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THE RELATIONSHIP OF GROWTH HORMONES AND FRUIT DEVELOPMENT

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Fruit development is normally the result of pollination and fertilization although numerous instances of parthenocarpy and parthenogenesis have been reported in the literature. It has been shown (Muir[11]) that considerable quantities of diffusible growth hormones are released in the style and ovary of *Nicotiana tabacum* following pollination and fertilization. The investigations of Fitting,[5] Laibach,[10] Thimann[14] and others have indicated that pollen is a relatively rich source of growth hormones. With this in mind Gustafson[7] postulated that the growth hormones from the pollen grains and pollen tubes initiate the early stages of fruit development and after fertilization the developing embryo provides additional growth hormones to other portions of the ovary for its development. Van Overbeek, Conklin and Blakeslee[17] have stated that the pollen of an ordinary pollination does not contain sufficient auxin to be the sole cause of fruit development. They suggest that the substance from the pollen which initiates enlargement of the ovary and ovule may be a prosthetic group which properly combined in the ovary forms an enzyme which activates the auxin precursor. The verification of either hypothesis requires the examination of the possible sources of the growth hormones involved in fruit development on a quantitative basis.

Experiments were performed to determine (1) the amount of growth hormones in the pollen grain which could be transported through the pollen tube to the ovary; (2) the production of growth hormones during the development of the pollen tube; (3) the amount of growth hormones in the ovary before fertilization; (4) the production of growth hormones in ovary tissues by the action of pollen extracts.

Bioassay of Growth Hormones.—Determinations of growth hormone concentrations were made according to the modification suggested by Skoog[12] of the standard *Avena* test as described by Went and Thimann.[18]

Curvatures are expressed as the arithmetical mean of the test row of plants with the standard error of the mean and are recorded for the same agar dilution in each instance. Direct comparisons of curvatures are valid in any given experiment. To correct for differences in the sensitivity of the test plants in different experiments the curvatures are translated into the number of micrograms of indoleacetic acid required to produce an equivalent amount of curvature of the *Avena* coleoptile as is produced by the growth hormones obtained from the test material. The calculation employs the following formula:

$$\frac{\text{Micrograms Indoleacetic Acid}}{\text{Milligram of Material}} = \frac{V_1 \times C_1 \times 2.5 \times 10^{-4}}{V_2 \times C_2 \times W}$$

V_1 = volume of agar dilution of the extract
V_2 = volume of agar block applied to each coleoptile = 0.0125 ml.
C_1 = average curvature of coleoptile induced by the extract
C_2 = average curvature of coleoptile induced by 2.5×10^{-4} micrograms of indoleacetic acid
W = fresh weight of material extracted in milligrams

Growth Hormones in Pollen Grains and Pollen Tubes.—A weighed quantity of viable pollen was ground with powdered glass and a few drops of a 10-ml. volume of 0.1 *N* HCl until examination under the microscope revealed no whole grains. The remaining acid was used to transfer the mixture quantitatively to a separatory funnel. The pH of the mixture was adjusted to 3.0–4.0 (glass electrode) before extraction with three separate 50-ml. portions of recently distilled chloroform. The chloroform was withdrawn and evaporated until only a few ml. remained which were transferred to a small vial and taken up in agar.

Excellent pollen grain germination and pollen tube growth for the various species tested occurred on a medium composed of 1% agar and 10% sucrose. The pollen was distributed uniformly over 2 ml. of sterile medium in a sterile petri dish. A piece of moist filter paper was inserted inside the cover and the dish was placed in a darkroom at 25°C. All cultures were examined microscopically before being ground with glass, acidified and extracted. Occasionally the cultures older than 24 hrs. were found to be contaminated and were discarded.

Representative experiments for each type of pollen are presented in table 1. *Nicotiana* pollen has a relatively small amount of free hormone and no marked increase in amount occurs upon germination. The pollen of *Datura* contains twice as much free hormone as the pollen of *Nicotiana* but no increase in amount occurs following germination. Upon germination of the *Antirrhinum* pollen the growth hormone concentration increased to an amount which was three or four times that present in the

TABLE 1

CONCENTRATIONS OF GROWTH HORMONES OBTAINED BY EXTRACTION OF POLLEN GRAINS AND POLLEN TUBES

SOURCE OF POLLEN	STAGE OF MATERIAL EXTRACTED	MG. OF POLLEN GRAINS	AVERAGE AVENA TEST CURVATURE	MICROGRAMS OF INDOLEACETIC ACID $\times 10^{-4}$ PER MG. OF POLLEN
Nicotiana	Grains	29.0	4.2 ± 1.2	0.7
tabacum	Grains	29.3	2.0 ± 0.8	0.3
	Tubes (15 Hrs.)	14.0	0.0	0.0
	Tubes (15 Hrs.)	30.0	0.0	0.0
	Tubes (25 Hrs.)	34.5	0.0	0.0
	Tubes (25 Hrs.)	35.0	8.6 ± 0.8	1.2
	Tubes (37 Hrs.)	29.2	2.0 ± 0.8	0.3
	Tubes (37 Hrs.)	28.7	5.0 ± 1.1	0.9
Antirrhinum	Grains	30.0	4.7 ± 0.9	1.1
majus	Tubes (8 Hrs.)	30.0	16.7 ± 1.2	3.9
	Tubes (8 Hrs.)	30.0	17.3 ± 1.8	4.0
	Tubes (14 Hrs.)	30.0	17.1 ± 1.8	4.0
	Tubes (14 Hrs.)	30.0	17.4 ± 1.4	4.0
	Tubes (14 Hrs.)	30.0	14.5 ± 0.7	3.4
	Tubes (25 Hrs.)	30.0	14.9 ± 1.5	3.5
	Tubes (25 Hrs.)	30.0	13.0 ± 1.1	3.0
Cyclamen	Grains	11.0	3.9 ± 1.5	2.4
persicum	Tubes (6 Hrs.)	12.0	3.4 ± 1.1	1.9
	Tubes (6 Hrs.)	13.0	7.9 ± 0.8	4.4
	Tubes (10 Hrs.)	12.0	4.7 ± 0.4	2.6
	Tubes (22 Hrs.)	12.0	7.2 ± 0.7	4.0
	Tubes (22 Hrs.)	12.0	9.9 ± 0.7	6.0
Datura	Grains	53.0	11.3 ± 1.1	1.4
suaveolens	Tubes (9 Hrs.)	70.0	16.5 ± 2.5	1.6
	Tubes (9 Hrs.)	63.0	14.3 ± 1.6	1.5
	Tubes (26 Hrs.)	62.0	16.4 ± 1.5	1.8

grain. The pollen grains of *Cyclamen* were the richest source of hormones. The concentration of free hormone in the germinated pollen was double or triple the concentration before germination. The considerable fluctuation in the concentration of free hormone was found to be characteristic for the pollen tube growth of both *Nicotiana* and *Cyclamen*.

Growth Hormones in Pollen Grains Subjected to Hydrolysis.—The increased concentrations of growth hormones in the germinated pollen of *Antirrhinum* and *Cyclamen* suggests the liberation of active hormones from inactive combinations during the growth of the pollen tube. The investigations of Skoog and Thimann,[13] Wildman and Gordon,[19] Gordon[6] and Avery, Berger and White[1] all indicate the existence of growth substances both as free, active entities and in bound, inactive combinations. To release the active hormones the pollen grains were subjected to acid and alkaline hydrolysis following the methods of Avery, Berger and White.[1] Thirty mg. of pollen grains were ground with powdered glass and trans-

ferred to a large test tube with 5 ml. 1.0 *N* NaOH or 10 ml. 0.1 *N* HCl. The mixtures were autoclaved 30 min. at 15-lb. pressure (120°C.) and allowed to cool. The pH was adjusted to 3.0–4.0 with 1.0 *N* HCl and the aqueous solutions were extracted with chloroform. The concentrations of free hormones following hydrolysis of the pollen materials are presented in table 2. A remarkable uniformity of yield following hydrolysis with

TABLE 2

CONCENTRATIONS OF GROWTH HORMONES OBTAINED BY EXTRACTION OF POLLEN GRAINS FOLLOWING HYDROLYSIS

SOURCE OF POLLEN	EXP. NO.	TYPE OF HYDROLYSIS	AVERAGE AVENA TEST CURVATURE	MICROGRAMS OF INDOLEACETIC ACID $\times 10^{-4}$ PER MG. OF POLLEN
Nicotiana tabacum	1	None	0.0	0.0
		None	0.0	0.0
		None	0.0	0.0
		Alkali	30.5 ± 1.8	3.6
		Alkali	27.8 ± 1.5	3.3
		Alkali	30.4 ± 3.1	3.6
		Acid	0.0	0.0
		Acid	0.0	0.0
		Acid	0.0	0.0
Antirrhinum majus	1	None	4.0 ± 0.7	0.6
		None	0.0	0.0
		Alkali	31.6 ± 1.3	4.6
		Alkali	32.0 ± 0.9	4.7
	2	Alkali	19.7 ± 1.0	3.0
		Acid	20.2 ± 1.3	3.1
		Acid	19.2 ± 1.0	3.0
Datura suaveolens	1	None	22.4 ± 1.3	3.3
		Alkali	22.6 ± 1.4	3.3
		Alkali	24.4 ± 2.1	3.6
		Alkali	22.7 ± 1.5	3.3
	2	Alkali	28.4 ± 1.9	4.3
		Alkali	27.6 ± 1.8	4.2
		Acid	0.0	0.0
		Acid	0.0	0.0

alkali is demonstrated. These yields are the same as those obtained following germination of *Antirrhinum* and *Cyclamen* pollen but are much larger than the yields obtained from germinated pollen of *Nicotiana*. Although larger amounts of hormones were obtained in these experiments with pollen of *Datura* collected in June than were obtained in the germination tests with pollen collected in April, the hydrolysis of the material did not increase the yield of free hormone, which fact is in agreement with the demonstration that no marked change in concentration of growth substances occurred following germination of the pollen. Acid hydrolysis did not increase the yield of free hormone from *Nicotiana* pollen and decreased the yield from *Datura* pollen. Acid hydrolysis of the pollen of

Antirrhinum increased the yield of hormone as much as hydrolysis with alkali increased it. These data indicate that the hormones of the *Nicotiana* pollen are in a bound, inactive state and are only partially liberated during the germination of the grain. The hormones of *Antirrhinum* pollen are in a bound, inactive state for the most part but are liberated during germination. The hormones of *Datura* pollen are all present in the free, active form.

Growth Hormones in Ovary Tissue.—The hypothesis of the enzymatic activation of the auxin precursor in the ovary as a result of pollination made the assumption that a similar condition of active and inactive forms of the growth hormones occurred in the ovary as had been demonstrated by van Overbeek[16] for the coleoptile tip of maize seedlings. Extractions of dried ovary tissue of *Nicotiana* and *Antirrhinum* were made to investigate the occurrence of both free and bound forms of the hormones in the unpollinated pistil. The ovary tissue was dried *in vacuo* at 60°C. and ground to pass through an 80-mesh screen. Determinations were carried out with 20-mg. samples of this material.

The conversion of the bound form of the hormone to the free form by enzymatic digestion was investigated by dispersing the tissue in 10 ml. of KH_2PO_4-NaOH buffer solution of pH 8.0 with 3 mg. of a commercial pancreatic preparation (Fairchild). Numerous tests of this preparation have shown it to have no growth effects in the *Avena* test. Controls were prepared in which the enzyme preparation was omitted. The development of microörganisms in the digestions was prevented by adding 15 drops of toluol every 24 hrs. and tightly stoppering the flasks. Following incubation at 37°C. for 48 hrs., the pH of the mixtures was adjusted to 3.0–4.0 and the hormones were extracted from the mixtures with purified ether. The conversion of the bound form to the free hormone by hydrolysis with 1.0 *N* NaOH and 0.1 *N* HCl was determined as in the experiments with the pollen materials. The concentration of free hormone was determined by an ether extraction of the ovary tissue dispersed in acidified water for a period of 8 hrs. at 4°C.

The results of representative experiments with each type of tissue are presented in table 3. They show that the unpollinated pistil of *Nicotiana* contains no detectable free hormone but that a considerable quantity of bound hormone is present which can be converted to the active form merely by incubation in a medium of pH 8.0 and to a lesser degree by acid and alkali hydrolysis. The unpollinated pistil of *Antirrhinum* contains a small amount of free hormone but a much larger amount of bound hormone which can be converted to the active form by incubation in a medium of pH 8.0 and by acid hydrolysis but not appreciably by alkali hydrolysis.

The Conversion of Bound Hormone to Free Hormone by Pollen Extracts.—The demonstration of considerable quantities of bound hormone in the

TABLE 3

CONCENTRATIONS OF GROWTH HORMONES OBTAINED BY EXTRACTION OF OVARY TISSUE

SOURCE OF TISSUE	TREATMENT	AVERAGE AVENA TEST CURVATURE	MICROGRAMS OF INDOLEACETIC ACID $\times 10^{-4}$ PER MG. OF TISSUE (FRESH WT.)
Nicotiana tabacum	Tryptic digestion	37.3 ± 2.5	1.5
	Tryptic digestion	32.0 ± 1.9	1.3
	Control incubation	38.0 ± 1.5	1.5
	Acid hydrolysis	10.0 ± 0.2	0.4
	Acid hydrolysis	10.2 ± 0.7	0.4
	Alkali hydrolysis	12.4 ± 0.8	0.5
	Alkali hydrolysis	9.1 ± 1.0	0.4
	None	0.0	0.0
	None	0.0	0.0
Antirrhinum majus	Tryptic digestion	26.2 ± 2.0	1.3
	Control incubation	19.5 ± 1.9	1.0
	Control incubation	14.3 ± 1.0	0.7
	Acid hydrolysis	14.0 ± 0.8	0.7
	Alkali hydrolysis	2.3 ± 0.4	0.1
	Alkali hydrolysis	3.9 ± 0.7	0.2
	None	5.4 ± 0.5	0.3
	None	3.1 ± 0.8	0.2

TABLE 4

THE CONVERSION OF BOUND HORMONE TO FREE HORMONE IN OVARY TISSUE OF NICOTIANA BY POLLEN EXTRACTS

EXP. NO.	MEDIUM	PREPARATION	AVERAGE AVENA TEST CURVATURE
1	Distilled water, pH 6.3	Ovary tissue + pollen extract	22.8 ± 1.2
		Ovary tissue	0.0
		Ovary tissue	0.0
		Pollen extract	0.0
		Pollen extract	0.0
	Buffer solution, pH 5.9	Ovary tissue + pollen extract	27.0 ± 2.6
		Ovary tissue + pollen extract	20.0 ± 1.1
		Ovary tissue	0.0
		Ovary tissue	0.0
		Pollen extract	0.0
		Pollen extract	0.0
2	Buffer solution, pH 5.9	Ovary tissue + pollen extract	15.0 ± 1.3
		Ovary tissue + pollen extract	14.7 ± 1.4
		Ovary tissue	5.5 ± 1.2
		Ovary tissue	4.5 ± 1.1
		Pollen extract	0.0
		Pollen extract	0.0
	Buffer solution, pH 8.0	Ovary tissue + pollen extract	22.7 ± 1.6
		Ovary tissue + pollen extract	40.2 ± 3.2
		Ovary tissue	34.1 ± 1.7
		Ovary tissue	34.6 ± 1.7
		Pollen extract	0.0

ovary of *Nicotiana* and *Antirrhinum* suggested the investigation of substances in pollen which might bring about the activation of the hormones as hypothesized by van Overbeek, Conklin and Blakeslee. Extracts were made by grinding 0.1 gm. of *Nicotiana* pollen grains with glass dispersing the material in distilled water and toluol, and agitating the mixture mechanically for 37 hrs. at 13°C. The suspension was centrifuged to remove the glass and pollen debris and 16 ml. of a yellowish, slightly turbid extract were obtained. Twenty mg. of dried ovary tissue of *Nicotiana* (unpollinated pistils) were dispersed in 10-ml. portions of distilled water and KH_2PO_4-NaOH buffer solutions of pH 8.0 and 5.9. Two ml. of the pollen extract were added to one set of dispersions, 2 ml. of distilled water were added to a control set and 2 ml. of the pollen extract were added to 10 ml. of water and buffer solutions which did not contain ovary tissue. All mixtures were incubated at 37°C. for 24 hrs. after 20 drops of toluol had been added and the flasks tightly stoppered. After incubation the pH of the mixtures was adjusted to 3.0–4.0 and extractions were made with ether.

The results of two experiments presented in table 4 show that in distilled water and buffer solution of pH 5.9 the mixture of ovary tissue and pollen extract yielded large quantities of free hormones whereas the ovary tissue alone and the pollen extract alone yielded none or little free hormone. In buffer solution of pH 8.0 the same yields of free hormones were obtained from the ovary tissue alone as were obtained from the mixture of ovary tissue and pollen extract. The conversion of the bound form of the hormone to the free form in an alkaline medium has been observed previously (see table 3). These experiments demonstrate that the extract of the pollen contains a substance or substances which can convert the bound hormones in the dried ovary tissue to free hormones.

Discussion and Interpretation of Experimental Results.—Since the pollen grains, pollen tubes and ovaries have all been shown to contain growth substances, the source of the diffusible hormones detected in the ovary following pollination and fertilization can be established only by a comparison of the amounts in the pollen and ovary tissue. Buchholz[4] has stated that 600–900 pollen grains may be regarded as a normal abundant pollination in *Datura* and van Overbeek, Conklin and Blakeslee cite this figure to support their view that the pollen has insufficient hormones to cause fruit development. Thus 1000 pollen grains would be a liberal figure for pollination. Determinations of hormone concentrations were made on a weight basis and the calculation of amounts of hormones involved in a normal pollination requires estimates of the number of grains per unit weight of pollen. Heyl[8] while studying the chemical composition of pollen estimated that there are 610 millions of grains in one gram of ragweed pollen and Ulrich[15] estimated 173 millions of grains per gram of

ragweed pollen. In this study estimates of the number of grains per gram of pollen were made by dispersing a weighed amount of pollen in a definite volume of 50% glycerol and water after wetting the grains with a drop of chloroform. Duplicate aliquots of the suspension were placed on a glass slide under a cover glass and counted with the aid of a mechanical stage. The pollen grain of *Nicotiana* has an average volume of 14,500 cubic microns and there are approximately 60 millions of grains per gram of fresh pollen. The pollen grain of *Antirrhinum* has an average volume of 5300 cubic microns and there are approximately 170 millions of grains per gram of fresh pollen.

Small yields of growth hormones were obtained from *Nicotiana* pollen grains and tubes but approximately 4.0×10^{-4} microgram of indoleacetic acid per milligram of pollen was obtained following alkali hydrolysis and as this concentration corresponds well with maximum yields from other types of pollen it will be acceptable as the maximum concentration in *Nicotiana* pollen. Calculation reveals that the maximum concentration of growth hormones in the pollen of a normal pollination is then 6×10^{-6} microgram of indoleacetic acid. In experiments with *Nicotiana tabacum* reported elsewhere (Muir[11]) it was found that immediately following fertilization there appear sufficient diffusible growth hormones in the ovary to produce curvatures of 30 degrees in the *Avena* test, a concentration of approximately 6×10^{-4} microgram of indoleacetic acid, 100 times as much hormone as is contained in the pollen of a normal pollination. Similarly, following pollination there appear sufficient diffusible growth hormones in apical and basal portions of the style to produce curvatures of 10 degrees, a concentration of approximately 2×10^{-4} microgram of indoleacetic acid, 30 times as much growth hormones as are contained in the pollen. Maximum yields of hormones from the ovary tissue of unpollinated *Nicotiana* pistils were 1.5×10^{-4} microgram of indoleacetic acid per milligram of fresh tissue. The ovary with an average fresh weight of 34 mg. thus contained approximately 5×10^{-3} microgram of indoleacetic acid, 800 times as much hormone as was found in the pollen. Yields of hormones obtained by extraction of unpollinated pistils were 8 times as great as the yields obtained by diffusion of fertilized ovaries.

Calculation of the amount of growth hormones in the pollen of a normal pollination for *Antirrhinum* gives a value of approximately 2×10^{-6} microgram of indoleacetic acid. The maximum yield of hormones from the ovary tissue of unpollinated *Antirrhinum* pistils was 1.3×10^{-4} microgram of indoleacetic acid per milligram of tissue. The ovary with an average fresh weight of 8 mg. thus contained approximately 1×10^{-3} microgram of indoleacetic acid, 500 times as much hormone as contained in the pollen.

The above comparisons of the amounts of growth hormones in the

pollen and ovary tissue and the demonstration that incubation of water extracts of pollen with ovary tissue yields large amounts of free growth hormones establish the fact that changes in concentration of active hormones in the pistil associated with pollination and fertilization are the result of a substance or substances, other than growth hormones, brought into the pistil by the pollen tubes.

The identity of the effective substance in pollen will be indicated more definitely when the mechanism of free auxin formation in the ovary following fertilization has been established. The investigations of Skoog and Thimann[13] and Wildman and Gordon[19] suggest that in some tissues the inactive growth hormones are bound to proteins and thus the effective substance in pollen may be part of an enzyme system which brings about the release of active hormone from protein combinations. Recently Bonner and Wildman[3] have suggested that the hormone protein complex in the leaves of spinach is an active entity with the enzymatic properties of a phosphatase and that the hormone, indoleacetic acid, is formed from tryptophane. The significance of phosphatase in the metabolism of reproduction in plants is indicated by the data of Ignatieff[9] who found that the phosphatase activity and the total phosphorous content of pistils and stamens are greater than in any other part of the flowering plant. In plant tissues other than leaves the inactive growth hormones may be true storage forms as indicated by the investigations of Gordon[6] on the hormone and protein combinations in wheat grains and the investigations of Berger and Avery[2] on the inactive hormone in maize endosperm. The possibility thus exists that the pollen substance is a coenzyme or activator of enzymatic systems present in the ovary which liberate active hormones from the storage forms.

Summary.—1. Determinations of growth hormone concentrations in pollen grains and pollen tubes have shown that free hormones are present in the pollen grains and that variable amounts are present during pollen tube growth. Larger quantities are present in a bound form that can be activated by alkali hydrolysis in the pollen of *Nicotiana* and by both acid and alkali hydrolysis in the pollen of *Antirrhinum*. The growth hormones in the pollen of *Datura* are all present in the active form. Maximum yields obtained by various procedures are in agreement.

2. Little or no growth hormone is present in the active form in dried ovary tissue of *Nicotiana* and *Antirrhinum* but large quantities are present in an inactive form that can be activated most completely by incubation at pH 8.0 and less completely by acid or alkali hydrolysis. A water extract of *Nicotiana* pollen contains a substance or substances which can bring about the conversion of the inactive growth hormones in the dried ovary tissue of *Nicotiana* to the active forms in an acid medium *in vitro*.

3. Comparisons of the amounts of growth hormones contained in the

pollen of a normal pollination with the amounts of diffusible hormones which appear in the ovary immediately after pollination and fertilization and with the amounts of inactive hormones in the ovary indicate that the changes in growth hormone concentrations following fertilization are the result of a substance in the pollen other than the growth hormones. It is suggested that this substance is part of an enzyme system.

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*NUTRITIONAL LIFE HISTORY AS INFLUENCED BY DIETARY ENRICHMENTS. II. BODY WEIGHT AND BODY CALCIUM IN CASES OF PROTEIN-ACCELERATED GROWTH**

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In a previous paper[1] we have reported that when a diet of about minimal adequacy in protein and calcium content was enriched in protein by the addition of poultry meat, growth was accelerated but calcium retention did not keep pace. During the resulting period of rapid growth with relatively low calcium content of body a minority of the experimental animals showed symptoms suggestive of calcium deficiency, while the