Summary

An improved spectrophotometric method for the determination of lipoxidase activity was developed and applied in studies of the purified enzyme and crude enzyme preparations from leguminous seeds, with linoleic acid solubilized in Tween 20 as the substrate. The optimum pH was found to be 7.0, 6.5, 6.0, and 5.5, for purified soybean lipoxidase and for the crude lipoxidases extracted from gram flour (*Cicer sp.*) soybean meal (*Glycine max* L.), and hydrated mung beans (*Phaseolus aureus* L.), respectively. The application of this test illustrates A) that the present method was free from the inherent limitations on pH present in the original methods, B) that increasing the amount of detergent, in a fixed concentration of fatty substrate, caused inhibition in the enzymatic activity; more pronounced inhibitions occurred when the concentrations of detergent as well as of fatty substrate were increased in equal proportions, and C) peroxide formation is proportional to time of reaction and to enzyme concentration.

Determination of lipoxidase activity in germinating mung beans indicated 3 periods of enzyme activity: a slow decline from 8 to 24 hours, a rapid decline from 24 to 40 hours, and a linear decline from 40 to 80 hours of germination.

Literature Cited

1. Bergström, S. 1946. On the oxidation of linoleic acid with lipoxidase. Arkiv Kemi Mineral Geol. Band 21A. No. 15.
2. Fritz, G. J. 1962. Studies on O_2-fixation by maize seedlings. Plant Physiol. 37: xxxii–xxxiii.
3. Holman, R. T. and S. Bergström. 1951. Lipoxidase or unsaturated-fat oxidase. In: The Enzyme Chemistry and Mechanism of Action, J. B. Sumner and K. Myrbäck, eds. Academic Press Inc., New York. vol. 2, Part 1, 559–74.
4. Holman, R. T. and G. O. Burr. 1945. Spectrophotometric studies of the oxidation of fats. IV. Ultraviolet absorption spectra of lipoxidase-oxidized fats. Arch. Biochem. 7: 47–54.
5. Holman, R. T. 1948. Lipoxidase activity and fat composition of germinating soy beans. Arch. Biochem. 17: 459–66.
6. Holman, R. T. 1955. Measurement of Lipoxidase Activity. In: Methods of Biochemical Analysis, D. Glick. ed. Interscience Publishers, New York. vol II, 113–19.
7. Tappel, A. L. 1962. Lipoxidase. In: Methods in Enzymology, S. P. Colowick and N. O. Kaplan, eds. Academic Press., New York and London. vol 5, 539–42.
8. Theorell, H., S. Bergström, and Å. Åkeson. 1946. Activity determination and further purification of lipoxidase. Pharm. Acta Helv. 21: 318–24.

Chemotropic Response of the Pollen of Antirrhinum majus to Calcium [1, 2]

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We reported earlier (A) our failure to identify among a large number of organic compounds active in biological systems any that exerted a chemotropic effect on the pollen tubes of snapdragon and (B) the results of extraction and fractionation procedures that suggested the chemotropic factor from gynoecia to be a quite small molecule, heat stable, water soluble, and associated with larger molecules from which it could be separated by various means (14). This led to a search among inorganic ions, particularly those reported at one time or another to enhance germination and tube growth of pollen. When we tested the chlorides of Ca, Mn, and Zn at several different concentrations, Mn and Zn proved to be inactive but Ca elicited a pronounced chemotropic response. The subsequent investigation of the chemotropic effect of Ca is reported in this paper. A brief summary of a part of these studies was previously published (15).

[1] Received May 6, 1963.

[2] Most of the research reported in this paper is part of a dissertation submitted by the first author to the Graduate Division of the University of California at Berkeley in partial satisfaction of the requirements for the degree of Doctor of Philosophy in the field of Plant Physiology. The work was supported by research grant G-7031 from the National Science Foundation.

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Materials and Methods

The basic procedures and materials are described in detail in the earlier report (14). Flowers of *Antirrhinum majus* (tetraploid) grown in the University Botanical Garden provided both gynoecia and pollen. For a brief period, when these plants were not available, cut flowers were purchased from a local florist.

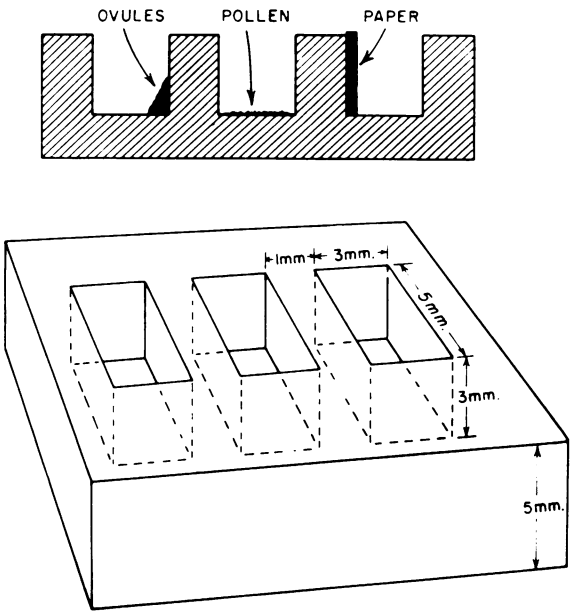

FIG. 1. Schematic diagram of the arrangement of the depressions in agar and the positioning of materials in the depressions for the assay of chemotropism.

The apparatus for the assay of chemotropic activity was an agar plate in which were formed 15 sets of 3 depressions as diagrammed in figure 1. The floor of the center (pollen) chamber was coated with pollen by means of a fine brush. The material to be tested was placed in one of the end (test) chambers against the wall facing the pollen chamber. The materials tested were of various forms. Solid materials such as plant parts, $CaCO_3$, and calcium malate were placed directly in the test chamber. Solutions were tested in either of 2 ways. Infrequently, agar was added to a solution to give a concentration of 1%. While still melted, a drop equal in volume to the test chamber was added to the chamber where it rapidly solidified. More often, measured amounts of solutions were absorbed into a 3 × 5 mm piece of Whatman No. 1 filter paper which was then dried with gentle heat and positioned in the test chamber against the dividing wall. The third or control chamber usually was left empty, particularly after it was found that matching the content of this chamber with that of the test chamber, except for the substance under test, gave results no different than when the control chamber was left empty. After the apparatus was assembled, it was incubated for 8 hours at 25° in the dark. Readings entailed counting the number (and frequently measuring the length) of the pollen tubes that grew into the agar walls separating the pollen chamber from the test and control chambers. A typical response to ovules is shown in figure 2a. The number of tubes growing into the test wall is proportional to the concentration of active material up to tube counts of approximately 200. The system apparently saturates a little beyond this level; moreover, the tubes are so crowded that accurate counting becomes impossible.

The basic nutrient medium (hereafter referred to as the plate medium) used in the bioassay consisted of 10 or 20% sucrose (depending on the lot of pollen), 1% yeast extract (Difco), 0.01% boric acid, and 1% agar (Difco)[4]. The optimum concentrations of sucrose and yeast extract for germination and tube growth were determined by appropriate tests.

The concentration of calcium in tissues was determined as follows. Tissues were dissected from flowers, dried in an oven at 85° for 24 hours, and weighed. The oven-dry material was then ashed in a muffle furnace at 550° until free of carbon. An acid extract of the ash was prepared and after removal of interfering ions, calcium was determined by titration with cyclohexanediamine-tetraacetic acid using calcein as the indicator (4).

Flowers containing Ca^{45} were obtained as follows. A snapdragon plant growing in the Botanical Garden was lifted from the ground on March 8, 1962 and the roots washed free of soil. The plant was then placed in a heated greenhouse in a jar containing 3.5 liters of nutrient solution [see Complete (FeEDTA) in table I of Machlis and Torrey (13)]. The nutrient solution was changed periodically until May 14, 1962 at which time the first floral buds were just beginning to appear. At this time the plant was transferred to a jar containing fresh medium but in which the $Ca(NO_3)_2$ was replaced with 1.0 mc of $Ca^{45}Cl_2$. During the next several weeks flowers were collected immediately after the dehiscence of anthers. The radioactivity of ashed samples was determined under a Micromil window tube in an atmosphere of Q gas.

Table I

The Effect of Calcium as ($CaCl_2$) on the Directional Growth of Pollen Tubes

The plate medium consisted of 10% sucrose, 0.1% yeast extract, 0.01% boric acid, and 1% agar. Nothing was placed in the control chamber. The readings were made after incubation for 8 hours at 25°. Each value is the average of 4 replicates.

Calcium µg per paper	Growth to calcium		Growth to control	
	Number	Length (mm)	Number	Length (mm)
0.0	21	0.3–0.4	20	0.3–0.4
0.8	46	0.3–0.5	23	0.3–0.4
1.6	70	0.3–0.6	27	0.3–0.4
2.4	90	0.3–0.6	26	0.3–0.4
4.0	110	0.3–0.6	26	0.3–0.4
6.0	198	0.4–0.8	33	0.3–0.5
8.0	226	0.5–0.9	45	0.3–0.5
9.6	260	0.5–0.9	47	0.3–0.6
20.0	269	0.5–0.9	127	0.3–0.6

[4] To eliminate any possibility of ambiguity over the use of concentrations expressed as per cent the procedure with sucrose, yeast extract, boric acid, etc., was to take the appropriate weight of solute and dilute (with solution) to the final volume, i.e., for 10% sucrose 10 g of sucrose was diluted with water to 100 ml. With agar, for technical reasons, the appropriate weight was added to the final volume of solution, i.e., for 1% agar 1 g of agar was added to 100 ml nutrient solution.

Results

The response of pollen tubes to calcium is presented in table I. The amounts of calcium indicated were added to rectangles of filter paper from a methanol solution of 1×10^{-2} M $CaCl_2$. The number of tubes growing towards the source of calcium is proportional to the amount of calcium until saturation is reached. Although the higher numbers of tubes could not be counted accurately attempts to do so were made and indicated that there was no further substantial increase in the number of tubes that grew into the wall. The data in table I also show that the higher amounts of calcium cause a growth of tubes towards the control chamber in excess of the background number. It is presumed that calcium diffused across the pollen chamber into the control wall where it then caused tubes to grow towards it. If the assay is made at the end of 3 hours instead of 8 hours, the number of tubes growing into the control wall is the same at all concentrations of calcium.

The cation calcium and not the anion associated with it is the cause of the response of the tubes; $CaCl_2$, $Ca(NO_3)_2$, $CaSO_4$, calcium gluconate, and calcium caseinate were all active. The chlorides of Mg, Ba, Sr, Na, and K over a wide concentration range (10 µliter per paper rectangle of solutions of 1×10^{-4}, 1×10^{-3}, 1×10^{-2}, 1×10^{-1}, and 1.0 M) failed to elicit a response, thus demonstrating the specificity of calcium. In these tests pH was not a factor. Noninhibitory (of germination) solutions of these salts (all except 1.0 M $MgCl_2$, 1×10^{-1} and 1.0 M $BaCl_2$, 1×10^{-2}, 1×10^{-1}, and 1.0 M $SrCl_2$) had pH values that overlapped extensively with those of active solutions of $CaCl_2$ and $Ca(NO_3)_2$ (pH 4.15–6.30).

Very few pollen tubes grow out of the pollen chamber unless calcium is supplied from the test chamber. It will be proposed later that the tubes stay in the pollen chamber in response to calcium released from the pollen grains. What happens if the plate medium itself contains calcium? As might be expected, when all of the agar surrounding the pollen chamber (bottom and all sides) contains sufficient calcium the tubes grow out of the pollen chamber in all directions. This response is shown in table II. Important parameters from these data to be used later are that growth of tubes out of the pollen chamber in all directions becomes high with concentrations of calcium in excess of 1×10^{-4} M, that the optimum concentration for such growth is about 1×10^{-2} M with the pollen used in this experiment, and that concentrations in excess of 4×10^{-2} M inhibit germination.

We next sought to determine what levels of calcium were present in various tissues of snapdragon, how these levels were related to the chemotropic activity of these tissues, and to what extent calcium was released from tissues into agar. About 350 flowers were collected and dissected into the parts listed in table III. All of these were analyzed for calcium and tested for chemotropic activity. The data show that there is a correlation between total concentration of calcium in the tissue and the chemotropic activity of the tissue. There is, moreover,

Table III

The Concentration of Calcium in the Floral Tissues of Snapdragon

Tissue	Dry wt	Ca	Activity*
	g	% dry wt	
Style, upper third	0.195	0.51	+
Style, middle third	0.135	0.50	+
Style, lower third	0.137	1.24	+
Ovules and placenta	0.332	2.17	+
Ovary walls	0.559	1.31	+
Pedicels	1.298	1.24	+
Sepals	3.066	1.51	+
Petals	19.152	0.19	—
Filaments	2.520	0.36	—

* This column indicates whether or not a chemotropic response was obtained when the fresh tissues of comparable flowers were tested in the bioassay apparatus.

Table II

The Effect of Calcium (as $CaCl_2$) in the Plate Medium on the Growth of Pollen Tubes Towards Empty Test and Control Chambers

The plate medium contained 20% sucrose, 0.1% yeast extract, 0.01% boric acid, and 1% agar. The readings were made after incubation for 8 hours at 25°.

Calcium Molarity	Growth to test chamber		Growth to control chamber	
	Number	Length (mm)	Number	Length (mm)
0	0	...	2	0.3
1×10^{-6}	0	...	0	...
1×10^{-5}	7	0.3	1	0.3
1×10^{-4}	44	0.3–0.4	25	0.3
1×10^{-3}	150	0.5–0.9	120	0.5–0.9
5×10^{-3}	~ 250	0.6– > 1.0	~ 250	0.6– > 1.0
1×10^{-2}	~ 300	0.7– > 1.0	~ 300	0.7– > 1.0
2×10^{-2}	150	0.8– > 1.0	170	0.8– > 1.0
3×10^{-2}	135	0.5–1.0	148	0.4–0.8
4×10^{-2}	105	0.5–0.8	130	0.5–0.8
5×10^{-2}	0	No germination	0	No germination
1×10^{-1}	0	No germination	0	No germination

an overall gradient amounting to a fourfold increase in concentration between the upper third of the style and the ovules and placenta. These data indicate that there is no gradient down through the first two-thirds of the style. It must be remembered, however, that the entire stylar tissue was analyzed whereas the pollen tubes presumably grow only in the stigmatoid tissue (5). Although attempted, no way was found to dissect the stigmatoid tissue from the rest of the style.

An unsuccessful attempt was made to observe the distribution of calcium by radioautography of longitudinal sections of the style. The gynoecia from a plant fed Ca^{45} were too heavily labeled to permit the necessary observations. The radioactive gynoecia and pollen were used, however, to establish the relationship between the content of Ca^{45} and chemotropic activity and the extent to which calcium diffused from the tissues into agar.

Ovules and placenta as a mass were removed from 3 flowers whose anthers had just dehisced. Each of these masses was divided approximately in half. One of each half was assayed for chemotropic activity and then dried, ashed, and counted. The results are given in table IV. Although there is not a strict proportionality between radioactive calcium content and chemotropic activity, the 2 measures do vary in the same direction. The other halves of the ovule and placenta masses were used to determine the extent to which calcium is released. In place of the paper shown in the upper diagram of figure 1, a piece of dialyzing membrane was inserted, extending downward to the bottom of the agar and 1 mm beyond each end of the chamber, to facilitate the subsequent removal of the tissue. The tissue was placed in the chamber against this membrane and allowed to stand for 6 hours at 25°. The ovules, placenta, and membrane were then removed, dried, ashed, and assayed for radioactivity. The agar to the left of the membrane was removed as a block 3 mm wide, 7 mm long (1 mm beyond each end of the chamber), and the full depth of the agar. It too was dried, ashed, and assayed for radioactivity. The results are tabulated in table V and indicate that almost 8% of the radioactive calcium moved from the tissue into the agar. This value is probably low because of the likelihood that not all of the agar containing Ca^{45} was cut out. In an earlier experiment 10% of the Ca^{45} was found in the agar after a diffusion period of only 3 hours.

Table IV

Comparison of Ca^{45} in Ovules and Chemotropic Activity*

Number of tubes to ovules*	Cpm
73	4,762
132	14,600
120	6,250

* Including placental tissue.

Pollen also contains calcium and readily gives it up to agar. Pollen collected from flowers grown in the Botanical Garden was found to contain 0.34% calcium on the dry weight basis. Pollen from a plant fed Ca^{45} was spread in a thin layer on a 1 square inch piece of dialyzing membrane which was then laid on the surface of a nutrient agar plate and left there for 3.5 hours at 25°. At the end of the diffusion period the membrane and pollen were removed and assayed for activity as was the agar beneath the membrane. The pollen and membrane contained 4,531 counts per minute of calcium and the agar 432 counts per minute. Thus approximately 10% of the calcium moved from the pollen into the agar.

Table V

The Release of Ca^{45} from Ovules

Flower No.	Counts per minute: Ovules*	Counts per minute: Agar	Ca released: %
1	11,098	958	8.6
2	5,542	547	10.1
3	14,273	884	6.2
Average	10,304	796	7.7

* Including placental tissue.

The next set of experiments was designed to substantiate our conclusion that calcium is a chemotropic agent for the pollen tubes of snapdragon. The first of these attempts was to distinguish between the enhancement of growth per se and a chemotropic response. The bioassay method does not permit observation of individual pollen tubes. Since it is known that calcium markedly enhances the growth of pollen tubes (2) it could be argued that those tubes in the pollen chamber initially directed toward the test chamber grow faster than those initially directed to the control chamber and hence are the only ones to register within the time allowed for growth (normally 8 hours). This cannot be clarified simply by extending the incubation period because sooner or later calcium from the test compartment will diffuse through the pollen chamber and into the control area. Three other substances are known to enhance the growth of pollen tubes: sucrose (8, 9, 10, 12, 17, 18); yeast extract (3, 7); and boron (11).

It was shown that the rate of growth of the pollen used in this study was enhanced by sucrose, yeast extract, and boric acid. Pollen was sown on agar plates containing sucrose, yeast extract, boric acid, and $CaCl_2$ at optimal levels (10% sucrose, 0.1% yeast extract, 0.01% boric acid, and 1×10^{-2} M $CaCl_2$) excepting that nutrient whose effect on the rate of growth was being tested. The concentration of the latter (sucrose, yeast extract, or boric acid) was varied. The minimal concentration of sucrose tested was 2% since the complete absence of sucrose prevented germination; with yeast extract and boric acid the minimal concentration was zero because reasonable although reduced germination of pollen did occur when these were not incorporated into the media. The ratio of the average length of the tubes in the optimal concentration of each nutrient compared to that in the minimal concentration was 3.8 for sucrose,

2.2 for yeast extract, and 5.2 for boric acid. When, now, sucrose or yeast extract gradients were established between the test chamber and the pollen chamber no pollen tubes in excess of the controls grew towards the test chamber. In the experiments with sucrose, the plate medium contained 2% sucrose with the other nutrients at optimal concentrations as specified just above. With yeast extract, the plate medium was devoid of yeast extract but with the other nutrients at optimal levels. In both series of tests, calcium was supplied in the plate medium as $CaCl_2$ at both suboptimal (2×10^{-4} M) and optimal (2×10^{-2} M). Thus, the enhancement of the growth of the pollen tubes by either yeast extract or sucrose is not involved in the chemotropic response indicated by the assay method.

In the tests with sucrose and yeast extract gradients it was assumed that there was sufficient time during the 8 hour incubation period for these substances to diffuse through the test wall and thus be capable of affecting the pollen tubes. Direct testing of this assumption was not done. However, the germination of the pollen in the pollen chamber as well as the average length of the pollen tubes growing into the test wall were greater with the higher concentrations of sucrose (10 and 15%) than with the lower ones; with yeast extract, which affects germination less markedly than sucrose, the evidence for diffusion rests only on the greater lengths of the tubes in response to the higher concentrations of yeast extract in the test chamber.

In contrast to these negative results with sucrose and yeast extract are those obtained with concentration gradients of boric acid. The initial experiment is recorded in table VI. Boric acid in the test chamber unaccompanied by calcium is without effect even though the plate medium contains calcium at a concentration just below that which causes tubes to grow out of the pollen chamber in all directions in the absence of any gradient of calcium. When this same concentration of calcium (2×10^{-4} M) is added together with boric acid to the test chamber there is a significant increase in the number of tubes growing to the test chamber compared to the control chamber. When there is a gradient of calcium (1×10^{-2} M in the test chamber and 2×10^{-4} M in the plate medium) the response to calcium is greatly increased by the optimum concentration of boric acid in the test chamber. A large number of additional experiments on this effect of boric acid were performed. The concentration of calcium that must be present to detect the effect of boric acid varies with the source of the pollen. With a different collection of pollen than that used in the preceding experiment no effect could be detected until the calcium concentration in the chamber and plate media reached 1×10^{-2} M. With pollen collected from commercially grown snapdragons no effect of boron was found even with 1×10^{-2} M calcium in the media. The results of all the experiments indicate that boric acid enhances the chemotropic response to calcium, that in the absence of calcium it has no chemotropic effect, and that the concentration of calcium necessary for this effect of boric acid to be detected depends on the physiological (nutritional ?) status of the pollen.

Table VI

The Effect of Concentration Gradients of Boric Acid on the Chemotropic Response of Pollen Tubes

The test media contained 10% sucrose, 1% agar, and 0.1% yeast extract in addition to the indicated amounts of boric acid and $CaCl_2$. The media were added to the test chamber as 30 µl drops which then solidified. The plate and control media contained 10% sucrose, 1% agar, 0.1% yeast extract, and 2×10^{-4}M $CaCl_2$.

Medium in test chamber		Number of tubes	
Boric Acid %	Ca M	Test	Control
0.000	0	21	24
0.005	0	15	17
0.010	0	21	19
0.020	0	28	22
0.030	0	31	26
0.050	0	33	36
0.100	0	28	36
0.000	2×10^{-4}	24	22
0.005	2×10^{-4}	34	16
0.010	2×10^{-4}	39	22
0.020	2×10^{-4}	46	26
0.030	2×10^{-4}	51	26
0.050	2×10^{-4}	79	21
0.100	2×10^{-4}	18	19
0.000	10^{-2}	58	36
0.005	10^{-2}	53	18
0.010	10^{-2}	61	21
0.020	10^{-2}	91	21
0.030	10^{-2}	115	27
0.050	10^{-2}	130	47
0.100	10^{-2}	56	47

The relationship between the spontaneous growth of pollen tubes into the test and control walls of the bioassay was earlier examined (table II) with the finding that a concentration of calcium in the plate medium in excess of 2×10^{-4} M was needed for such spontaneous growth (with the particular pollen used) and that 1×10^{-2} M was optimum for the growth of the tubes out of the pollen chamber in all directions in the absence of a gradient of calcium. In these tests the plate medium contained 0.01% boric acid. The finding that boric acid effects the response to calcium necessitated a testing of the appropriateness of the concentration of boric acid used in the earlier experiments. Plates were therefore prepared with the usual nutrients, 2×10^{-2} M $CaCl_2$, and different concentrations of boric acid. The maximum number of tubes grew toward the test and control chambers, which were empty, when the boron concentration in the medium was 0.01%. This result is another indication of an interaction between boric acid and calcium and confirms the use of 0.01% boric acid as optimal in all the preceding experiments.

To further confirm that calcium was exerting a chemotropic effect, tests were made involving methods other than the standard bioassay. In these tests

$CaCO_3$ and later, platelets of calcium DL-malate were used since they made it possible to maintain a constant concentration of calcium at the source because of their low solubility. It was presumed that this more closely simulated the conditions prevailing when tissue was used as the source of the chemotropic factor. When $CaCO_3$ was used for the standard bioassay, it was introduced into the test depression against the inner wall as the powder approximately 4 to 5 hours prior to introducing the pollen. With the malate, no time difference was necessary for optimum results and the malate was introduced as a sheet laid against the inner wall of the test compartment.

Figure 2 shows a series of tests conducted with $CaCO_3$ and the comparisons of the responses observed with those elicited by ovules. Figures 2a and b illustrate the growth of pollen tubes in the standard assay apparatus to $CaCO_3$ and to ovules placed in the left depression. No additions were made to the control depressions. In figures 2c and d the pollen was spread in a row along the edge of each side of the pollen depression. Many tubes grew into the wall towards the test depressions which contained $CaCO_3$ or ovules whereas very few grew into the opposite wall. In figures 2e and f pollen grains were placed in a slit in the agar approximately 1 mm from the long outer edge of the test depressions in which $CaCO_3$ and ovules had been placed. Finally, in figures 2g and h the traditional method of displaying pollen tube chemotropism was used. $CaCO_3$ and ovules were placed 1 mm away from pollen grains spread on the surface of the agar. When ovules or $CaCO_3$ are omitted from the types of tests illustrated by 2a, b, c, and d, the tubes tend to remain, but not completely, in the pollen chamber. When the attractants are omitted from tests 2e, f, g, and h, then the pollen tubes grow randomly in all directions.

These illustrations show that the pollen tubes respond to Ca as they do to ovules; however, the response is almost always less intense to the calcium than to the ovules. In those tests where the pollen grains are spread in rows, it is possible to observe the individual tubes and to note that whatever the direction of emergence of the tubes, most of them turn and by a convoluted pathway eventually grow toward the source of the chemotropic agent whether it is ovules or calcium.

An attempt was made to see if ovules would attract pollen tubes in the presence of calcium. The procedure used was the usual method in which ovules are placed on the surface of a nutrient agar plate with the pollen placed individually about 1 mm from the ovules. Although we never were able to obtain fully consistent results with this method [see discussion in (16)], the usual spectacular response as shown in figure 2g was obtained in more than half of the tests. When the agar contained 2, 3, and 4×10^{-2} $CaCl_2$ no such chemotropic effect was detected in 31 trials. Thus, adequate calcium in the plate medium erases the chemotropic effect of ovules.

The pollen of *Narcissus pseudonarcissus* and *Clivia miniata* also responded to calcium in the bioassay apparatus in the same manner as that of snapdragon.

Discussion

A variety of evidences have been presented leading to the conclusion that calcium exerts a chemotropic effect on the pollen of snapdragon. A comprehensive review of the literature on pollen tube chemotropism (16) cites all work done on pollen tube chemotropism and is the basis for the following discussion.

The chemotropic factor is widely distributed in plant tissues or else there are several factors to which pollen tubes can react tropically. Chemotropism of pollen tubes of one species to nongynoecial tissues of the same and other plant species has been repeatedly observed as has chemotropism between pollen and gynoecial tissue from different plant species, even when the plants were widely separated taxonomically. Thus the chemotropic substance is of common occurrence and not a very specific compound. Positive chemotropic responses to specific compounds and crude preparations were obtained by the early workers—to compressed yeast, sucrose, glucose, fructose, lactose, diastase, and egg albumin. However, although more recent work has not confirmed these results, they cannot be altogether discounted. It is possible that unlike the compounds obtainable at the present time, the compounds used in the 1890's contained impurities that were chemotropic, probably calcium. It is known that diastase which is one of the materials earlier found to have chemotropic activity does contain one or more gram atoms of calcium per mole of enzyme (6).

The chemotropic factor(s) is known to be heat stable, resistant to dilute acid hydrolysis, small enough to pass through a dialysis membrane, and soluble in water and alcohol, but not in acetone or ether. Calcium possesses all these properties.

The active substance from gynoecial tissues is known to have in addition to a tropic effect also a growth-stimulating effect on pollen tubes. When pollen is sown in sufficiently large colonies on nutrient agar, growth begins in the center and the tubes grow towards the periphery and go a short distance beyond the limits of the colony. Then with only a few exceptions, the tubes recurve towards the colony. Sometimes tubes that grow beyond the limits of a colony grow into a neighboring colony. Evidently there is some substance within the colonies which influences the direction of growth of the pollen tubes. As the number of grains in the colony increase, the pollen germination percentage and tube length proportionately increase. Recent work by Brewbaker and Kwack (1) has shown that the factor causing this population effect is calcium.

Calcium, which has been found to have a tropic effect in addition to a growth effect, appears to possess all the properties of the tropic substances for pollen tubes that have been reported in the literature.

The conclusion that calcium is a chemotropic fac-

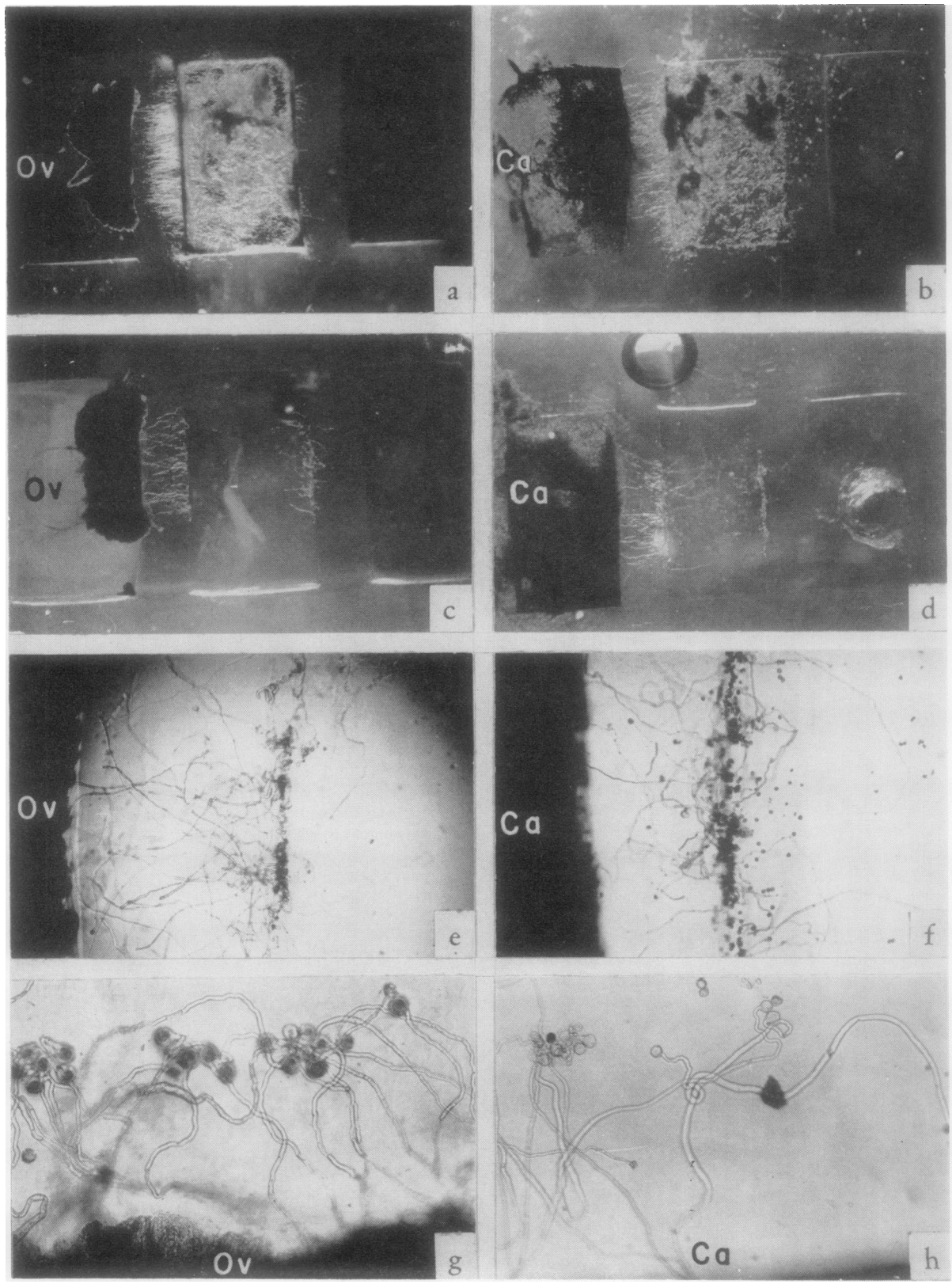

FIG. 2. The chemotropic response of pollen tubes to ovules (left) and to $CaCO_3$ (right). a and b: Standard bioassay. c and d: The pollen grains were spread in a single-grain row along each edge of the pollen chamber. e and

tor is further supported by the distribution of calcium in floral parts, the readiness with which calcium diffuses from pollen and floral parts into agar, and the inability to obtain a chemotropic response by ovules in the presence of ample calcium. There was shown to be a very substantial overall gradient in calcium from the stigma to the ovules. As pointed out earlier, the lack of a gradient in the upper part of the style may not reflect the actual distribution of calcium affecting the pollen tubes in their growth through the style because they are presumably restricted to the stigmatoid tissue. The actual situation in snapdragon was not investigated. It would be desirable to work with a plant where the distribution in stigmatoid tissue itself could be examined. Although we were unable to observe chemotropism to ovules in the presence of calcium using the surface test, Brewbaker and Kwack state that they do get a response in their test procedure (2).

The ready diffusion of calcium from pollen grains offers a possible explanation for both the recurvature of pollen tubes back to a colony of pollen referred to above and for the failure of pollen tubes to grow out of the pollen chamber in the bioassay apparatus unless the plate medium contains high levels of calcium. In both cases the released calcium could be presumed to act chemotropically. Quantities of pollen, all releasing calcium into the agar, would result in a localized concentration of available calcium tending to keep the pollen tubes in the vicinity.

Evidence was presented that the optimum response of pollen tubes to calcium was conditioned by the presence of boron, depending on the particular lot of pollen used in the tests. Although calcium elicits a response in the bioassay plate in the absence of added boron, the reverse does not happen. Not enough is known about this interaction to explain it.

Summary

Evidence is presented that the pollen tubes of snapdragon (*Antirrhinum majus*) grow chemotropically towards a source of calcium. Analyses of the parts of the gynoecia of snapdragon indicate the highest concentration of calcium to be in the ovules. Boric acid enhances the chemotropic response to calcium.

Acknowledgment

We wish to thank Miss Linda Simons for her technical assistance.

Literature Cited

1. BREWBAKER, J. L. AND S. K. MAJUMDER. 1961. Cultural studies of the pollen population effect and the self-incompatibility inhibition. Am. J. Botany 48: 457–64.
2. BREWBAKER, J. L. AND B. H. KWACK. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. Am. J. Botany 50: 859–65.
3. BRINK, R. A. 1924. The physiology of pollen. IV. Chemotropism; effects on the growth of grouping grains; formation and function of callose plugs; summary and conclusions. Am. J. Botany 11: 417–36.
4. CARLSON, R. M. AND C. M. JOHNSON. 1961. Chelometric titration of calcium and magnesium in plant tissue. Method for the elimination of interfering ions. J. Agr. Food Chem. 9: 460–63.
5. ESAU, K. 1953. Plant Anatomy. John Wiley and Sons, Inc., New York. 735 pp.
6. FISCHER, E. H., W. N. SUMERWELL, J. JUNGE, AND E. A. STEIN. 1958. Calcium and the molecular structure of α-amylases. Proc. 4th Internatl. Cong. Biochem., Vienna. 8: 124–37.
7. GLENK, H. 1960. Keimversuche mit Oenothera-Pollen in vitro. Flora 148: 378–433.
8. HRABETOVA, E. AND J. TUPY. 1961. Respiration of apple pollen on different sugar substrates and the problem of the role of sucrose in pollen-tube growth. Biol. Plantarum (Praha) 3: 270–76.
9. IWANAMI, Y. 1956. Physiological researches of pollen. IX. The starch and the sugar in the pollen grain (Japanese with English summary). Botan. Mag. Tokyo 69: 91–5.
10. IWANAMI, Y. 1956. Physiological researches of pollen. X. The absorption of sugars and some enzyme reactions (Japanese with English summary). Botan. Mag. Tokyo 69: 198–202.
11. JOHRI, B. M. AND I. K. VASIL. 1961. Physiology of pollen. Botan. Rev. 27: 325–81.
12. LINSKENS, H. F. 1958. Zur Frage de Entstehung der Abwehr- Korper bei der Inkompatibilitats-Reaktion von Petunia. I. Mitteilung: Versuche zur Markierung der Griffel mit P^{32}-und C^{11}-Verbindungen. Ber. Deut. Botan. Ges. 71: 3–10.
13. MACHLIS, L. AND J. G. TORREY. 1956. Plants in Action. W. H. Freeman and Co., San Francisco. 282 pp.
14. MASCARENHAS, J. P. AND L. MACHLIS. 1962. The pollen-tube chemotropic factor from *Antirrhinum majus:* bioassay, extraction, and partial purification. Am. J. Botany 49: 482–89.
15. MASCARENHAS, J. P. AND L. MACHLIS. 1962. Chemotropic response of *Antirrhinum majus* pollen to calcium. Nature 1962: 292–93.
16. MASCARENHAS, J. P. AND L. MACHLIS. 1962. The hormonal control of the directional growth of pollen tubes. Vitamins and Hormones 20: 347–72.
17. O'KELLEY, J. C. 1955. External carbohydrates in growth and respiration of pollen tubes in vitro. Am. J. Botany 42: 322–26.
18. TUPY, J. 1960. Sugar absorption, callose formation and the growth rate of pollen tubes. Biol. Plantarum (Praha) 2: 169–80.

f: The pollen grains were inserted in a row in a slit made in the agar 1 mm from the outer edge of the control chamber. The test materials were placed against the outer wall of the control chamber. g and h: The pollen grains were placed on the surface of nutrient agar and the test materials 1 mm awav.