 2 (0·162 — ·055) or ·213 grm. CuO. The invert sugar arising from the inversion of 1 gram of cane sugar reduces 2·321 grms. CuO, therefore ·213 grm. CuO corresponds to ·091 grm. cane sugar. As this quantity was found in 2·346 grms. pollen, the latter contained 3·9 per cent.

The other sugar was most likely (though not certainly) maltose, judging from the

* "Ueber die chemische Zusammensetzung des Blüthenstaubes der Haselstaude," 'Landwirth. Versuchs.,' 6e Reshe, 1884 and 1885.

† Kindly sent me by Mr. Horace T. Brown.

presence of starch, a little dextrin, and diastase in the pollen grain. Computing it as maltose, there would be in the total extract a quantity corresponding to a reduction of 2 (·055) or ·111 grm. CuO. 1 gram of maltose reduces 1·345 grm. CuO, therefore the extract contained ·083 grm., which equals 3·54 per cent. of the dry weight of the pollen used.

An examination was also made of the sugars present in the pollen of *Lilium tigrinum*. This pollen contained but little starch, and was very strongly impregnated with resinous sticky matter, the grains being thereby coloured a dark red-brown.

The parcel of pollen taken weighed 2·24 grms. It was repeatedly extracted with ether as before till the solvent came away colourless. Dried and weighed, it was found to have lost ·7 grm., or 31·2 per cent. It was then extracted as before several times with boiling absolute alcohol, the several extracts mixed and evaporated to dryness on the water bath. The residue was dissolved in 300 cub. centims. water and neutralized with a drop or two of weak ammonia.

The extract was now divided into three: 100 cub. centims. were boiled at once with excess of FEHLING'S fluid and the CuO ascertained as before. The final weight was ·041 grm. CuO, or ·123 grm. computed for the whole quantity.

100 cub. centims. were warmed with an appropriate quantity of pure invertase in the incubator at 38° C. for several hours, and then treated as the first 100 cub. centims. This gave no evidence of inversion by increase of reducing power.

The third 100 cub. centims. were boiled for two hours with 2 per cent. H_2SO_4 in a flask provided with an inverted condenser, neutralised and titrated as the other two. The final weight of the CuO in this case was ·064 grm. or ·192 grm. computed for the whole quantity.

There being no increase brought about by the action of invertase, cane sugar was not present in this sample of pollen. The increase in the quantity boiled with acid was due to the transformation of a certain quantity of maltose to glucose; the reduction in the unchanged extract to the presence of this maltose with probably some glucose.

The increase in reducing power is ·192 − ·123 = ·069 grm. The maltose corresponding to this is ·069 ÷ 0·976 = ·071 grm. (0·976 representing the difference between the amount of CuO reduced by 1 grm. of maltose, and the CuO reduced by the glucose to which 1 grm. of maltose gives rise when hydrolyzed). This amount of maltose computed on 2·24 grms. equals 3·17 per cent. As ·071 grm. maltose reduces ·0955 grm. CuO, and the original reduction was ·123 grm., we have left ·0275 grm., which must have been reduced by glucose. As 1 grm. glucose reduces 2·205 grm. CuO, this corresponds to ·0125 grm. of this sugar, or ·56 per cent.

VAN TIEGHEM states that the pollen of Narcissus, Crocus, Cheiranthus, and Viola, among others, does not contain cane sugar.*

* "Inversion du Sucre de Canne par le pollen," *loc. cit.*

The sugars in different pollen grains are thus seen not to be constant; cane sugar, maltose, and glucose all being present, separately or together, in different species.

Before dealing with the question of nutritive materials in the styles, it may be well to state what can be seen of the fate of these different reserves in the pollen grain as the tube grows. The microscope is of no use to us in the case of the sugar. No doubt a study of the various sugars present in cultures of tubes at various stages of germination would lead to valuable results as to the metabolic changes involving sugars, but such study has still to be made.

In the case of the starch, some observations made upon tubes of *Lilium pardalinum* are worth quoting. The ungerminated grains, as already said, when treated with iodine in chloral-hydrate solution, showed minute granules of starch, generally filling the cell, but mixed here and there with grains staining like dextrin. As the tube was put forth from the grain, these granules were gradually carried over with the protruding portion, and they flowed slowly down the tube as it extended. When the tube was as long as twice the diameter of the grain, they were found to be gradually changing in colour, becoming slightly purple with the iodine. The tube still elongating and the grains travelling forward, this change was more and more marked, particularly near the tip of the tube. When the latter had reached a length of 20 or 30 times the diameter of the grain, the general effect of the iodine was markedly changed. There were but few blue granules, and those in the part nearest the pollen grain. The greater part of the length of the tube was studded thickly with purple grains, and towards the tip they become nearly red. The starch was evidently in process of digestion by the diastase, ministering to the great formation of cellulose composing the wall of the tube. The granules did not change their shape and showed no corrosion, even when magnified very highly, but were gradually being digested in the usual fashion of translocation.

It has already been pointed out that there is an excretion of the enzymes from the pollen-tube into the culture fluid, indicating the same thing as probable when a grain is germinating in the normal way upon the surface of the stigma. The gradual progress of the tube down the conducting tissue of the style appears to be attended by the absorption of nourishment as it passes, for in the case of such flowers as the Lily, the length of the tube is far too great for its cellulose to be supplied from the comparatively small store of carbohydrate in the pollen grain itself. We must look, therefore, to the tissue of the style as the seat of some metabolism, having for its purpose the feeding of the pollen tube during, at any rate, the latter part of its growth.*

When examined with the microscope, the centre of the style of the Lily is seen to be hollow, and continuous with the cavity of the ovary. The cells are many layers in thickness around this cavity, the external layer being an epidermis with stomata.

* Mangin and Van Tieghem both state that they could conduct the process of germination of pollen in a culture medium longer when the fluid contained nutritive matter than when it did not.

The central canal in the style is small, but well defined, and lined by an extremely well-marked epithelium, the cells of which are rounded or papilla-like towards the cavity, recalling very much the appearance of the epidermis of the stigma, with which they are continuous. The style has usually three fibrovascular bundles running up it, placed symmetrically.

When the sections are mounted in chloral-hydrate and iodine solution, the epithelium cells and the cells of several layers under them are found to be full or nearly full of minute starch granules, of about the same size as those found in the pollen grain. The outer layers are free from these. The path of the tube is down the canal or the cells abutting on it, the so-called "loose conducting tissue," where the starch is plentiful. A longitudinal preparation of the style, made by soaking one in the chloral-hydrate solution till it is transparent, which usually takes five or six days, shows that the distribution of the starch is still more significant. Besides being in the conducting tissue, it is plentiful in the outer soft tissue of the fibrovascular bundles, indicating a definite deposit or reserve store placed in the conducting tissue after formation in the leaves. The deposit does not extend to the stigma, but falls short just before the style opens out to form that structure, indicating that the store of reserve material here is intended for the growth of the pollen tube after it has exhausted the special store of the pollen grain.

The amount of this deposit of starch varies in different styles.

Besides starch the tissue of the gynæcium evidently may be expected to contain sugar, even if we only consider the sticky nature of most stigmas. An investigation of the nature and amount of this sugar was made on two species of *Lilium*.

L. tigrinum.—165 styles of various ages were collected. They were rapidly washed in water to remove adherent sugar from the stigmas, dried on blotting paper, and weighed while turgid, being found to weigh 37·73 grms. They were then dried, first at moderate temperature, and then at 100° C., and, after cooling, weighed again, being then 3·403 grms. They were ground up in a mortar and extracted repeatedly with boiling absolute alcohol, as in the case of the pollen estimation already detailed on p. 402. The final residue from evaporation of the alcoholic extracts, consisting of the sugars of the styles, was dissolved in 300 cub. centims. of water; 100 cub. centims. were titrated at once, 100 cub. centims. warmed with invertase for 23 hours, and 100 cub. centims. boiled for two hours with 2 per cent. H_2SO_4 in a flask provided with an inverted condenser. This was then neutralized. After titration the weights of the CuO were found to be as under.

100 cub. centims. original extract.	100 cub. centims. + invertase.	100 cub. centims. + acid.
Gross 1·3245 Tare 1·251	1·375 1·246	1·435 1·251
·0735 or ·2205 in the whole	·129 or ·387 in the whole	·184 or ·552 in the whole

The difference between—

(2) and (1) is ·387 — ·220 = ·167 = CuO due to inversion of cane sugar.

(3) „ (2) „ ·552 — ·387 = ·165 = CuO „ grape sugar formed from maltose.

As 1 grm. of cane sugar inverted reduces 2·321 grms. CuO, ·167 grm. CuO indicates ·072 grm. cane sugar present in the styles, or 2·1 per cent. of their dry weight of 3·403 grms. So, also, ·165 grm. CuO is equivalent to ·17 grm. maltose (·165 ÷ ·976 = ·17),* which gives 5 per cent. in the weight of the dried style tissue. The original reduction of the fluid is given by (1) and amounts to ·2205 grm. Now, ·17 grm. maltose would reduce ·228 grm. CuO, which agrees fairly well with this quantity; glucose, therefore, was not present in the styles. Taking the sugars found in conjunction with the water evaporated, we have for the concentration of the sap ·21 per cent. cane sugar and ·49 per cent. maltose.

L. pardalinum.—105 styles were collected, found to weigh ·566 grm. when dried, and then treated as before. The final residue after solution in water was divided into two, and half warmed for 24 hours with an appropriate quantity of invertase. Both were then boiled with excess of FEHLING's fluid, and the CuO collected and weighed as in other cases. The weights were—

Original solution.	Solution + invertase.
Gross 1·279	1·292
Tare 1·249	1·249
·030 or ·06 in the whole	·043 or ·086 in the whole

The increase in (2) = ·026 grm., which corresponds to ·0112 grm. cane sugar inverted, or 1·96 per cent. of the ·566 grm. dry weight of the styles. Also taking the reducing sugar in the residue as maltose, ·06 grm. CuO = ·0446 grm. maltose, or 7·9 per cent. of the same dry weight.

Besides these analyses of the styles of the two species of Lily, experiments were made with those of *Narcissus pseudo-Narcissus*, which proved to contain cane sugar, and a reducing sugar the nature of which was not satisfactorily determined. The cane sugar amounted to about 6 per cent. of the dry weight of the styles examined.

The quantity of starch present in styles of different ages was found to vary, the maximum observed being in those flowers which were just ready for pollination. As it diminished after this, and in old styles, whose attached ovaries were swelling into the stage of fruit, there was often but little to be found, it appeared possible that the style not only stores starch for the pollen tube, but may go further and present some of this at least to the advancing organ in the shape of the maltose found. Experiments were made to ascertain the presence or absence of diastase in the style, as well as in the pollen grain. The species taken for these experiments was *L. auratum*, the season of flowering of the others being over. Two stages were selected, one from flowers

* *Cf.*, p. 403.

which had been fertilized and showed the fruit in course of formation; the other from those whose stamens were just mature.

To secure the action of all the enzyme that might be present, the fresh styles were bruised to pulp in a mortar, and the pulp mixed, as it was, with the usual thin starch paste (1 per cent.). Half of each mixture was then boiled for several minutes, and the four quantities were allowed to digest in the incubator at 40° C.

Action was noticeable in 21 hours, the unboiled tubes having become limpid, while the controls were opalescent, as at first. After four days the difference in colour in samples treated with iodine was very marked, and the action was thereupon stopped. The digestions were then boiled with excess of FEHLING's solution, and the CuO determined as usual. The older styles gave a reduction which, when divided by the number of styles taken, amounted to ·0045 grm. CuO each; the younger, similarly computed, reduced ·014 grm. CuO each. Diastase consequently appears to be present normally in the styles, side by side with the starch, the quantity diminishing after fertilization.

Invertase was also tested for in a similar manner, but none could be detected.

In the course of development of the pollen tube, we have thus clearly two stores of reserve material for its nutrition. Part is deposited in the pollen grain itself, its nature and amount varying considerably in different plants. The grains also contain at some time or other the enzymes necessary for the transformation of these reserves into plastic material. At the same time, and particularly in styles which are of some length, the style contains a subsidiary store, part of which is transformed by enzymes also present in the style, and part by the excreted enzyme of the pollen tube. The action of the diastase is partly intracellular, as shown by the gradual transformation of the starch granules as they pass down the tube, and partly extracellular, hydrolysing the starch granules in the cells of the style. The relative times of action of the two portions of the diastase are indicated by the locality of the distribution of the starch in the latter, none being present in the portion just below the stigma. The extracellular action of the invertase is evident when we remember VAN TIEGHEM's observation that cane sugar is not in the pollen of Narcissus, while we have seen that it exists in the style.*

The secretion of enzymes in this case does not appear to be a starvation phenomenon, as noted in many other cases, especially in the hyphæ of Botrytis,† which show in many respects a similar mode of growth to that of the pollen tube. On the contrary, well-nourished grains show a greater formation than starved ones, indicating that the absorption of food material is a strong stimulant, much as has been determined in the case of the peptic secretion of the stomach. Pollen of Narcissus allowed to germinate in water did not yield so much invertase as the same quantity germinated in cane-sugar solution, the proportion being as 13 : 24.

* Compare pp. 403 and 406.

† MARSHALL WARD, *loc. cit.*

In other experiments on the same point the absorption of sugar led to a still larger increase. In *Zamia* no enzyme could be detected in the resting grains, but, on the absorption of cane sugar or glucose, even before visible extrusion of the pollen tube had taken place, a small amount of diastase was found to have been secreted. The temporary decrease of diastase noticed in the case of *Lilium pardalinum* as germination began may perhaps be explained by the assumption that the transformation of some of the reserve starch of the grain takes place before the protrusion of the tube, and that this involves a partial consumption of the enzyme. The secondary increase would then follow the absorption of food material from the culture medium as soon as the thin-walled intine allowed this to take place. At the same time it must be mentioned that the increase is not altogether dependent on such nutrition, for there was an increase of the quantity in grains germinating in water only (see p. 393).

Whether the enzymes exist in the pollen grains in the state of zymogen is a question of some interest. Only a little evidence was obtained on this point, drawn from a study of the *Zamia* pollen. The culture medium which best suits this pollen is, as already shown, the expressed juice of the apple or pear. Even the cane-sugar solution gives less satisfactory results than the juice, or the pulp of the fruits. As these juices both contain malic acid, we have in them just the condition needed to transform zymogen into enzyme. A small quantity of pollen was extracted with chloroform water for two days and filtered. Half of it was then made faintly acid with malic acid and warmed for twenty-four hours in the incubator. After careful neutralization with very dilute ammonia the two were mixed with starch paste, and half of each mixture was boiled to serve as a control. After forty-eight hours' action the four tubes were tested with iodine, when the one that contained the extract that had been warmed with acid showed very slight evidence of diastatic action; the one with the extract as prepared showed none. The controls were both unchanged. The quantities used in the experiment were only small, and the experiment can hardly be taken as certain evidence on the point, though the results were confirmatory of the hypothesis so far as they went. More experiments on the point are, however, necessary.

The course of events in the germination of the pollen grain appears to be the following. When it falls upon a suitable substratum, it absorbs water from the moist surface of the stigma, and swells, becoming generally more granular. This is followed by the absorption of whatever food material may be present. In presence of the water, intracellular digestion of the reserves at once begins and the ferment is rapidly increased after absorption of food. In some cases this increase only takes place after a temporary diminution, but ultimately a much larger quantity is present than at first. Very soon an excretion of the enzyme takes place, the reserves of the style thenceforward being attacked and affording the tube the plastic material for its further growth. In many pollens the absorption of the sugar is followed by the temporary increase of the starch in the tube, notably so in the case of *Zamia*, which contains none at starting.

At the same time the style takes part in the nutritive processes, by itself transforming part of its starchy reserves.

Though it would, perhaps, be at present premature to say that the process of pollen germination is altogether dependent on the presence of enzymes in the ripe grains, it is a very significant fact that as the grains lose their power of germination with increased age, this loss of power is attended by a very marked diminution in the quantity of diastase that can be extracted from them.

Summary.

The results of the above-described experiments may be briefly summarized as follows :—

1. Diastase and invertase are both present in pollen grains, and can be extracted from them by the same treatment as has been found effectual in the cases of seeds and foliage leaves. The relative quantities differ a good deal; while some pollens contain both, others possess only one, which may be either of the two. Various solvents may be used for extraction, 5 per cent. solution of NaCl being the most generally useful.

Though the presence of a cytolyst is suggested by the growth of some pollen tubes, it has not yet been demonstrated.

2. At the onset of germination, usually the amount of both diastase and invertase is considerably increased. In one species examined, this increase was preceded by a primary diminution. When the pollen grain has lost the power of germinating the quantity of diastase has materially decreased.

3. The pollen tube is nourished during its growth by plastic material derived from two sources, the store of reserve matter deposited in the grain itself, and a further store deposited in the style.

4. The reserve store of the pollen grain consists of different materials in different species; starch, dextrin, cane sugar, maltose, and glucose being the forms in which it is found.

5. The store in the style consists usually of the same carbohydrates, with the exception of dextrin.

6. The style itself contains enzymes to assist in preparing the reserve materials for absorption by the pollen tube, while the latter excretes the same ferments during its progress down the conducting tissue.

7. The absorption of food materials appears to be one cause of the increase of enzyme found to occur during the germination.

8. The absorption of food material is usually so active that the reserve store of the pollen grain is often largely increased by a temporary deposition, either in the grain or its tube, of some of the absorbed sugar in the form of starch.

9. There is a certain amount of evidence pointing to the existence of zymogens in some pollens, particularly such as germinate best in a faintly acid medium.

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