BIOCHEMICAL STUDIES OF POLLEN FROM ZEA MAYS

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PREFACE

The work to be described was undertaken as a cooperative project of the departments of Chemistry, Horticulture, and Agricultural Chemistry at Michigan State College. It was initiated as a result of the interest of Dr. S. H. Wittwer in the reproductive physiology of the corn plant. The primary aim of the study was to isolate and characterize, if possible, the plant growth regulating substance or substances which could be extracted from corn pollen with ether..

Early in the investigation it was apparent that the ether extracts also contained a pigment, which could be purified readily and which had not previously been identified. Although the identification of this pigment is only remotely related to the original aim of the experimental work, it represents another addition to man's store of knowledge concerning the corn plant. For this reason, the identification of this pigment will also be described. Thus, the thesis will be divided into two chapters, the first dealing with the study of the growth regulators in corn pollen, and the second concerning the identification of one of the yellow pigments in the pollen.

The author wishes to express his sincere thanks

to Dr. S. H. Wittwer for generously assisting with the biological assays, without which a study of the growth regulators in corn pollen would have been an impossibility, and to Professors H. M. Sell and C. D. Ball for their friendly counsel and encouragement.

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CHAPTER I

CHEMICAL STUDY OF PLANT GROWTH REGULATORS IN POLLEN FROM ZEA MAYS

I. HISTORICAL BACKGROUND

The concept of naturally occurring plant growth regulators is a comparatively recent development. It began with the observations of Fitting (6), who in 1909 reported that application of aqueous extracts of the pollen of Phalaenopis amabilis or of the pollen of Aerides odoratum to the stigmas of the blossoms from either plant would cause the gynostemia to swell.

Growth regulators from sources other than pollen.

Shortly after Fitting had described his experiments with orchid pollen, Boysen-Jensen (3), (4) published his experimental observations of the decapitated coleoptiles of Avena sativa seedlings. His work showed clearly that phototropic movements of the coleoptile occurred through a mechanism involving production at the site of illumination of some chemical substance which was then transmitted down the stem to the elongating cells.

Subsequent progress was slow until Kögl and co-

workers isolated auxin a (12), (13) and heteroauxin, or 3-indoleacetic acid (16), from human urine. The starting material for this isolation obviously was not a plant material, but Kögl and his associates soon demonstrated that the auxin (or heteroauxin) content of human urine probably arose from ingested foodstuffs of vegetable origin (14). Later, these same investigators obtained auxin a and auxin b from corn oil (15).

The more recent isolation of 3-indoleacetic acid from immature corn kernels by Haagen-Smit, Dandlicker, Wittwer, and Murneek (10) can leave no doubt that 3-indoleacetic acid is indeed a growth regulator normally present in plant tissues.

Unfortunately, the position of auxin a and auxin b has remained somewhat confused. Only Kögl and his associates have reported isolating auxin a or auxin b from any source. Natural products which might serve as precursors for auxin a and auxin b in the plant are not known. No analytical method independent of biological assay methods has been developed for auxins a or b. The "acid stability test", which was devised by Kögl and co-workers to demonstrate the presence of auxin a in the tips of corn seedlings (17) has been

shown to be unreliable*. For these reasons, the presence of auxin a and auxin b in plant materials is disputed by some workers.

Growth regulators from pollen.

Several investigators besides Fitting have studied the effects of application of pollen extracts to various plant tissues. Laibach (20) found that extracts of pollen of orchids would stimulate the elongation of the Avena coleoptile as well as initiate the enlargement of the gynostemium and ovary of the orchid blossom. Laibach and co-workers found that application of a lanolin paste of the extract of orchid pollen to internodal cut surfaces of Coleus or of Tradescantia stems would cause callus formation (22) and initiate root development (23). La Rue (24) observed that a lanolin paste of the pollen from Populus grandidentata or the spores of Equisetum arvense delayed abscission of the petioles of Coleus leaves from which the leaf blades had been removed. Gustafson (9) found that a lanolin paste of the chloroform soluble material from corn pollen applied to the cut surface of the pistil of a

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* Unpublished observation of A. J. Haagen-Smit and C. T. Redemann.

Salpiglossis blossom would cause the ovary to swell. And more recently, Mitchell and Whitehead (26) found that application of the ether extractible material from corn pollen to the intact epidermis of the first internodes of Phaseolus seedlings would cause cell elongation at the site of application.

The studies of Mitchell and Whitehead indicated that the cellular responses to the corn pollen extract were not identical with those which resulted from the application of 3-indoleacetic acid. Since the task of collecting large quantities of corn pollen is not impossibly difficult, it seemed that corn pollen should be a fruitful material to study chemically in the hope of re-isolating auxin a or b, or perhaps of even isolating a new plant growth regulator.

II. METHOD OF BIOLOGICAL ASSAY

Experimental details.

The first question to be answered concerned the choice of an assay method. The ideal biological assay would be simple, rapid, and specific for one substance. It was decided that an adaptation of the assay method for growth promoting substances described by Laibach (21) would be satisfactory, although it did not represent

the ideal assay method.

The procedure used was simple, for it consisted of applying unilaterally to the first internodes of bean seedlings (Phaseoleus vulgaris, variety Tendergreen) a lanolin solution of the test substance. Four mm. diameter loops of #22 B. and S. gauge nichrome wire served as applicators, permitting treatment of each plant with between 5 and 10 mg. of lanolin solution.

The procedure was also rapid. The maximum curvatures were reached within a period ranging from three to twenty-four hours, depending upon the intensity of illumination and the greenhouse temperature at the time. Intense illumination and high temperatures favored the more rapid response.

The specificity of the assay method is open to question. The possibility of confusing the responses of the assay method to more than one compound during the course of the isolation studies seemed great. Since no more specific assay method was available, however, this risk of confusion was accepted as unavoidable.

During the early experimental work, Merck's anhydrous U.S.P. lanolin was used as a diluent for the material tested. Lanolin had been used repeatedly as a diluent for growth regulators by various workers

since the original suggestion of Laibach (21), and we accepted its use without question. The observation that every method of fractionation attempted seemed to result in appreciable loss of activity was attributed to the chemical instability of the growth regulator. Finally, qualitative tests showed that the lanolin used contained appreciable quantities of an oxidizing agent which was probably responsible for the inactivation of the growth regulator. It was found that replacing the lanolin with that portion of the lipid from corn pollen which was more soluble in petroleum ether than in 75% ethanol (see p.30) avoided this loss. Since the presence of the oxidizing agent in lanolin was not detected until very nearly all the isolation studies had been completed, no attempt will be made to establish quantity vs. specific activity balances.

Interpretation of assays.

After a solution containing the proper concentration of a growth-stimulating substance has been applied to the first internode of a bean seedling, the cells on the same side of the stem to which the material was applied develop more rapidly than those on the opposite side. The resulting effect is a bending of the stem away from the site of application, or the

development of a "negative curvature".

When a solution containing a concentration of growth-promoting substance considerably higher than that required to produce a negative curvature is applied to the first internode of a bean seedling, another effect may be observed. The rate of diffusion through the tissues, the rate of elongation of the plant cells, and the rate of conduction along the vascular system of the plant interact is such a way that the bean stem curves toward the side on which the test substance was applied. This type of response is spoken of as a "positive curvature".

It is also possible for a substance which is a growth inhibitor to cause positive curvature of the stem of the bean seedling. If the compound responsible for this positive curvature is only an inhibitor, no dilution, however great, will initiate a negative curvature in the assay plant; if it is a stimulator, some dilution should give rise to a negative curvature. Thus, by assaying a series of dilutions of the test substance, one may distinguish a growth inhibitor from a stimulator.

Photographic evidence for the relatinship between concentration and type of curvature produced by the growth regulators in corn pollen ether extractive

is shown in Figure 1.

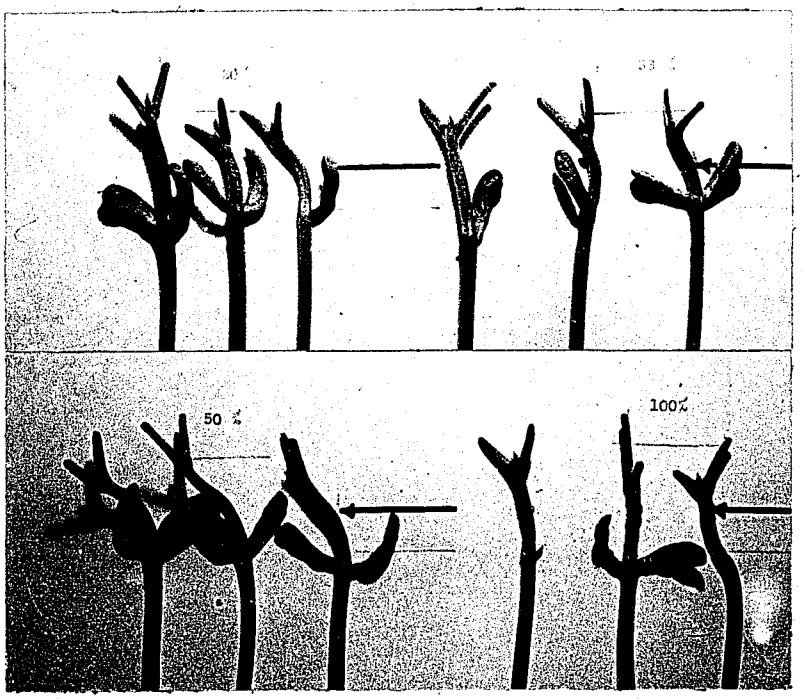

Figure 1. Curvatures induced on bean seedling internodes six hours following application of ether extractive from corn pollen to first internodes. Upper left: Plants treated with a 20% solution in lanolin. Upper right: Plants treated with a 33% solution in lanolin. Lower left: Plants treated with a 50% solution in lanolin. Lower right: Plants treated with undiluted material.

III. SOURCE OF MATERIAL

Pollen collection.

Mitchell and Whitehead (26) had employed the bagging method of obtaining corn pollen from the tassels. As this method was rather laborious, another, simpler method was desirable. The method of Anderson (1) seemed like a suitable possibility, for it consisted of removing the nearly mature tassels from the plants just before the anthers shed their pollen, placing the tassels on a clean sheet of paper indoors, and shaking off the pollen after a time. This method would permit a great quantity of pollen to be collected with a minimum of effort.

Fifty tassels of sweet corn, Zea Mays (variety Golden Cross Bantam) were collected and placed indoors on wrapping paper on the same day that 50 tassels were bagged. On the following day, the bags were removed from the plants, the pollen samples combined, and the tassels which had remained indoors were shaken over the paper on which they had been placed, and the discharged pollen was separated by sifting. From the 50 bagged tassels were obtained 18.5 gm. of pollen. From the 50 tassels removed from the plants before collection of the pollen were obtained 9.3 gm. of pollen.

Thus, it appears that collection by bagging gives a higher yield of pollen per tassel than collection by the "tray method".

Following collection, each sample of the pollen was covered with ethyl ether (about 50 ml.) and permitted to stand 2 days at room temperature. The pollen was filtered off from each ethyl ether extract with suction and the filtrates individually evaporated to dryness over a hot water bath. Almost twice as much residue remained in the flask containing the bag-collected pollen extractive as did in the flask containing the tray collected pollen extractive. When the residues were applied to the bean plants, that from the bag collected pollen produced a strong negative curvature, whereas that from the tray collected produced only a slight negative curvature. The growth regulatory substances were either present in considerably smaller concentrations in the tray collected pollen than in the bag collected, or else they were less readily extracted. This observation, combined with the differences in quantity of pollen obtainable by each method led to the adoption of the bag collection method of Mitchell and Whitehead in our further work.

Extraction of pollen.

The choice of a solvent with which to extract the pollen was based primarily on the findings of other workers. Gustafson (9) had used chloroform to extract the ovary-enlarging factor from corn pollen. Wittwer (33) had used 95% ethanol to extract the stem cell-elongating factor, while Mitchell and Whitehead (26) had used ethyl ether. Since ethyl ether extracted a smaller portion of the total weight of the pollen than did 95% ethanol, and since it was more readily removed from the extracts than chloroform, it was selected as a solvent.

In a typical extraction, 2 kg. of freshly collected pollen were placed in a 5 l. Florence flask within 6 hours of the time of collection. The pollen was covered with peroxide-free ethyl ether and permitted to stand for a day at room temperature. Periodically during extraction the ether was kneaded into the dough-like pollen mass with a stout glass rod. The extract was then decanted and replaced with fresh ether, and the entire process repeated. This extraction and decantation was subsequently repeated at daily intervals until ten fresh portions of ether had been used for each flask of pollen. The combined ether extracts were concentrated by distilling off the ether

through a Vigreux column, the last traces of solvent being removed by evacuating the flask containing the extract. In this manner, there were obtained 126 gm. of ether extractible lipid from 6.26 kg. of Golden Cross Bantam and 304 gm. from 7.89 kg. of Ohio M-15 hybrid field corn. This ether extractible lipid will be referred to in the future as "ether extractive".

The activity of the crude ether extractive obtained from both varieties of corn was about equal, although the bean assay suggested that the extract of the pollen from Golden Cross Bantam might be slightly more active. Since a larger quantity of pollen from field corn was available than from sweet corn, the majority of the experiments to be described were carried out on extracts from Ohio M-15 hybrid field corn.

IV. STABILITY TESTS

Previous experience had suggested that the growth regulators in corn pollen were of a somewhat fugitive nature. In order to obtain definite information concerning the risk of losing the growth regulators in the course of isolation processes, a series of experiments was carried out.

Thermal stability.

One-hundred mg. of the crude ether extractive were placed in a stoppered test tube and heated at 100° for 6 hours. A sample of the heated material produced about the same result on the bean assay as did the material not heated. This observation was interpreted to indicate that the growth regulators were not appreciably sensitive to heating in the absence of water.

A similar experiment was carried out with a sample of the corn pollen ether extractive to which had been added an equal volume of water. The growth regulator proved moderately heat-stable, even in the presence of water.

Stability toward irradiation.

Irradiation of a sample of the crude ether extractive from corn pollen for ½ hour with the light from a quartz mercury vapour arc lamp placed 4 inches away resulted in no detectable loss of growth regulatory activity. Apparently, the growth regulators were not sufficiently sensitive to irradiation to make it imperative to carry out laboratory operations in the dark.

Stability toward acids.

The crude ether extractive contained amounts of organic acids detectable with moistened wide-range indicator paper. If some rearrangement, oxidation, or hydrolytic cleavage of the growth regulator molecule into an inactive substance were promoted by acids, it seemed possible that these naturally occurring acids could contribute to a loss of activity.

Three hundred mg. of crude ether extractive were suspended in 3 ml. of 6 N hydrochloric acid. The mixture was then permitted to stand with occasional shaking at room temperature for 20 hours. At the end of this time, the oil was taken up in 10 ml. of ether. The residue left after evaporation of the ether caused but very little curvature when applied to the bean assay plants; the material not treated with acid caused a strong positive curvature. Taken along with information obtained later about the relative insolubility of the growth regulators in acids, this result suggests the wisdom of avoiding prolonged contact of the extracts with strong acids. Admittedly, it does not demonstrate that contact with any acid whatsoever would harm the corn pollen growth regulator or regulators.

Stability toward alkalis.

A test for stability of the growth regulators in the presence of aqueous alkali was carried out by suspending 300 mg. of crude ether extractive in 3 ml. of 1 N sodium hydroxide and heating for 6 hours in a water bath maintained at 95°. The material obtained by ether extraction of the cold alkaline solution and subsequent evaporation of the ether was not active as a growth regulator toward the bean plant. However, acidification of the aqueous phase with dilute hydrochloric acid, followed by ether extraction and evaporation of the ether phase to dryness left a residue which produced a strong response even following twenty-five fold dilution with lanolin. Since experiments to be described later showed that the growth regulators in the crude ether extractive were not base-soluble substances, it appears that treatment with hot aqueous alkali changes the growth regulators into other compounds having growth-regulating activity.

Stability toward oxidizing agents.

Mitchell and Whitehead (26) stated that no difficulty was experienced in obtaining highly active extracts from corn pollen by extraction with ordinary ether. Nevertheless, the possibility that the compounds

would be inactivated by traces of peroxides in ether used for extraction seemed great.

A 3% aqueous solution of hydrogen peroxide was selected as representing an oxidizing agent which was readily available, and which would quite probably cause many of the same types of oxidation that organic peroxides present in solvents might produce.

Three hundred mg. of crude ether extractive from corn pollen were suspended, with shaking, in 1 ml. of 3% aqueous hydrogen peroxide. The suspension was permitted to stand at room temperature for 3 days. Then the resulting suspension was shaken with 10 ml. of ethyl ether and the phases were separated. The residue which remained after evaporation of the ether under vacuum produced a slight positive curvature when applied to the first internode of a bean seedling. Diluted 5 fold and also 25 fold, it produced no curvature at all. Apparently, the treatment with dilute hydrogen peroxide had inactivated the growth stimulator present. As a consequence of this finding, only ethyl ether which had been freshly freed from peroxides was used in further work.

V. METHODS OF ENRICHMENT

Salt formation.

Since the growth regulators with which we were dealing had been extracted from the pollen with ethyl ether, any growth regulators in the crude extract would necessarily be ether soluble. This solubility in ethyl ether offered a possible simple means of enrichment of the extracts by dissolving the material in ethyl ether and then shaking out the ether solution with aqueous solutions of different strength acids or bases.

About 3.5 gm. of crude ether extractive were suspended in 7 ml. of ethyl ether. Fifteen mg. of the bright yellow solid failed to dissolve. This was filtered off with the aid of suction, and after it had been sucked as dry as possible, it was assayed for growth regulating activity on the bean plants. Because this substance produced only a slight curvature (much less than did a sample of the filtrate), it is assumed that the main activity arose from the oily filtrate adhering to the particles rather from the solid material itself.

Salt formation with bases.

The filtrate remaining was dissolved in 25 ml. of ethyl ether and then shaken out with 20 ml. of a saturated solution of sodium bicarbonate. Little carbon dioxide was evolved. The aqueous phase was acidified to litmus with hydrochloric acid and shaken out with two successive 10 ml. portions of ethyl ether. The combined ether extracts of the acidified bicarbonate extract yielded only 300 mg. of oil on evaporation. This oil induced very slight curvature of the bean seedlings. The ether solution containing those materials which were insoluble in aqueous sodium bicarbonate was shaken out with two successive 30 ml. portions of 1 N sodium hydroxide, followed by 10 ml. of a saturated solution of sodium chloride in water and then by 5 ml. of water. The ether phase, which contained neutral and basic constituents, was evaporated to dryness and assayed. It induced strong curvature in the bean plant.

The sodium hydroxide extracts and the salt wash solution were combined, acidified to litmus with hydrochloric acid, and extracted with three successive 10 ml. portions of ether. On evaporation of the ether there remained behind less than 200 mg. of material producing very slight curvature on the bean plant.

This first series of assays indicates that although some of the growth promoting substances present in corn pollen might be carboxylic acids, there are present in the ether extractive significant amounts of growth regulating substances which are either neutral or basic in nature.

Salt formation with acids.

In a second and somewhat similar study, 500 mg. of crude ether extractive was dissolved in 10 ml. of ethyl ether, filtered, and shaken out with 10 ml. of 1 N hydrochloric acid. The ether phase was dried over anhydrous sodium sulphate and evaporated to dryness. Bean assays indicated the presence of growth regulating activity in the residue from this ether solution.

Next, the acid extract was made just alkaline to litmus by the addition of 6 N sodium hydroxide and extracted with three 8 ml. portions of ethyl ether. Evaporation of the combined ether extracts to dryness left about 10 mg. of material. Since this small a quantity of material was very difficult to assay, it was diluted with 0.07 mg. of lanolin and applied to the test plants. No curvature was produced. Since the total volume of the diluted material was only a fraction of the volume of the starting material, this

test demonstrates that if any of the growth stimulating substances present in the pollen extract is basic in nature, it is but an insignificant portion of the total growth stimulating substances.

Since only a very small fraction of the total material contained in the crude ether extractive is soluble in either acid or base, the use of partition between ethyl ether and acid or between ethyl ether and base seemed of little value in effecting even a partial purification of the growth regulator.

The observed solubility behavior, combined with the fact that treatment of the crude ether extractive with hot sodium hydroxide solution transforms it into a material soluble in base suggests that the growth regulator sought could be an ester, a lactone, an amide, or a nitrile. However, the simultaneous presence in the molecule of such functional groups as alcoholic hydroxyls or methoxyls or carbonyl groups is not eliminated from further consideration.

Reaction with sodium bisulphite.

If by chance there were a highly reactive carbonyl group in the molecule, the possibility of concentrating the growth regulator by shaking out an etheral solution of the ethyl ether extractive with

sodium bisulphite solution seemed promising.

Accordingly, 500 mg. of the crude ether extractive were dissolved in 8 ml. of ethyl ether and shaken out with two successive 8 ml. portions of 40% sodium bisulphite. After separation of the two phases, the ether solution was evaporated to dryness and assayed. It showed little, if any, change in activity. The sodium bisulphite solution was made strongly alkaline by the addition of 15 ml. of 10 N sodium hydroxide. After standing at room temperature for 15 minutes, the alkaline solution was extracted with two 10 ml. portions of ether; the ether phase was then washed with 5 ml. of water and evaporated to dryness. There remained about 10 mg. of residue. After dilution with 100 mg. of lanolin, this residue was assayed by the bean test. It induced no curvatures in the bean plants.

One may conclude that if a part of the growth regulatory effect of a crude ether extract of corn pollen is due to a compound containing a functional group capable of reacting with sodium bisulphite, it is a minor part. If a sodium bisulphite addition compound formed, it was not regenerated by treatment with cold sodium hydroxide solution. Therefore, the

use of sodium bisulphite as a concentrating agent was not further considered.

Distillation.

The possibilities of utilizing distillation as a method of enrichment of the growth regulator content of the crude ether extractive were next to be investigated.

Steam distillation.

The first type of distillation studied was steam distillation. A knowledge of the behavior of the growth regulators in the presence of steam was important for two reasons. First, this information would indicate the possibility of separation of the growth regulator from the crude ether extractive by steam distillation. Second, it would show whether any risk of loss of the growth regulator was involved in concentrating solutions containing both the growth regulators and residual water.

One gram of the crude ether extractive was placed in a 50 ml. distilling flask with 25 ml. of water. The contents of the flask were boiled over a free flame, the vapour issuing from the side-arm of the

flask being collected in an ice-cooled receiver. When approximately 10 ml. of distillate had been collected, the distillation was discontinued and the distillate was shaken out with 5 ml. of ether. Evaporation of the ether left behind about 10 mg. of a fragrant oil. This oil, after dilution with 50 mg. of lanolin, showed no activity toward the bean assay. The boiler residue was applied directly to the assay plants. It showed marked growth regulatory activity. This experiment demonstrates that the growth regulator is so involatile and the proportion of volatile material in the crude ether extractive is so small that steam distillation would be of little value in purification of the growth regulator.

Molecular distillation.

In view of the success achieved in the molecular distillation of such relatively non-volatile materials as vitamins A and D, it seemed worth while to attempt distillation of the growth regulators from corn pollen ether extractive in a small short-path still.

A pot-type still similar to that designed by Washburn (28) was constructed. The distillation path length was approximately 1 cm. in the first model.

Two gm. of crude ether extractive from corn pollen were placed in a small Claisen flask fitted with a 1 inch long water cooled condenser and a receiver. The material was distilled at a bath temperature of 70° and a pressure of 2 mm. in order to remove the last traces of volatile solvent. Less than one drop of material distilled under these conditions. This small quantity of distillate showed no activity.

The residue was transferred with the aid of a medicine dropper to the boiler of the short path still. The condenser of the still was then filled with dry ice, the still attached to a dry ice trap and mercury vapour pump, and the pressure within the still was lowered to 10^{-4}mm. A heated oil bath was then raised about the bottom of the still, and the temperature of the bath was gradually increased. At suitable intervals, the bath would be lowered, and after the still had cooled to room temperature, the vacuum would be released and samples of both boiler content and distillate removed for assay. The still would then be re-connected to the vacuum system, and the distillation process resumed. The results of this experiment are listed in Table I.

Since the distillate obtained with a bath temperature between 135° and 195° did not seem as potent as

TABLE I

DISTRIBUTION OF ACTIVITY FOLLOWING DISTILLATION OF CORN POLLEN ETHER EXTRACTIVE AT 10^{-4}mm. PRESSURE

PATH LENGTH: 1 cm.

Bath temperature	Estimated weight of distillate	Activity of boiler residue	Activity of distillate
Below 25°	None	Low	----------
25° to 70°	500 mg.	Low	High
70° to 115°	250	----	Low
115° to 135°	100	High	Very low
135° to 195°	200	Very low	Medium

the boiler charge at the beginning of the 135° to 195° distillation, it appears that a considerable amount of decomposition took place at this high a temperature.

Subsequent to the first attempt at molecular distillation of the ether extractive, the path length of the pot-type still was shortened to approximately 1/2 cm. A 1 gm. sample of crude ether extractive was, as before, stripped of solvent in a small Claisen flask and then transferred to the short path still.

Fewer fractions were collected than in the first

attempt at molecular distillation.. The results of this second experiment are presented in Table II.

TABLE II

DISTRIBUTION OF GROWTH REGULATORY ACTIVITY FOLLOWING DISTILLATION OF ETHER EXTRACTIVE FROM CORN POLLEN AT 10^{-4}mm. PRESSURE

PATH LENGTH: 0.5 cm.

Bath temperature	Activity of boiler residue	Activity of distillate
Starting material	Strong	----------
Below 150°	Strong	Weak
150° to 170°	Medium	Medium

Inspection of Tables I and II leads to two conclusions. The first is that the growth regulators in the ether extractive may actually consist of more than one compound. The second is that even with the distillation path length reduced to 0.5 cm., the use of a pot-type of molecular still at a pressure of 10^{-4}mm. leads to considerable decomposition of the higher boiling of the growth regulators.

The use of pressures far below 10^{-4} mm. in the still might enable a pot type apparatus to be employed satisfactorily. Pressure measuring devices suitable for use at such high vacuums are costly, though, and none of the pumps locally available would have a satisfactory pumping speed at pressures below about 10^{-4}mm.

The difficulties involved in the use of a pot-type molecular still might well be overcome by substitution of a falling-film type of still or a centrifugal type of still for the pot type (11). At the present time, a representative of neither of these types of apparatus is available on the campus. Furthermore, the cost of purchasing one or the labour involved in the construction were so great as to make it highly desirable to find a satisfactory method of isolation not dependent on molecular distillation. For this reason, our attention was turned to additional possible isolation procedures.

Solvent distribution.

Distribution of the growth regulatory material between two immiscible organic solvent phases proved a considerably more effective method of partial purification than did distillation. Five groups of

solvents employed in preliminary experiments were petroleum ether and aqueous acetic acid, carbon disulphide and acetic acid, petroleum ether and aqueous acetone, benzene and aqueous ethanol, and petroleum ether and aqueous ethanol.

In each of these exploratory experiments, 500 mg. of crude ether extractive were dissolved in 7.5 ml. of the hydrocarbon or carbon disulphide. This was subsequently shaken out with an equal volume of the aqueous or acidic phase. Following separation, the phases were evaporated to dryness under reduced pressure and the residues assayed. The results are presented in Table III.

Those solvent combinations of which acetic acid was one component were tried several times on larger samples of crude ether extractive. However, each attempt, even when all evaporations were carried out under an atmosphere of nitrogen in vacuo, resulted in considerable darkening of the product and apparently in appreciable loss of activity. Therefore, acetic acid was finally discarded from consideration as a possible solvent.

The petroleum ether and aqueous acetone combination was also eliminated. The reason for this action was that the acetone extracted only one or two

TABLE III

DISTRIBUTION OF GROWTH REGULATORS IN CORN POLLEN ETHER EXTRACTIVE BETWEEN IMMISCIBLE SOLVENT PAIRS

Solvent combination	Phase containing most of activity	Phase containing most of lipid
Carbon disulphide and glacial acetic acid	Acetic acid	Carbon disulphide
Petroleum ether (B.P. 35-70°) and 95% acetic acid	Acetic acid	Petroleum ether
Petroleum ether and 60% aqueous acetone	Petroleum ether	Petroleum ether
Petroleum ether and 80% ethanol	Aqueous ethanol	Petroleum ether
Benzene and 60% ethanol	Aqueous ethanol	Benzene

percent of material from the crude ether extractive, leaving most of the active material together with most of the extraneous substances.

Petroleum ether and 80% ethanol as well as benzene and 60% ethanol showed considerable promise. Each of these combinations would permit about a ten-fold concentration of the growth regulator. Further, the substances extracted by each solvent combination were

not identical, so that by employing the two solvent combinations in succession a considerable enrichment in activity should be obtained.

The possibility remained that 80% ethanol was not the optimum concentration to employ. Accordingly, a series of four distributions between petroleum ether and aqueous ethanol were performed, a different concentration of ethanol being used in each experiment. Approximately 500 mg. of crude ether extractive were dissolved in 5 ml. of petroleum ether. The resulting solution was shaken out with five successive 1 ml. portions of aqueous ethanol of the chosen concentration. The combined ethanol extracts were evaporated to dryness under vacuum, weighed, diluted five fold with lanolin, and assayed. The petroleum ether residues were evaporated to dryness under vacuum, weighed, and assayed undiluted. The results of these experiments are presented in Table IV.

From Table IV, it may be seen that an aqueous solution of ethanol containing much less than 75% ethanol extracts the growth regulator from a petroleum ether solution incompletely. The difference in amount of contaminating material extracted by 50% and by 75% ethanol appeared to be so small that it did not seem worth while to carry out further explora-

TABLE IV

DISTRIBUTION OF CRUDE ETHER EXTRACTIVE FROM CORN POLLEN BETWEEN AQUEOUS ETHANOL AND PETROLEUM ETHER

% Ethanol	Wt. of aqueous phase	Wt. of pet. ether phase	Activity of aqueous phase	Activity of petroleum ether phase
0	20 mg.	435 mg.	Some	Much
25	8	503	Some	Much
50	40	516	Much	Slight
75	53	413	Much	None

tory experiments using concentrations of ethanol intermediate between 50% and 75%. Therefore, 75% ethanol was adopted for use in further petroleum ether and aqueous ethanol solvent partition studies.

Multiple counter-current extraction.

Because the simple solvent distribution experiments had been successful in concentrating the growth regulators in the ether extractive, the multiple counter-current solvent extraction method of Craig (5) seemed worth trying. The apparatus for performing

exactly the same type of an extraction described by Craig was unobtainable, but an equivalent process was carried out with apparatus already on hand.

Petroleum ether and 75% ethanol.

The first study dealt with the distribution of crude ether extractive between petroleum ether (B.P. 35-65°) and 75% ethanol. As a preliminary step of this trial, two volumes of petroleum ether were shaken out with one volume of 75% ethanol, so that the resulting solvents were mutually saturated. These saturated solvents were employed exclusively in the rest of the experiment.

One gm. of crude ether extractive was dissolved in 10 ml. of the petroleum ether. A small amount of insoluble pigment was filtered off, and the filtrate was shaken out with 5 ml. of the 75% ethanol in an 18 mm. X 150 mm. test tube. The ethanol phase was removed with the help of a medicine dropper to another test tube of the same size, and 10 ml. of petroleum ether was added. Five ml. of fresh 75% ethanol were added to the petroleum ether phase in the original test tube, and both tubes were shaken to insure mixing of the solvents. This process was then repeated, transferring the original 75% ethanol

phase to fresh petroleum ether each time and the original petroleum ether to a fresh portion of 75% ethanol, until a total of ten test tubes were involved in the scheme, and the original ethanolic phase had been extracted with ten portions of petroleum ether, while the original petroleum ether phase had been extracted with ten portions of 75% ethanol. The intermediate tubes in this counter-current scheme had, of course, been extracted with fewer than ten portions of solvent which had already been used one or more times in the solvent extraction.

If the test tube containing the original petroleum ether phase at the end of this process is designated as tube #1, and the test tube containing the petroleum ether phase which had been extracted with nine portions of 75% ethanolic extract from the original petroleum ether phase as tube #2, and so on, the tube containing the original 75% ethanol phase becomes tube #10. This designation will be used in reporting the results of assaying the residues obtained from vacuum evaporation of the contents of the various test tubes.

Immediately after evaporating the solvents, 50 mg. of lanolin were added to the contents of each

of the last eight test tubes in order to bring the volume up to a sufficiently large value to permit assaying. The results of the assays are presented in Table V. Under the conditions of this experiment, the greater part of the growth regulators migrated to tube #10.

TABLE V

GROWTH REGULATING ACTIVITY OF FRACTIONS FROM COUNTER-CURRENT DISTRIBUTION OF ETHER EXTRACTIVE BETWEEN 75% ETHANOL AND PETROLEUM ETHER

Fraction from tube #	Curvature produced in bean test
1	None
2	None
3	None
4	None
5	None
6	None
7	None
8	Positive
9	Positive
10	Very strong positive

Benzene and 65% Ethanol.

A second multiple countercurrent solvent extraction experiment was carried out on the crude ether extractive from corn pollen, using benzene and 65% ethanol which had been mutually saturated. Five ml. of benzene and 5 ml. of 65% ethanol were employed instead of 10 ml. of petroleum ether and 5 ml. of 75% ethanol. Otherwise, the experimental details were identical with those of the experiment just described. The results of the assays carried out on the residues obtained by evaporating the solvents from the constents of the various test tubes are shown in Table VI.

Precipitation.

Since none of the active residues obtained from the multiple counter-current solvent extractions were crystalline, it seemed evident that yet other purification means would have to be studied if any of the growth regulators were to be isolated in a pure form. The use of precipitating agents of various sorts either to separate non-active substances from the concentrates or to separate the active substances was an obvious possibility to consider.

TABLE VI

GROWTH REGULATING ACTIVITY OF FRACTIONS FROM COUNTER-CURRENT DISTRIBUTION OF ETHER EXTRACTIVE BETWEEN BENZENE AND 65% ETHANOL

Fraction from tube #	Curvature produced in bean test
1 (Original benzene)	None
2	None
3	None
4	Positive curv.
5	Strong positive curvature
6	Positive curv.
7	Negative curv.
8	None
9	None
10 (Original ethanol)	None

Digitonin precipitation.

The first reagent tested for precipitation of the plant growth regulators in the crude ether extractible was digitonin. One half gm. of the ether

extractive was suspended in 12 ml. of ether, and the insoluble particles of solid were centrifuged off. The clarified ether solution was dissolved in 5 ml. of absolute ethanol. Thereupon, a saturated solution of digitonin in alcohol was added until no further precipitate formed. The digitonide which separated was first filtered off; then the filtrate was evaporated to dryness under vacuum. Both the digitonide and the residue from the filtrate were applied to the bean test plants. The residue from the filtrate caused about the same amount of curvature as did the original crude ether extractive, while the digitonide produced no curvature. Apparently, then, the growth regulators were not compounds which would be precipitated by digitonin.

The use of digitonin at first seemed a practical means of removing contaminating sterols, for an early attempt at chromatographic adsorption of the crude ether extractive indicated that the sterols and the growth regulators were adsorbed in approximately the same region of a column of silica gel. However, later experiments showed that by the use of solvent distribution methods, the growth regulators could be freed from all impurities which would be precipitated

by digitonin. This observation led to abandoning the use of the relatively costly digitonin.

Lead acetate precipitation.

Partition experiments had indicated that the plant growth regulators were not acidic substances, and therefore would not be expected to form the usual types of heavy metal salts. Nevertheless, the possibility of utilizing insoluble salt formation as a means of removing contaminating substances from the enriched growth regulators seemed worthy of consideration.

Chronologically, these studies preceded the studies of multiple countercurrent solvent distribution. Therefore, the tests were carried out on ether extractive which had been enriched by extraction from a petroleum ether solution with 75% ethanol, vacuum evaporation of the (unneutralized) ethanolic solution to dryness, solution of the residue in petroleum ether, extraction of the petroleum ether solution with 90% equeous acetic acid, vacuum evaporation of the aqueous acetic acid solution to dryness, partial solution of the residue in acetone, and evaporation of the acetone solution. One gram of the acetone soluble residue was subsequently dissolved in 3 ml. of 95%

ethanol. To this solution was added 10 drops of a saturated aqueous solution of lead acetate. The copious orange colored precipitate was centrifuged off an 10 more drops of lead acetate solution were added. Little additional precipitate formed. This solution was re-centrifuged to effect clarification and decanted from the solid phase.

The decanted solution was made alkaline by the dropwise addition of 5 drops of concentrated ammonium hydroxide. A large volume of orange colored precipitate formed. This was centrifuged off and 5 more drops of concentrated ammonium hydroxide were added. little additional precipitate formed. The resulting suspension was re-centrifuged, and the clear liquid decanted from the solid. Each of the two lead salts was regenerated by suspension in 2 ml. of water followed by the addition of 10 drops of glacial acetic acid and extraction with 4 ml. of ethyl ether. The ether solutions were evaporated to dryness under vacuum, 1/2 ml. of water was added to each residue, the evaporation was repeated, and the final residues diluted in lanolin were applied to the assay seedlings. The greater part of the activity appeared in the material regenerated from the lead salt precipitated by neutral lead acetate. Neutralization with acetic acid of the ammoniacal

supernatant liquid from the basic lead salt precipitation, followed by ether extraction and evaporation of the ether left a residue possessing little or no activity toward the bean test. Thus, it appeared that the growth regulators had actually formed insoluble lead salts.

Unreliability of method.

Attempts to repeat the above experiment seemed unpredictable. At one time, the growth regulators would be found to have formed an insoluble lead salt; at another time, they would not.

The first suspected source of trouble was the concentration of the alcoholic solution from which the lead salt had been precipitated. When the experiment was repeated, precipitating the lead salt from solution in absolute ethanol with a solution of lead acetate in absolute ethanol, it was found that the growth regulators formed lead salts insoluble in absolute ethanol. Addition of water to the ethanol solution caused the formation of additional precipitate, but the active substances were not in this precipitate. Apparently, the presence of alcohol in the solution from which the lead salt had been precipitated was not a source of irregular behavior.

The second source of trouble was thought to be the use of acetic acid in the preliminary enrichment prior to the precipitation. Difficulty was still experienced, however, when the distribution between petroleum ether and 90% acetic acid was omitted.

The third source of error was the method of regeneration of the lead salt. It was thought that perhaps the excess of acetic acid used to regenerate the lead salt might also be a good solvent for the growth regulator, preventing its being extracted by the ether. Substitution of hydrochloric acid, in which the regulator had been shown to be relatively insoluble, resulted in no improvement. Similarly, the use of hydrogen sulphide as a means of regenerating the lead salt did not eliminate the difficulty.

The fourth source of irregularity to be considered was the solvent distribution between 75% ethanol and petroleum ether, which was carried out prior to all the attempts at salt precipitation. The ethanolic phase resulting from such a distribution proved to be weakly acidic, and it seemed remotely possible that some sort of a modification in the structure of the growth regulator could take place during evaporation

of this solution. The extent to which such a decomposition occurred would probably be a function of the temperature to which the solution was heated during evaporation, and the length of time required for concentration.

Two gm. of crude ether extractive were dissolved in 20 ml. of petroleum ether. The undissolved pigment was filtered off, and the filtrate was subsequently shaken out with five successive 10 ml. portions of 75% ethanol. The resulting ethanolic phases were combined, and then divided into two equal portions. One portion was evaporated to dryness in vacuo, nitrogen being introduced through the customary capillary. The other was adjusted to a pH of about 7 by the addition of 0.01 N sodium hydroxide and evaporated to dryness similarly. The residual sodium salt was then taken up in 2 ml. of water, acidified by the addition of 3 drops of 6 N hydrochloric acid, and extracted with 2 ml. of ethyl ether. The residue from evaporation of the ethyl ether was diluted ten fold with lanolin and applied to the test plants. The substance remaining from evaporation of the 75% ethanolic solution not neutralized before evaporation was also diluted and applied to the test plants. The diluted residue obtained from the ethanol phase which had been neutral-

ized before evaporation produced approximately the same magnitude of positive curvature as did the original crude ether extractive. The diluted residue from the ethanol phase not neutralized prior to evaporation produced only a negative curvature when applied to the test plants. This observation was interpreted as an indication that some chemical change took place during evaporation of the 75% ethanol solution of growth regulator if this solution was not first neutralized.

A second observation confirmed this supposition. Addition of aqueous lead acetate to a 75% ethanol solution of the residue from evaporation following neutralization of the 75% ethanol extract resulted in precipitation of a colorless lead salt, which on regeneration with hydrogen sulphide gave no active substance. Addition of aqueous lead acetate to a 75% ethanol solution of the resiude from evaporation without neutralization of the 75% ethanol extract resulted in the precipitation of an orange colored solid, which on regeneration with hydrogen sulphide yielded a substance which would exert a regulatory effect on the growth of the bean internode.

As a consequence of this observation, the 75%

ethanol solution was neutralized with dilute sodium hydroxide before concentration in all further experiments.

Prior to finding that the regulators were undergoing some alteration in their structure during concentration of the 75% ethanolic phase from the solvent partition, several attempts were made to use other than lead salts as a means of concentrating the growth regulator. Since all these experiments were carried out on material obtained from evaporation, without neutralization, of the 75% ethanol concentrate, they probably relate to degredation products of the growth regulators rather than to the intact substances. Therefore, the experiments will not be described in detail. Table VII sumarizes the types of salts studied, together with the observed behavior of the growth regulator(s).

Chromatographic adsorption.

A total of approximately two dozen attempts at isolation of the growth regulators from the ether extractive by chromatographic adsorption were made. Obviously, these studies did not exhaust all the possible combinations of adsorbent, solvent, and

TABLE VII

APPARENT SOLUBILITY OF SALTS OF GROWTH REGULATORS FROM CORN POLLEN ETHER EXTRACTIVE

Salt	Solvent	Remarks
Lead salt	Ethanol	Active salt insoluble in absolute ethanol
Lead salt	Ether (ethyl)	Active salt insoluble in ether
Lead salt	Water	Active salt insoluble in water
Sodium salt	Water	Sodium salt very soluble in water
Calcium salt	Water	Calcium salt soluble in water
Lithium salt	Water	No precipitate with lithium acetate
Lithium salt	Water	No precipitate with lithium acetate and ammonium hydroxide
Thallous salt	Water	No precipitate with thallous acetate
Cadmium salt	Water	No precipitate with cadmium chloride
Picrate	95% Ethanol	No precipitate with picric acid
Zinc salt	50% Ethanol	Precipitate froms with $ZnCl_2$. Zinc salt of regulator appears soluble.

processing of the extract prior to adsorption. They will not be described in great detail, although a summary of the results of the adsorption studies is presented in Table VIII.

Adsorbents used.

A study of the data in Table VIII shows that only two adsorbents tried possessed satisfactory adsorption characteristics. Anhydrous sucrose (dried over phosphorus pentoxide) is too weak an adsorbent to be used for chromatographic adsorption of the growth regulators. Aluminum oxide ("Alorco" activated alumina, F-20 grade), "Special Filtrol" and "Super Filtrol" all proved to be such powerful adsorbents that only a small fraction of the total growth regulators present could be eluted. Calcium carbonate reacted with the organic acids present in the crude ether extractive, evolving carbon dioxide and breaking the column packing. This latter difficulty could perhaps have been avoided by suitably treating the extract before adsorption. However, the adsorptive capacity of calcium carbonate seemed too small to warrant further experimentation with this adsorbent. Calcium sulphate and silica gel both proved to be suitable adsorbents. The silica gel used

TABLE VIII

BEHAVIOR OF VARIOUS SOLVENT AND ADSORBENT COMBINATIONS IN CHROMATOGRAPHIC ADSORPTION OF CORN POLLEN ETHER EXTRACTIVE

Starting material	Adsorbent	Solvent	Developer	Eluent	Location of activity
Crude ether extractive	Silica gel	Pet. ether	Pet. ether	Acetone	Top 1 1/2" of column
Pet. ether soluble part from distribution with 80% ethanol	Silica gel	" "	" "	"	"
80% Ethanol soluble part from distribution with pet. ether	Silica gel	" "	" "	"	"
Crude ether extractive	Alumina	" "	" "	. . . . .	. . . . .
Crude ether extractive	$CaCO_3$	" "	" "	. . . . .	. . . . .
75% Ethanol soluble part from distribution with petroleum ether	Silica gel	" "	" "	Ethanol & ethyl ether	Top 2" of column
Material from top section of preceding column	Silica gel	Benzene	80% Benzene 20% Ethyl acetate	Ethanol & ethyl ether	Center of column

TABLE VIII, Continued

Starting material	Adsorbent	Solvent	Developer	Eluent	Location of activity
Ether extractive	Sucrose	Pet. ether	Pet. ether	Ethyl ether	Not localized
" "	Alumina	" "	" "	Acetone, then acetic acid	. . . . . (Not recovered)
" "	$CaCO_3$	" "	" "	. . . . .	Column broke
75% Ethanol soluble from distribution with pet. ether	Sucrose	" "	" "	Pet. ether	Not localized
Ether extractive	Calcium sulphate	" "	" "	Ether & ethanol	Part in percolate, part at top.
" "	"Super Filtrol	" "	" "	Acetone, then ethanol	Little could be eluted
" "	"Special Filtrol"	" "	" "	Acetone, then ethanol	None could be eluted

TABLE VIII, Continued

Starting material	Adsorbent	Solvent	Developer	Eluent	Location of activity
Eluate from top of silica gel chromatogram	Silica gel	Pet. ether	Benzene	Ethanol & ethyl ether	Localized at top 1" of column
75% Ethanol soluble part from counter-current solvent ext.	"	"	Pet. ether	Ethanol & ethyl ether	Localized at top 1" of column
75% Ethanol soluble part from counter-current solvent extraction	"	Benzene	Benzene	Ethanol & ethyl ether	Localized at top 1 1/2" of column
Regenerated insoluble lead salt	"	"	"	Ethanol	None recovered
Regenerated insoluble lead salt	"	"	Benzene & ethyl acetate	Ethanol	None recovered

(Davison, through 325 mesh) was so finely divided that a solvent could be passed through a column of the material only with great difficulty. Admixture of two parts of the silica gel with three parts, by weight, of "Hyflo" filter aid produced a mixture which still possessed good adsorptive properties, yet which would permit a solution to pass readily through a column of the packed material.

Solvents employed.

Petroleum ether gave satisfactory results as a developer for use with the calcium sulphate column. Benzene or a mixture of benzene and ethyl acetate served as a satisfactory developer for use with silica gel.

Experimental observations.

Several facts learned from these experimental efforts are not evident in Table VIII.

The first of these is that the location of the growth regulator on the adsorption column could not be correlated invariably with a colored zone. Colored zones did appear, but the growth regulator might or might not be located in the same part of the adsorbent as one of the colored compounds.

The second is that the localization of the growth regulators on the column improved with partial purification of the extracts prior to adsorption. On most of the adsorbents studied, the growth regulators were adsorbed from a solution of the crude ether extractive over a rather wide band. If the ether extractive were enriched by some method, such as a distribution between 75% ethanol and petroleum ether prior to adsorption, the zone on which the regulators were adsorbed was made considerably narrower.

The third, and perhaps the least encouraging, observation was that none of the eluates containing most of the growth promoting activity, even after repeated adsorption, showed any tendency to crystallize. They were generally very viscous glass-like substances, which did not crystallize even following such measures as cooling in a bath of solid carbon dioxide, scratching, or even permitting to stand undisturbed in the refrigerator for a period of weeks.

If the growth regulators had shown some tendency to crystallize, chromatographic adsorption undoubtedly would have proved a useful method of isolation, for the growth regulator(s) appeared to have been concentrated about 500 times by the best of these

adsorption schemes.

Even if the growth regulators studied were not solids, chromatographic adsorption would have been valuable provided that sufficiently accurate assay methods were available. The photographs shown in Figure 1 (p. 8) illustrate that the bean assay method employed was not sufficiently accurate to detect small changes in concentration. It would be difficult to tell by the bean assay alone whether a repeated adsorption had increased the purity of a partly purified growth regulator, for example, from 20% to 33%, or whether the adsorption had resulted in a purity decrease from 33% to 20%.

If the increase in growth regulatory activity during the concentration processes were correlated with some property of each concentrate, such as its apparent molecular weight, its nitrogen content, or its adsorption spectrum, it is altogether possible that solvent partition methods, combined with chromatographic adsorption could lead to the isolation of the pure compound, even if this substance should be a liquid at room temperature.

The difficulty of manipulating small quantities of liquid is so much greater than that involved in

handling small quantities of crystalline solids that it seemed highly desirable to devise some method of transforming the growth regulator into a crystalline substance and subsequently isolating this substance. Such a derivative could easily be purified, and it might possibly yield a great deal of information concerning the chemical nature of the original growth regulator.

The information thus far obtained eliminated certain possibilities from the structure of the compound, although it did not point to any one type of structure which would be likely to form a solid derivative. In order to establish with a greater degree of certainty whether alcoholic hydroxyl groups or carbonyl groups were present in the compound, several experiments were devised.

Reaction with Girrard's reagent P.

The failure of the growth regulator to react with sodium bisulphite still did not exclude the possibility that at least one of the growth regulators present was a ketone, or even a relatively unreactive aldehyde. The possibility of forming a water-soluble derivative of any ketone or aldehyde present in the ether extractive by causing it to react with Girrard's

reagent P (acetylhydrazidepyridinium chloride) (8) seemed a helpful way of deciding whether the chief growth regulator was a carbonyl compound. Accordingly, 1 gm. of crude ether extractive was dissolved in a solution of 1 ml. of glacial acetic acid in 10 ml. of 95% ethanol. To this was added 1 gm. of Girrard's reagent P. The resulting solution was heated under a reflux condenser for 30 minutes, cooled to room temperature, and adjusted to a pH of about 7 by the addition of a saturated solution of sodium carbonate. The insoluble material which separated was then taken up in 30 ml. of ether. The residue obtained by evaporation of the ether from this extract showed good growth regulatory activity in the bean test.

The aqueous phase containing any pyridinium salts which might have formed in the reaction with Girrard's reagent P was made 0.5 N in hydrochloric acid and permitted to stand at room temperature for 1 hour. It was subsequently shaken out with 30 ml. of ethyl ether. Evaporation of this ether extract left a few milligrams of residue which possessed no growth regulating power.

While these experimental results do not prove conclusively that the growth regulators are not

aldehydes or ketones, they indicate that such such is probably the case.

Reaction with phthalic anhydride.

The relatively great solubility of the growth regulators in alcohol, as compared with their solubility in hydrocarbons, would seem to suggest that the material might be an alcohol. The device of causing an oil soluble and water insoluble alcohol to react with phthalic anhydride, forming alkali soluble hydrogen phthalates, was used by Stephan (31), (32) with considerable success. This method enabled Stephan, as well as many investigators since Stephan's time, to separate alcohols from mixtures with other compounds. It therefore seemed that the formation of hydrogen phthalates might prove a helpful means of detecting alcoholic hydroxyl groups in the growth regulator molecules.

Consequently, 500 mg. of the crude ether extractive was dissolved in 5 ml. of toluene, and to the resulting solution was added 500 mg. of finely powdered phthalic anhydride. This mixture contained in a tightly stoppered test tube was placed in a water bath and heated at 90° for six hours. At the end of the heating period, the tube was removed from the water

bath, cooled, and the toluene solution was shaken out with two successive 3 ml. portions of 1 N sodium hydroxide, followed by 3. ml. of water. The resulting toluene solution, containing many crystals of phthalic anhydride, was evaporated to dryness and the residue was applied to the internodes of young bean plants. No growth regulatory effect could be noticed. It would appear, then, that the phthalic anhydride had transformed the growth regulators into base soluble compounds.

In order to confirm this observation, the wash water, combined with the sodium hydroxide extracts, was shaken out with two 5 ml. portions of ethyl ether to remove any growth regulators, as such. The alkaline solution remaining was then heated at 90° for another six hours to effect hydrolysis of the hydrogen phthalates, acidified, and extracted with three 5 ml. portions of ethyl ether. The ether extracts of the hydrolysis products were diluted with 100 mg. of lanolin to aid adherence and applied to the first internodes of young bean seedlings. The plants developed distinct positive curvatures.

It is certain that these curvatures were not produced by phthalic acid, for no curvatures resulted from application of a paste of phthalic acid in lanolin

to the bean internodes. It therefore appears very probable that the growth regulators in corn pollen contain at least one primary hydroxyl group in each molecule, for the period of heating with phthalic anhydride was too short to permit the appreciable conversion of a secondary or a tertiary alcohol to a hydrogen phthalate (25).

Reaction with 3-nitrophthalic anhydride.

Since the position of aromatic nitro compounds adsorbed on a column of alumina dyed with morin may be seen with the aid of the fluorescence extinction phenomenon (2), the possibility of converting the alcohols in the crude ether extractive into 3-nitrophthalates seemed a hopeful method of enrichment. The solubility of the 3-nitrophthalates in aqueous alkali would permit separation of the 3-nitrophthalates from the larger part of the lipid material. The 3-nitrophthalates could then be separated from each other by chromatographic adsorption, the separated 3-nitrophthalates saponified, and the saponified products assayed in order to determine which of the crystalline products were the 3-nitro hydrogen phthalates of the growth regulators.

When 3-nitrophthalic anhydride was substituted for phthalic anhydride in the procedure just outlined, the activity no longer remained in the material more soluble in toluene than in aqueous alkali. However, saponification of the resulting 3-nitrohydrogen phthalate mixture did not yield a material which would show a growth regulatory effect on the bean seedling. It is altogether possible that the presence of the nitro group of the 3-nitrophthalic acid, in the alkaline medium, resulted in the oxidation of the growth regulator, which has already been shown to be subject to oxidative destruction.

Preparation of p-phenylazobenzoates.

The p-phenylazobenzoates of all alcohols are bright colored esters, and they are reported (29) to be easily hydrolyzed. If the alcohol fraction of the ether extractive from corn pollen could be converted into p-phenylazobenzoates, then the possibility seemed great that these substances could readily be purified by chromatographic adsorption.

Five gm. of crude ether extractive were carried through the single partition between 75% ethanol and petroleum ether described on page 30, followed by a single partition between benzene and 65% ethanol.

The resulting solution in 65% ethanol was neutralized to a pH of 7 by the addition of 0.01 N sodium hydroxide, and was evaporated to dryness under reduced pressure, the evaporation being interrupted when the volume was about 1/10 of its original value, in order to permit checking the pH of the solution. The addition of a few drops of 0.01 N hydrochloric acid brought the pH back to 7.0 from 7.5. The evaporation was then continued to dryness. The residue was subsequently dissolved in 5 ml. of anhydrous pyridine and 0.8 gm. of p-phenylazobenzoyl chloride was added. The solution was then permitted to stand at room temperature for 24 hours. At the end of this period, 15 ml. of 6 N hydrochloric acid was added, and the orange colored precipitate which formed was filtered off from the solution immediately.

A portion of the mixed precipitate of p-phenylazobenzoic acid and p-phenylazobenzoates was hydrolyzed by refluxing for 10 minutes with a 1 N solution of sodium hydroxide in 95% ethanol. At the end of this time, the alcoholic solution was cooled, diluted with an equal volume of water, and acidified to Congo red paper with 6 N hydrochloric acid. The resulting suspension of orange colored solid was shaken out with

three equal volumes of ether, and the ether extracts were evaporated to dryness, the residue being mixed with an equal volume of lanolin before application to the bean plants. This hydrolysate from the p-phenylazobenzoates induced a strong positive curvature in the test plants.

The remaining portion of the mixture of p-phenylazobenzoic acid and p-phenylazobenzoates was dissolved in benzene and passed through a chromatographic adsorption column packed with "Alorco F-20 Grade" activated alumina. The adsorption on alumina appeared to be very strong. The separate bands did not begin to move down the column until the developing solvent had been changed from benzene to ethyl acetate. Furthermore, the alumina appeared to permit poor resolution of the p-phenylazobenzoates. It was very difficult to elute the adsorbed derivatives from the alumina, hot 95% ethanol containing acetic acid failing to remove them completely.

A trial adsorption on silica gel, as recommended by Reich (29), indicated that silica gel (in this instance mixed with 1.5 times its weight of "Hyflo Super-Cell") would be a very suitable adsorbent for the particular p-phenylazobenzoates under consideration.

VI. METHODS OF ISOLATION

Isolation of pure p-phenylazobenzoates.

Considerable difficulty was experienced in obtaining pure p-phenylazobenzoates in the earlier experiments. The trouble proved to be due partly to the fact that the concentrate treated with p-phenylazobenzoyl chloride contained enough water to result in the formation of appreciable quantities of p-phenylazobenzoic acid, and the acid would then be adsorbed over such a large portion of the adsorption columns that all the esters were contaminated with p-phenylazobenzoic acid. By taking care to dry the concentrate well before preparing the esters, this difficulty was overcome to a considerable extent. Several p-phenylazobenzoates were obtained pure, among these being the p-phenylazobenzoates of two of the growth regulating substances in corn pollen ether extractive.

The following procedure is typical of one of the more successful ventures. Fifty gm. of crude ether extractive were dissolved in 400 ml. of 35-70° petroleum ether, and the insoluble pigment was filtered off. With the aid of large separatory funnels, a "multiple counter-current solvent extraction"

(see page 32) was carried out. Two hundred ml. portions of 75% ethanol and 400 ml. portions of petroleum ether, b.p. 35-70° were employed in each extraction. A total of ten fresh portions of petroleum ether were employed. The 75% ethanol phase from the separatory funnel containing the material most readily soluble in aqueous ethanol was adjusted to a pH of about 7 with 0.01 N sodium hydroxide and evaporated in vacuo. The pH was checked after 75% of the solvent had evaporated, and a few drops of 0.01 N hydrochloric acid were then added to return the pH to about 7 before continuing the evaporation. There remained about 150 mg. of non-volatile residue which shall, for convenience, be called "residue A".

The 75% ethanol phases from the funnels containing the three portions next most readily soluble in ethanol were combined and treated in the same fashion. The 500 mg. of substance obtained from evaporation of these extracts shall, for convenience, be called "residue B".

Both residues A and B were dried for 24 hours in a vacuum desiccator over phosphorus pentoxide, and subsequently each was dissolved in 25 ml. of anhydrous pyridine. Each solution was next cooled in an ice bath. When the temperature had fallen to 5°,

1.0 gm. of p-phenylazobenzoyl chloride was added to the solution of residue A and 3.5 gm. to that of residue B. Thereafter, the flasks were tightly stoppered and placed in the refrigerator, where they remained at 5° for one hundred hours. By this time, numerous orange crystals had formed in each flask. These were filtered off from the contents of each flask, rinsed with 10 ml. of water, and dried in a vacuum desiccator before further treatment.

The pyridine insoluble material formed from residue A was dissolved in 160 ml. of chloroform free from ethanol, and the resulting solution was divided into two equal parts. Each half was adsorbed on a 43 x 175 mm. chromatographic adsorption column packed with a mixture of 1 part silica gel and 2 parts "Hyflo-Super-Cell". The chromatograms were developed by passing chloroform through the column until the bottom colored zone had completely passed through the column into the percolate. This required about 400 ml. of solvent for each column.

Following development of the chromatogram, the packing was extruded from each column. There appeared to be two separate zones of adsorbed colored compounds on the column, although these zones were somewhat indistinctly defined. The packing was cut into

two almost equal sections in an effort to separate these different zones, and each section was eluted by stirring the adsorbent into 250 ml. of 95% ethanol and filtering. On evaporation, the eluate from the top section yielded about 50 mg. of orange colored needles, which following recrystallization from ethyl acetate proved to be p-phenylazobenzoic acid. The eluate from the bottom section of adsorbent yielded 150 mg. of orange needles which also proved to be p-phenylazobenzoic acid.

The percolate, on evaporation, yielded 320 mg. of orange needles. These, after three successive recrystallizations from ethyl acetate, reached the constant melting point of 203-204°. Unlike p-phenylazobenzoic acid, they were insoluble in dilute base.

When 5 mg. of the purified p-phenylazobenzoate was boiled for five minutes with 3 ml. of 50% ethanol 1 N in sodium hydroxide, the product completely dissolved. Acidification of the cooled solution to a pH of 4 with 10% hydrochloric acid produced a voluminous precipitate. The acid solution was shaken out with three successive 5 ml. portions of ethyl ether and the combined ether extracts were evaporated to dryness. The residue which remained, diluted 1,000 fold with lanolin produced a response in the assay plants about

equal to that caused by application of the crude ether extractive. Since the p-phenylazobenzoate had been recrystallized to a constant melting point, it seems unlikely that this growth regulatory activity was due to a trace of impurity present in the p-phenylazobenzoate. Unfortunately, as earlier experiments have shown, alkaline hydrolysis of the intact growth regulator changes it into another substance still having growth regulatory properties. Since the ratio of the activity of this hydrolysis product to the activity of the unchanged growth regulator is not known, it cannot be said with certainty what portion of the total growth regulatory activity of the 50 gm. of crude ether extractive is represented by the 320 mg. of p-phenylazobenzoate isolated.

It is certain, however, that the activity of the saponified p-phenylazobenzoate is not due to an artifact arising during the alkaline treatment of p-phenylazobenzoic acid. A saturated paste of p-phenylazobenzoic acid in lanolin is completely without effect on the bean seedling, before as well as after heating with alcoholic sodium hydroxide.

The pyridine insoluble p-phenylazobenzoates from residue B weighed about 500 mg. They were dissolved in 1 liter of benzene and the solution was divided

into four equal parts. Each part was adsorbed on a 43 x 175 mm. column of the 1:2 silica gel and "Hyflo" mixture, development being carried out by passing 1 liter of benzene through each column. The resulting chromatogram appeared to consist of a very broad orange zone extending from the bottom of the adsorbent to within 35 mm. of the top, together with a 30 mm. broad zone at the top of the adsorbent. Extrusion of the packing and elution with 95% ethanol showed the bottom zone to consist almost entirely of p-phenylazobenzoic acid. The material from the top zone was light orange in color, had a melting point range of about 50°, and was obviously quite impure. However, alkaline hydrolysis of the crude eluate followed by assaying showed that the active substance in these chromatograms was associated with the narrow zone at the top of the column. Because the total material obtained from this top zone amounted to only 15 mg., it was not investigated in great detail. Since it appeared to be very soluble in cold ethyl acetate, whereas the active p-phenylazobenzoate obtained from residue A was not, and since the two substances were adsorbed on different parts of columns packed with the same adsorbent, it is very likely that the active material found in residue B is a different substance from that found in residue A.

Inasmuch as both residue A and residue B received identical treatment with pyridine and p-phenylazobenzoyl chloride, it is unlikely that one of these substances should be a partial esterification product and the other a complete esterification product of the same alcohol. It seems rather more probable that the original crude ether extractive contained at least two different substances having growth regulatory action on the elongating bean stem. This supposition is in agreement with the results of the attempted short-path distillation of the ether extractive.

The pyridine solutions resulting from formation of the p-phenylazobenzoates were combined, diluted with 75 ml. of water and acidified to a pH of 5 by the addition of 10% hydrochloric acid. Extraction with five successive 50 ml. portions of ether followed by evaporation of the solvent from the combined ether extracts left a residue consisting mainly of p-phenylazobenzoic acid. By taking the residue up in 200 ml. of ethyl ether and shaking the solution out with 100 ml. of a saturated aqueous solution of sodium bicarbonate, most of the interfering acid was removed. Evaporation of the ether from the solution of p-phenylazobenzoates and other non-acidic substances left 1,200 mg. of

orange colored semi-solid residue.

This product was dissolved in 200 ml. of benzene and adsorbed on a 44 x 215 mm. column packed with a mixture of 1 part silica gel and 2 parts "Hyflo". Development with chloroform resulted in the formation of several distinct colored zones of adsorption. The percolate was collected in separate portions, corresponding approximately to these zones, as the adsorbed materials were washed through the column. Designating the first portion of percolate collected as fraction #1, the second as fraction #2, etc., the quantities of percolate, together with the weight of non-volatile material obtained following evaporation of the percolates are shown in Table IX. Fraction #6, the last of these, was obtained by passing acetone through the column to remove most of the adsorbed material.

Hydrolysis of fractions 1 through 6, as previously described for the other p-phenylazobenzoates, showed compounds having growth regulating activity to lie principally in fractions 1, 2, 3, and 6. Only in fraction 2 was this effect quite strong. After recrystallization from ethyl acetate, the p-phenylazobenzoate from fraction 2 melted at 117-119°. Whether this p-phenylazobenzoate represents a different alcohol from that corresponding to the p-phenylazobenzoate

TABLE IX

QUANTITIES OF SUBSTANCE OBTAINED FROM CHROMATOGRAPHIC ADSORPTION OF PYRIDINE SOLUBLE p-PHENYLAZOBENZOATES

Fraction #	Volume of eluate	Weight and appearance of residue
1	300 ml.	166 mg. yellow oil
2	180	173 mg. red-orange crystals
3	270	19 mg. orange wax
4	550	19 mg. orange wax
5	475	288 mg. red crystals
6	645	500 mg. red-brown gum

melting at 203-204°, or whether it is only a partial esterification product of the same compound is a matter which can be settled only by further study of the problem.

The fractions other than fraction #2 seemed too weakly active to warrant further consideration. However, it is remotely possible that these fractions contained yet other growth regulators which would be worthy of study if large quantities of pollen extract were available.

VII. PROBABLE STRUCTURE OF GROWTH REGULATOR FORMING p-PHENYLAZOBENZOATE MELTING AT 203-204°

The p-phenylazobenzoate melting at 203-204° appeared to be obtainable in larger quantity than the other esters obtained from the ether extractive. Therefore, further efforts were directed toward elucidating the constitution of this p-phenylazobenzoate.

Elementary analysis..

Analysis of the p-phenylazobenzoate showed it to contain 71.2% carbon, 4.0% hydrogen, and 12.9% nitrogen. Its molecular weight, by the Rast method (using camphor as a solvent), was found to be of the order of 527. These values are in agreement with those calculated for a compound having the empirical formula $C_{32}H_{23}N_5O_4$, which would contain 71.0% carbon, 4.3% hydrogen, 12.9% nitrogen, and have a molecular weight of 542.

Probable empirical formula.

Now, if there were only one esterified hydroxyl group in the molecule, thirteen carbon atoms, nine hydrogen atoms, two nitrogen atoms, and one oxygen

atom would belong to the p-phenylazobenzoyl residues, and the growth regulator itself would have the empirical formula $C_{19}H_{15}N_3O_3$. If there were two esterified hydroxyl groups in the molecule, twenty-six carbon atoms, eighteen hydrogen atoms, four nitrogen atoms, and two oxygen atoms would belong to the p-phenylazobenzoyl residues, and the growth regulator would have the empirical formula $C_6H_7NO_2$.

If the p-phenylazobenzoate having the empirical formula $C_{32}H_{23}N_5O_4$ contained one esterified hydroxyl group in each molecule, saponification should yield 458 mg. of sodium p-phenylazobenzoate for each gram of ester. If two esterified hydroxyl groups were present in the molecule, saponification should yield 916 gm. of sodium p-phenylazobenzoate for each gram of ester. As sodium p-phenylazobenzoate has a maximum absorption of light at a wavelength of 3650 Å, it should be possible to determine by spectrophotometric measurements at this wave length the quantity of sodium p-phenylazobenzoate formed on saponification of the ester and consequently the number of p-phenylazobenzoyl residues associated with each molecule of the growth regulator.

When 1.12 mg. of the p-phenylazobenzoate were

saponified by boiling for 10 minutes with 1 ml. of a 50% ethanolic solution 1 N in sodium hydroxide, and the hydrolysate was cooled and made up to a volume of 10.0 ml. with 50% ethanol, the resulting solution was found to contain 0.99 mg. of sodium p-phenylazobenzoate. This would correspond more closely to the 1.02 mg. of sodium p-phenylazobenzoate theoretically obtainable from 1.12 mg. of the ester having two p-phenylazobenzoyl residues in each molecule than to the 0.521 mg. obtainable from 1.12 mg. of the ester having one p-phenylazobenzoyl residue in each molecule. Apparently, the empirical formula of the growth regulator is $C_6H_7NO_2$, rather than $C_{19}H_{15}N_3O$.

Methoxyl content.

A methoxyl determination indicated that there were no methoxyl groups in the molecule.

N-Methyl group content.

Similarly, an N-methyl determination showed that N-methyl groups were absent from the structure of the p-phenylazobenzoate, and therefore that N-methyl groups were absent from the molecule of the growth regulator.

C-Methyl group content.

A C-methyl determination indicated that the molecule contained about 0.5 C-methyl groups. However, an equivalent quantity of p-phenylazobenzoic acid also appeared to contain 0.5 C-methyl groups. Therefore, the apparent C-methyl content of the p-phenylazobenzoate may be attributed to interference arising from the presence of the p-phenylazobenzoyl residues rather than to the actual presence of a C-methyl group in the growth regulator molecule.

Function of the oxygen atoms.

Since there are two residues in the molecule capable of acylation, one may conclude either that the molecule contains two hydroxyl groups (the nitrogen atom being involved in some relatively unreactive structure) or else that the molecule contains one hydroxyl group and one primary or secondary amino group. Since the intact growth regulator appears to be nearly neutral in nature , it is improbable that the molecular structure includes a primary or a secondary amino group. It is therefore likely that the molecule of growth regulator contains two hydroxyl groups.

Function of the nitrogen atom.

Since both oxygen atoms of the growth regulator molecule can be accounted for on the basis of the two hydroxyl groups present, the possibility of the nitrogen's being present as a part of an acyclic amide, an oxime, a nitro group, a nitroso group, or an amine oxide may be excluded from further consideration.

The experimental evidence thus far presented does not exclude a nitrile from the list of possibilities. However, when the p-phenylazobenzoate was saponified in 50% alcohol 1 N in potassium hydroxide, a strip of moistened red litmus paper placed in the vapour arising from the boiling solution showed no change in color. This test indicated that the nitrogen in the growth regulator was probably not involved in a nitrile structure.

As a result of a process of elimination, it appears that the nitrogen of the growth regulator molecule is quite probably a part of a tertiary amino structure, and that this group is modified through resonance to show few of the basic properties characteristic of many amines.

Possible structural formula of the growth regulator.

Admittedly, the magnitude of the molecular weight of the p-phenylazobenzoate introduces considerable uncertainty into the number of hydrogen atoms present in the growth regulator molecule. From just the elementary analyses and molecular weight data, one could easily conclude that the regulator might be an aliphatic compound. The molecule, however, has been shown to contain two hydroxyl groups, no N-methyl groups, and probably a tertiary amino group. Therefore, all aliphatic structures must be excluded, for it is not possible to devise a stable six carbon atom acyclic molecule which would fulfill all these requirements.

Furthermore, it is not possible to devise an isocyclic structure having fewer than six carbon atoms in the ring which would agree with the requirements of the quantitative analytical data.

Any of the dihydroxy anilines would have agreed with the formula $C_6H_7NO_2$, and would have fulfilled the rest of the requirements set by quantitative analytical data. In the event that the amino group and the hydroxy groups were unfavorably oriented, it is altogether possible that one of these groups would have failed to react with the p-phenylazobenzoyl

chloride. However, all the dihydroxy anilines would be expected to be readily soluble in cold dilute aqueous alkali. Since this condition is contrary to the experimentally observed behavior of the growth regulator, the dihydroxy anilines may be excluded from the list of probable structures.

Several possible structures containing a five membered heterocyclic ring could be drawn. However, if one makes the assumption that the empirical formula $C_6H_7NO_2$ is indeed correct, then he must eliminate the five membered heterocyclic compounds from further consideration, for the empirical formula, combined with the few structural requirements already outlined, does not agree with any possible compound having a five-membered heterocyclic ring.

One remaining possibility is that the growth regulator is made up of a substituted pyridine ring, having the one spare carbon atom attached to another of the carbon atoms in the pyridine nucleus. The structures shown in Figure 2 would have the proper empirical formula.

Since the C-methyl determination showed the absence of a C-methyl group from the molecule of the regulator, structures I through XIV, inclusive, may be regarded as quite unlikely.

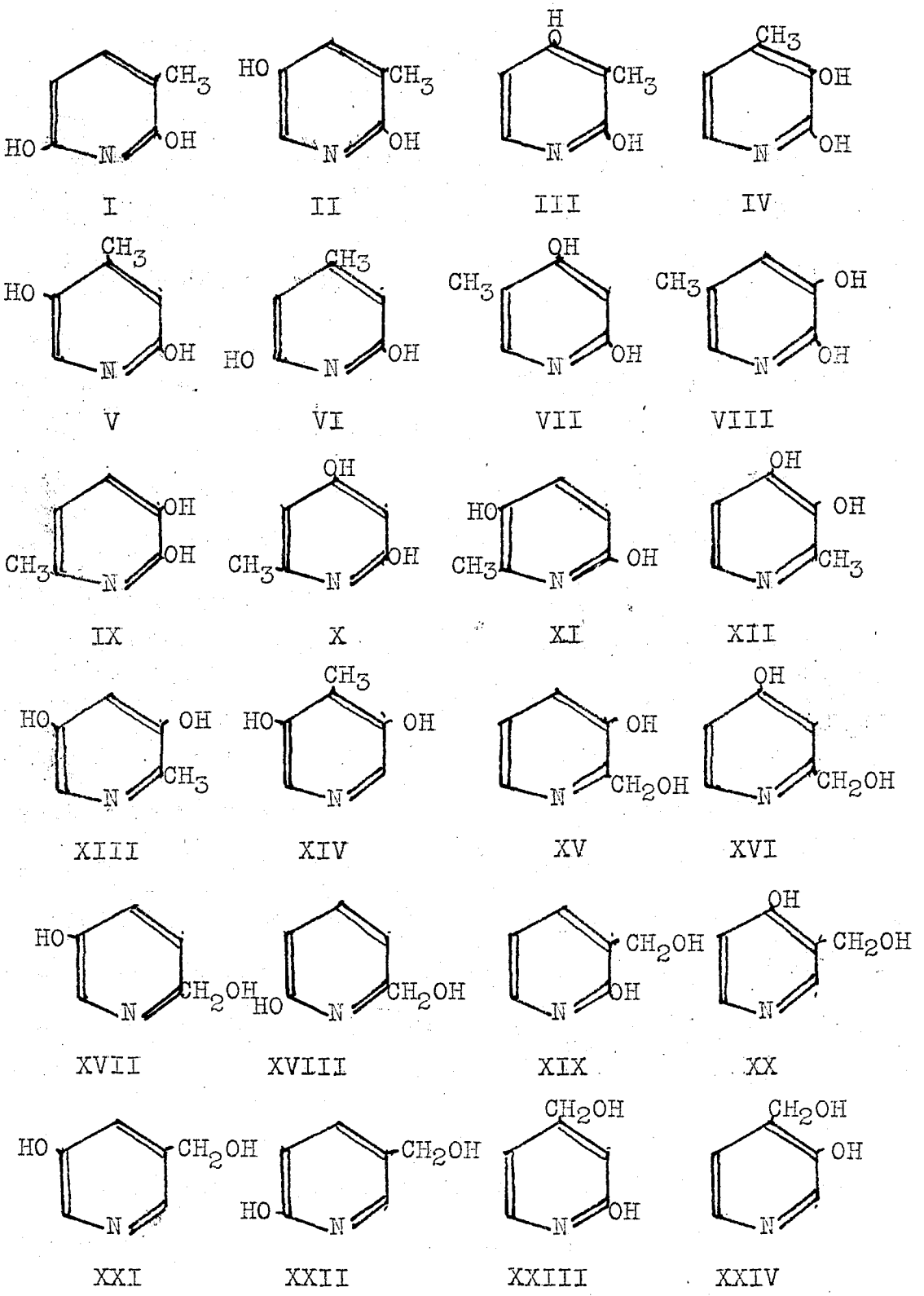

Figure 2. Dihydroxy pyridines having the formula $C_6H_7NO_2$.

It will be recalled that the growth regulators in corn pollen ether extractive reacted with phthalic anhydride under conditions sufficiently mild to cause no appreciable reaction between phthalic anhydride and a typical secondary or tertiary alcohol. It is improbable that compounds having the structures I through XIV would react under these conditions, while compounds of structures XV through XXIV would quite probably react. Thus there are not just one, but two reasons for eliminating structures I through XIV from further consideration.

It is a commonly observed fact that alpha- and gamma- pyridones either do not form colored substances with aqueous ferric chloride solution or else form yellow to orange colored products, whereas beta-pyridones produce blue to green colors when treated with ferric chloride (27). A 5 mg. sample of the p-phenylazobenzoate was boiled with 2 ml. of a 50% solution of ethanol 1 N in potassium hydroxide for five minutes, and the resulting solution was cooled, made acid to litmus with 6 N hydrochloric acid, and shaken out with three 3 ml. portions of ethyl ether. Evaporation of the ether extracts left behind an orange colored residue which produced only an orange

color when treated with either aqueous or alcoholic ferric chloride. Thus, the ferric chloride test suggests that the growth regulator might be either an alpha- or a gamma-pyridone.

A further observation adds support to this supposition. It has been observed (27) that most beta-pyridones are acidic substances, forming water soluble sodium salts, whereas the alpha- and gamma-pyridones are not. Thus, the lack of any tendency of the growth regulator to be extracted from a solution in ethyl ether by aqueous alkali would favor the possibility of the regulator's being an alpha- or a gamma- pyridone.

It is true that these tests are not very rigorous, but they do make structures XV, XVII, XXI, and XXIV seem more improbable than structures XVI, XVII, XIX, XX, XXII, and XXIII.

From this latter group of possibilities, structures XVIII, XIX, and XXIII may be eliminated by the following considerations. According to Gibbs (7), mildly alkaline aqueous solutions of the great majority of phenols having unsubstituted hydrogen atoms in the position para- with respect to the phenolic hydroxyl will react with 2,6-dichloroquinonechloroimide to form brightly colored compounds. When 5 mg. of the

p-phenylazobenzoate were saponified as described on page 79, and the residue was suspended in 3 ml. of sodium tetraborate buffer (pH 9.2), treatment with 5 drops of a saturated aqueous solution of 2,6-dichloroquinonechloroimide resulted in no color production. Apparently, then, the one phenolic hydroxyl group is in a position para- to some substituent or to the ring nitrogen atom. This would be the case with a compound having the molecular structure indicated by either formula XVI, XX, or XXII.

Skraup found (30), in studying a large number of pyridones, hydroxy-quinolines, and pyridine and quinoline carboxylic acids, that only those compounds having a hydroxyl or a carboxyl group in the alpha-position with respect to the ring nitrogen would produce a red to orange color on treatment with aqueous ferrous sulphate. When a 5 mg. portion of the p-phenylazobenzoate was saponified as described on page 79, the ether soluble saponification products produced a distinct orange coloration after treatment with a drop of 5% aqueous ferrous sulphate. As a check of this observation, a solution of the orange colored sodium salt of p-phenylazobenzoic acid, as well as a drop of an alcoholic solution of p-phenylazobenzoic

acid were treated similarly. Neither of these substances produced an orange colored product in any way resembling that formed from the saponification products and ferrous sulphate. The outcome of this test would suggest that the most probable structure of the growth regulator encountered was that shown in formula XXII. However, it is altogether possible that a gamma-pyridone having a hydroxymethyl group in the alpha position might also produce an orange color similar to that observed for alpha-hydroxy or alpha-carboxy pyridines. Until information concerning the behavior of alpha-hydroxymentyl pyridines toward ferrous sulphate is obtained,, formula XVI must also be considered as a possibility.

VIII. CONCLUSIONS

Admittedly, none of the experimental work described leads to a positive conclusion regarding the structure of the growth regulatory substances present in the crude ether extract of corn pollen. However, it does permit the following statements to be made:

1. There exist, in all probability, more than one ether soluble plant growth regulator in corn pollen.

2. Most of the ether soluble growth regulators in corn pollen are neutral substances. In this respect they differ from auxin a, auxin b, and heteroauxin, the three plant growth stimulating acids which have been isolated from plants or plant products.

3. One of the growth stimulating substances in corn pollen is a nitrogenous alcohol, quite possibly 2-hydroxy-5-hydroxymethyl pyridine, a compound which has not yet been synthesized in the laboratory.

A conclusive solution of the problem of the nature of all the plant growth regulators in corn pollen must necessarily await much further study.

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CHAPTER II

ISOLATION OF QUERCETIN FROM THE POLLEN OF ZEA MAYS

It is a commonplace observation that the pollen of most flowering plants is colored yellow. Yet the identity of the compounds responsible for this color has been determined for less than a half dozen species of plants.

I. NATURE OF THE PROBLEM

Although the pollen from corn, Zea Mays, is readily obtainable (6), the exact nature of its pigment, or pigments, has remained unknown. Vinson (8) obtained a yellow crystalline substance, which he did not identify, from corn pollen. Uber (7) studied the absorption spectrum of corn pollen, but he concluded that he was unable to correlate his observations with the chemical composition of the pollen. Because of this lack of knowledge concerning the pigments of corn pollen, the yellow solid which separated on evaporation of the ether extracts of the pollen from Zea Mays, varieties Golden Cross Bantam and Ohio M-15, was studied in considerable detail.

II. CHEMICAL STUDY OF THE PIGMENT

Purification of the pigment.

Fifty gm. of the crude ether extractive (see page 12) were stirred into 250 ml. of petroleum ether (b.p. 35-70°) and the flask containing the suspension of yellow pigment was then permitted to stand at 5° for 24 hours. At the end of this time, the pigment was collected on a Büchner funnel and washed with 50 ml. of petroleum ether. The filter cake weighed 2.25 gm. The entire quantity of crude pigment thus obtained was dissolved in 100 ml. of boiling 95% ethanol, and filtered to remove insoluble impurities. To the filtrate was added dropwise a hot solution of 10 gm. of neutral lead acetate in 25 ml. of 95% ethanol. Enough 12 N sodium hydroxide was added to adjust the pH of the solution to 8 or 9.

After the alcoholic suspension of the lead salt had cooled to room temperature, the orange solid was collected on a Büchner funnel and washed with 25 ml. of 95% ethanol. When the filter cake was sufficiently dry to be handled with a spatula, it was dissolved in 25 ml. of boiling glacial acetic acid, and the solutin was diluted with 75 ml. of water. Upon cooling, lemon yellow crystals formed. These were filtered off, recrystallized three times from 50% aqueous acetone and

dried over phosphorus pentoxide in a vacuum at 90° for 72 hours. M.P. 312-315° (dec.). The reported m.p. of quercetin is 313-314° (dec.) (3).

Anal. Calculated for $C_{15}H_{10}O_7$: C, 59.6%; H, 3.3%; Mol Wt., 302.

Found: C, 59.7%; H, 3.6%; Mol. Wt. (Rast, in camphor), 288.

Qualitative tests.

The purified yellow pigment reduced Fehling's solution. The substance formed a green iron salt when treated with aqueous ferric chloride. Reduction of the pigment with magnesium amalgam in ethanolic hydrochloric acid led to the production of a bluish-red pigment extractible in amyl alcohol. All these properties suggest that the compound is a flavonol. They are all in agreement with the published characteristics of quercetin (4).

Acetylation of pigment.

Five hundred and ten mg. of the purified pigment were boiled under a reflux condenser with 10 ml. of acetic anhydride and 5 mg. of concentrated sulphuric acid for 1½ hours. The reaction mixture was then

poured into 20 ml. of methanol, and the volatile components of the reaction mixture were evaporated *in vacuo*. The residue was recrystallized three times from hot 95% ethanol. Decolorizing carbon was added during the first recrystallization.

After drying over phosphorus pentoxide at 90° in a vacuum, the 150 mg. of colorless silky needles obtained melted at 199-199.5°. The reported melting point of quercetin pentacetate is 200°(2). The acetylated product showed no melting point depression when mixed with an authentic sample of quercetin pentacetate.

Anal. Calculated for quercetin pentacetate, $C_{25}H_{20}O_{12}$: C, 58.6%; H, 3.9%; Acetyl, 42.0%; Mol. Wt., 512.

Found: C, 58.4%; H, 4.1%; Acetyl, (From C-methyl determination), 41.9%; Mol. Wt. (Rast, in camphor), 530.

Methylation of pigment.

Five hundred mg. of purified pigment were dissolved in 60 ml. of acetone. To this solution were added 6 ml. of water, 5 ml. of dimethyl sulphate, and 6 gm. of powdered sodium carbonate monohydrate. The

mixture was boiled under a reflux condenser for 1½ hours, cooled, and poured into 100 ml. of water. After standing at 4° for one hour, the slurry was filtered through a suction filter. The filter cake (350 mg.) was recrystallized three times from hot 75% acetone. A small quantity of decolorizing carbon was added during the first recrystallization.

The product melted at 161-161.5° after drying over phosphorus pentoxide at 90° in a vacuum. The reported m.p. of quercetin 3,7,3',4'-tetramethyl ether is 159-160° (1).

No depression of melting point was noted when this methylated product was mixed with an authentic sample of quercetin 3,7,3',4'-tetramethyl ether.

Anal. Calculated for quercetin tetramethyl ether, $C_{19}H_{18}O_7$: C, 63.7%; H, 5.1%; Methoxyl, 34.6%; Mol. Wt., 358.
Found: C, 63.5%; H, 5.3%; Methoxyl, 34.5%; (Rast, in camphor), 349.

III. CONCLUSION

It is evident that the yellow ether soluble pigment in corn pollen is indeed quercetin. It was isolated in yields of about 0.2%, both from the pollen

of sweet corn, variety Golden Cross Bantam, and from the pollen of Ohio M-15, a hybrid field corn. It was identified not only by qualitative tests, but also by conversion to the 3,7,3',4'-tetramethyl ether and to the pentacetate. Inasmuch as quercetin has been isolated from the husks of Emerson's brown husked type of maize (5), it is not surprising that it should also be present in maize pollen.

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