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Growth hormone in embryo dormant seeds

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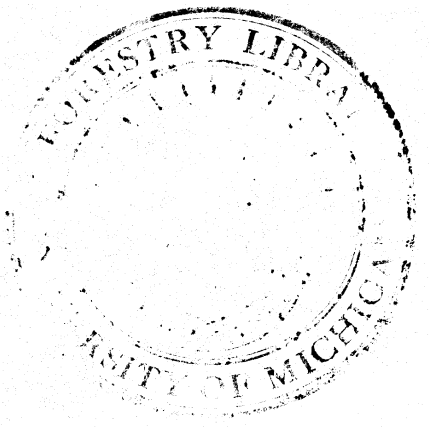
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A STUDY OF GROWTH HORMONE
IN
EMBRYO DORMANT SEEDS

Submitted in Partial Fulfillment of

the Degree of Master of Forestry

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I. INTRODUCTION

In many plant species, delayed germination of the seed is caused by a dormant state of the embryo. Before growth can occur, the seed must undergo a period wherein this dormancy is broken. More information is undoubtedly needed concerning this pre-germination period, about which there exists only hypotheses integrating the known physical and chemical changes. In the past few years, the role of plant hormones in controlling and instigating growth has received marked attention, and increased significance has been attached to the function of the hormone in the growth process. It is the purpose of this investigation to study the growth-hormone production and relations of some embryo-dormant seeds during the period in which the dormancy is lost.*

1. Seed Dormancy

There occur, especially in the nursery, problems which evolve from the delayed germination of seeds from trees and shrubs. Since the turn of the century, many workers have fixed their attention on the study of seed dormancy and methods of breaking it. These investigations have covered an extensive range of species found under varied climatic and physiographic conditions, and the literature upon the subject is voluminous.

In general, the seeds of cultivated plants have been found to be less dormant than those of the wild species (19).

* I wish to thank Professor F. G. Gustafson for his suggestions and criticism during the course of this investigation.

According to Spaeth (29), the causes of dormancy are hereditary characters, but the intensity with which some manifest themselves, particularly such a character as impermeability of the seed coat, may be modified by the climate in which the seed crop is produced. For instance, cool temperatures, and the alternate freezing and thawing that is found in some regions play an important part in the overcoming of persistent embryo dormancy through their effect on seed-coat permeability (ibid.).

Crocker (11) has classified the types of seed dormancy, and his grouping has been followed by most recent workers. The main causes of delayed germination are:

1. Rudimentary embryos that must mature before germination can begin
2. Inhibition of water absorbtion by an impermeable seed coat
3. Mechanical resistance to the expansion of the embryo and seed contents by enclosing structures
4. Incasing tissues interfering with oxygen absorbtion by the embryo, and possibly its carbon-dioxide elimination, resulting in the limitation of dependent processes
5. A state of dormancy in the embryo itself, or some organ of it, in consequence of which it is unable to grow when naked and supplied with all germinative conditions
6. Combinations of two or more of the above causes
7. The assumption of secondary dormancy

It is with members of the fifth class, seeds inherently embryo-dormant, that this investigation is primarily concerned. Into this category fall members of certain genera of particular interest to forestry, notably Juniperus, Tsuga, Pinus, Taxus, Hamamelis, Cornus, Liriodendron, Acer, Crataegus and other Rosaceae.

The dormant embryo type of seed must undergo a period of what is known as "afterripening" to terminate the dormancy and instigate growth. Afterripening may be defined as the series of chemical or physical changes, occurring within the plant or plant part, which brings to a close the dormant period and makes growth again possible (14). More specifically, afterripening may be thought of as a series of physiological changes within the kernel which are preliminary to germination. A broader use of the term applies it to the causes of delayed germination other than embryo-dormancy. Thus Crocker(10), Shull (26), Atwood (1), Sifton (27) and others have discussed the afterripening of seeds whose dormancy is due to gas exchange limitation, or in which secondary dormancy has been induced. Crocker (10), considers lowering of the breaking strength of the seed-coat colloids (allowing free swelling of the kernel) to be afterripening.

Many treatments, of which chemical stimulation and cold storage are the most effective, have been used in efforts to shorten the time required for afterripening. Extensive work at the Hull Botanical Laboratories, the National Seed Laboratories and the Boyce Thompson Institute has established the fact that moist storage at temperatures slightly above freezing, under conditions of good aeration, is the most effective means known for bringing about the afterripening of most species, particularly those where dormancy is inherent in the embryo; the fact that some seeds afterripen best at alternating temperatures whereas low even temperatures are best for others also is established but not explained (9). An interesting characteristic of the process is that it does not obey the Vant Hoff temperature law

for chemical reactions, but procedes faster at the lower temperatures.

Accompanying physical and chemical changes have been extensively studied in seeds, (a comprehensive bibliography may be found in Spaeth (29) and Miller (19)) but the mechanism whereby the dormancy is broken has not been satisfactorily formulated. Coville (9) and others have advanced the theory that afterripening serves to decrease the vital activity of the living cell membranes. This would make them more permeable to diastatic enzymes whose activity in increasing sugar content would raise the osmotic pressure and initiate growth. Some aspects of this theory will be subsequently discussed.

2. Growth Hormones

A hormone may be defined as a substance which, being produced in any one part of an organism, is transferred to another part and there influences a specific physiological process (38). Since the substances causing cell enlargement have been found to comprise a separate heterogeneous group, the term "auxins", suggested by Kögl and Haagensmit in 1931 (16), will be used to indicate those compounds which bring about the specific growth reaction which is conveniently measured by bioassay, viz., Avena coleoptile or Pisum stem curvatures.

There are three known chemically different auxins occurring in plants whose physiological action is, except for quantitative differences, the same. These are auxin a, auxin b, and

heteroauxin. The first is a monocyclic, trihydroxy-carboxylic acid with one double bond, with the empirical formula $C_{18}H_{32}O_5$. It is stable in acid, but not in alkali, has a molecular weight of 328 and is heat and light stable. Auxin b, $C_{18}H_{30}O_4$, is a monocyclic hydroxy-keto-carboxylic acid with one double bond. It is also heat and light stable, has a molecular weight of 310, and is destroyed by both acids and alkalis. Heteroauxin, $C_{10}H_9O_2N$, which has been found to be identical with 3-indole acetic acid, is destroyed by acids but is stable in alkali and has a molecular weight of 175. Though many workers consider all plant auxins to be one of the above three, there is evidence that other substances found in the plant function as growth hormones (3). Until more qualitative information is obtained, I believe one is not justified in restricting the identity of all plant growth hormones to the three hitherto isolated.

Growth substances may be obtained from plants by diffusion and by extraction. In the diffusion method, which is based on the presence of a free moving form of the auxin, the tissue containing the growth substance is smoothly cut, and the cut end is placed on an aqueous gel into which the auxins diffuse; generally agar is used. After the diffusion has progressed a sufficient time, the agar is cut into cubical blocks of a standard size and then assayed.

In the extraction method, not only the free moving auxin is obtained, but also a form of active auxin bound in some manner in the cell (15)(28). For this purpose, water, alcohol,

ether, and chloroform have been used. Water as the solvent is open to the objection that its use is accompanied by enzymic oxidative inactivation (30); the organic solvents, when used, must be freed of inactivating peroxides and chlorine (6). Following the extraction, growth substance is then taken up in agar to be assayed.

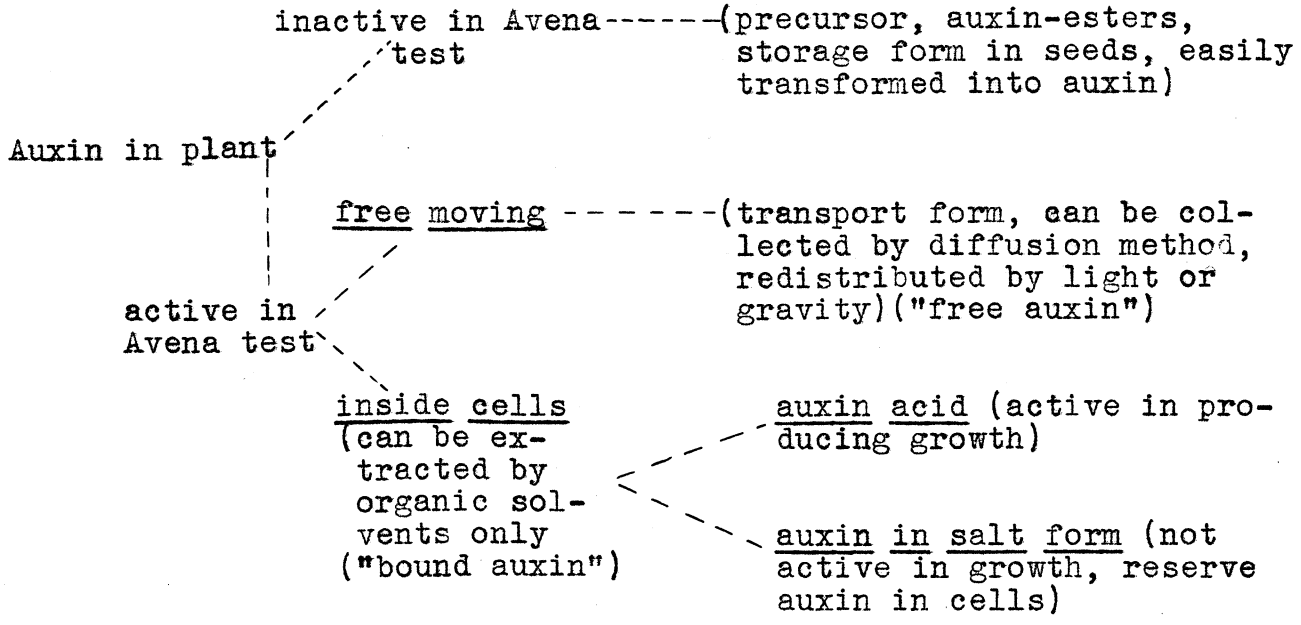
There is strong evidence that the active auxin of a plant is produced from a storage or precursor form, which serves to supply auxin to the various organs or tissues undergoing physiological activity (4)(15)(28). It has been suggested that the precursor could be in the form of an ester; this is supported by the work of Kögl, Erxleben, and Haagensmit (15) who were able to obtain considerable activity from Arachis oil and some other vegtable oils by hydrolyzing them with a lipase preparation or with sodium ethylate.

Where the auxin is heteroauxin, the precursor may well be in the form of an amine or amino acid which is either in the free state or as part of a protein complex. Thus Skoog (28), applying tryptamine to the Avena coleoptile, could obtain no curvature at first. Six hours later, however, a strong response set in; the transformation of the amine to indole acetic acid was postulated. Went and Thimann (38) found tryptophane to behave the same way. It was discovered by Avery and Sargent (3) that compounds like tryptophane and tryptamine, when placed in agar some time before applying the block, would produce an active compound or compounds. The original substances were inactive and the possibility of enzyme action in the agar was precluded.

Bonner (4) and Thimann and Dolk (35) found that growth substance production by Rhizopus growing on a peptone-dextros culture medium is greatly increased by aeration. They, and Thimann (31), explained this as an oxidation of tryptophane to heteroauxin. Thimann also showed that culture media containing tryptophane is superior to those that do not, since auxin is more readily produced. The auxin was identified as indole acetic acid. Aspergillus was found by Boysen-Jensen (6) to be able to convert tryptophane and the 6-carbon-containing amino acids: lysine, leucine, tyrosine and phenylalinine to growth hormone.

Went and Thimann (38) have given a partially hypothetical table of the various possible states of auxin in the plant. I believe its presentation will facilitate the understanding of some aspects of the subsequent work.

Table I. States of Auxin in the Plant



The small quantities of growth substances in plants make difficult the direct proof of their presence by chemical means. It is generally satisfactory to obtain indirect evidence of their existence by their activity in certain measureable growth reactions. As put by Boysen-Jensen (6), "growth substances, in common with other hormones and activators, produce in the living organism responses out of all proportion to the size of the stimulus". Although auxins have been extracted and purified from many plant sources, the usual method of detecting them is by means of biological indicators.

There are a number of techniques used in the quantitative bioassay: straight growth and curvatur response of the Avena coleoptile, the "pea test", epinastic responses, callous formation, and several others. However, the Avena coleoptile is more extensively used than any other organism. For a full discussion of these methods, the reader is referred to Boysen-Jensen (6) and Went and Thimann (38).

The bioassay to be here used is known as the "Avena coleoptile method", first reported by Went (38) and since then technically modified; it is based on a determination of the degree of curvature of a decapitated oat coleoptile on which has been placed the substance being tested. Blocks of agar containing the growth substance are applied unilaterally on coleoptiles and the resulting curvatures photographed after a constant time interval. (For specific details of the modified Avena bioassay used, cf. Appendix A). It was established by Thimann and Bonner

(34), that the rate of curvature is proportional (within certain limits) to the concentration of growth substance applied.

Various concentration units have been proposed for expressing the degree of curvature caused by the auxin, viz., Avena Einheit, Plant-Unit, Wuchsstoff-Einheit. However, since it has been shown that one of the auxins is 3-indole acetic acid, which is readily available in pure form, the activity may be expressed in comparison with the effect of an indole acetic acid control run at the same time as the unknowns tested. The advantages of this are multifold: diurnal and seasonal variations in Avena coleoptile sensitivity and constant variations in technique are automatically compensated for, and the indole acetic acid equivalents are independent of the test method and the test plant species. Van Overbeek (20) has expressed the indole acetic acid equivalents of a curvature in a convenient mathematical form:

$$C = \frac{C_a \cdot I_1^o \cdot V_{agar}}{W}$$

Where

C = auxin concentration in gamma indole acetic acid equivalents per kilogram material under investigation

W = weight in grams of material

C_a = average curvature obtained from extracted material in the Avena test

I_1^o = concentration of indole acetic acid in gamma per liter required to give a curvature of 1 degree upon the control coleoptiles

V_{agar} = volume of agar in cc. in which the residue is taken up

3. Auxins and Seed Dormancy

The presence of auxins in seeds has been noted by a number of investigators. Kögl, Erxleben, and Haagensmit, (15) found small amounts of auxin in ungerminated barley seeds, the quantity increasing as germination progressed. Samples of wheat germ and its oil, corn meal and rice polishings were found by Thimann (30) to contain considerable amounts of auxin, some up to four milligrams per kilogram. The auxin content of corn and Helianthus plants during their whole life cycle was studied by Laibach and Meyer (17). In both species they found that acidified alcohol extracts of the seeds contained a substance that stimulated the growth of the Avena coleoptile; it was present in greatest concentration during the early stages of germination. Pohl (23) showed a decrease in the growth of germinating Avena when the seed coat and aleurone layer was removed or punctured so as to allow the growth auxins to diffuse outward from the endosperm into the water.

Cholodny (7) could find no auxin in the seeds of Avena until water was absorbed. An interesting observation was that the auxin production was in the endosperm and not in the embryo which, in fact, appeared to absorb the growth substance produced. He states that the hormone does not exist in the embryo, and the germination of seeds is only possible in the case where the hormone, called "Blastinin" by him, is transfered from the endosperm to the embryo. The work of Shibuya (25) tends to corroborate

Cholodny's observations; the former succeeded in forcing the germination of dormant strain of peanut by applying heteroauxin to the radicle of the embryo, which was exposed by partially chipping the seed coat.

On the other hand, Thimann (32) found that the application of indole acetic acid to isolated oat embryos did not promote the out-growth of young roots, but only inhibits them. Apple embryos, which, like the endosperm, contain much auxin, will not germinate unless freed from the endosperm. Though Thimann (30) found the embryo of Triticum to contain abundant growth substances, the hypothesis of Cholodny is not necessarily negated: that is, that the auxin is produced in the endosperm and transferred to the embryo.

In the embryo dormant seed we have a definite organization and differentiation of the embryo, yet there is a reluctance to undergo the initial cell elongation which characterizes the awakening of the primordial meristems. This enlargement takes place largely through distension by water uptake, the amount (dry weight) of protoplasm increasing little, if at all. In turn, the absorbing power of the cell <u>per se</u> is dependent upon its turgor and wall pressures; the increase of absorption necessitated by growth can be attained by either a rise in osmotic tension or a lowering of the wall pressure. There is doubt concerning the <u>direct</u> relationship of osmotic pressure and growth (cf. Hrsprung and Blum (39); however, it can be seen that even with an increase in osmotic force concurrent with initial growth, cell extension can be limited, within the bounds of cell bursting (plasmoptysis), by the inflexibility of the cell wall.

That auxin is utilized in growth has been shown by Bonner and Thimann (5); that cell elongation is dependent upon and controlled by the presence of auxin, and that without auxin there is no growth has been shown (30)(38). However, the mechanics of the process whereby auxins function through increasing the plasticity, intussusception and apposition, and perhaps permeability of the cell wall is not definitley known. Therefore, one can logically query as to the precise role auxins play in initiating and maintaining growth in the afterripening of dormant seeds, wherein the growth processes begin. Are auxins utilized in this period? Is there a movement of auxin to or from the embryo? The presence of large amounts of auxin has been shown (33) to exert an inhibiting action on shoots and buds. Might this be true also of seeds?

A knowledge of the auxin content of the endosperm and embryo at various stages of the period wherein the dormancy is broken would answer the above questions. From the literature on seed dormancy, moist cold storage stratification is the most facile means of afterripening inherently embryo-dormant seeds. The basic experimental plan is therefore: To determine the active and precursor auxin content of embryo-dormant seed endosperms and embryos before, during, and after a period of afterripening by stratification.

EXPERIMENTAL

As can be seen from the preceding plan, the problem entails a quantitative differentiation between the free auxins and the inactive precursor. Since in the standard methods of auxin extraction from plant materials there is no way of quantitatively demarcating the source or amount of the Avena active hormone, and no way whereby the amount of precursor can be conveniently estimated, the preliminary work has revolved about the determination of a suitable technique.

The experiments performed can be broadly classified into three groups:

A. Comparison of a number of extraction methods
B. The extraction of active auxin
C. The auxin precursor

A. Extraction Methods

Seeds of Cornus paniculata were used in the experiments to be detailed, since the fruits of the 1939 crop were readily obtained in quantity. The fleshy pericarp was removed by vigorous rubbing against a water immersed screen, and adhering bits of the pulp were eliminated by a number of subsequent washings. After they had dried, the endocarps were stored at room temperature.

In a recent paper, Avery, Creighton and Shalucha (2) found that with maize endosperms, water extraction yielded the most auxin while alcohol extractions gave the most consistent

results. At the beginning of this investigation, extractions were tried with water, alcohol, petroleum ether, and the lower boiling-point fraction of redistilled petroleum ether. All of these solvents gave unsatisfactory results.

1. Extraction with chloroform

Method a. Approximately one-half of a gram of the fruit, without the pericarp, was thoroughly ground in a mortar. The crushed tissue was weighed, placed in the cellulose thimble of a Soxhlet condenser and extracted with chloride and peroxide free chloroform as the solvent. (Chloroform of reagent grade was distilled, several cc. of absolute ethanol added, and the liquid stored in the dark at 0-5°C. (6)). At intervals, the chloroform was removed for assaying, and replaced by fresh solvent. When the extraction period was terminated, the thimble was immersed in fresh chloroform and stored in the dark at 15°C. for about five days, when Soxhlet extraction was repeated. The samples taken for assay were reduced in volume and drop-pipetted to the bottom of vials partially immersed in boiling water; thus the extract containing the growth substance was left on the bottom of the vial. Where the bioassay could not be run on the same day as the extraction was made, the vials were stored overnight at 0-5°C. Agar of 1.5% concentration was added and stirred with the extract by a glass rod. The growth substance was allowed to diffuse into the agar for at least two to three hours before being assayed.[1]

1. Unless this is done, the extract will not be uniformly mixed with the agar. This was shown by an experiment in which the agar was poured into the mold immediately after stirring in the vial. The oils and extraneous materials which floated to

The standard Avena assay then followed. Controls of 21.5 gamma indole acetic acid were run, and the concentration of auxin calculated in terms of indole acetic acid activity by the formula of van Overbeek on a basis of dry weight of seed. The values obtained are listed in Table II. After 198 hours of Soxhlet extraction, the chloroform was evaporated at room temperature from the thimble containing the seed. When no odor of the solvent could be detected, 0.5cc. of redistilled water was added to the seed. Upon the elapse of three hours, the thimble was placed in fresh chloroform and extraction continued.

Method b. Before extraction, the seeds were pulverized with quartz sand which was freed of impurities by acid-alkali washings (2). Procedures were the same as in Method a, except that, in the reduction of solvent volume, the chloroform was reduced to dryness on a bed of glass beads. The auxin was then taken up in 4 cc. water that had been freshly redistilled over $KMnO_4$ (37). An aliquot was mixed directly with an equal amount of three percent agar, allowed to diffuse, and then assayed. The dilution factor was incorporated into the formula of van Overbeek's. Table III. shows the heteroauxin equivalents obtained from each extraction. At 95 hours, 0.5 cc. water was added as in chloroform method a.

Method c. Since 4 cc. of water soon brought the concentration of auxin in the aliquot below the threshold sensitivity of Avena, i.e.,

1. (cont.) the top were cut off and assayed; they yielded about eight percent more auxin than the agar below. Where diffusion for three hours took place before pouring, the floating material gave a curvature of about one and one-half percent over the lower agar.

Tables II to VI Auxin yields of Cornus paniculata seed as obtained by Soxhlet extraction. Concentrations given in terms of gamma indole acetic acid equivalents per kilogram dry weight

Table	Method	Extraction Time in Hours																			Total
II	Chloroform$_a$	5	7	9	4	3	13	4	14½	31	36	7¾	16¾	20½	26¼ #	24	24	40¼			286
		20.0	53.2	46.2	69.4	22.6	25.7	5.8	5.3	0	0	0	0	0	0	35.0	16.0	0			299.2
III	Chloroform$_b$	8	24½	15½	20½	26¼ #	24	24													142¾
		0	143.8	125.4	0	0	114.1	0													383.3
IV	Chloroform$_c$	7¾	17¾	24	24	24	40½														138
		114.0	94.1	120.0	42.2	31.4	0														401.7
V	Ether$_a$	5	7	9	4	3	13	4	14½	31	36	7¾	16¾	15½	20½	26¼	3¼	7¾	17¾	24	40¼	306¼
		30.1	16.9	29.2	52.5	7.5	8.8	15.6	6.6	18.4	18.8	14.2	41.2	33.7	23.3	18.3	8.3	28.0	35.1	43.0	60.2	509.7
VI	Ether$_b$	5	7	9	4	3	13	7¾	16¾	20½	26¼	3¼	7¾	17¾	24	24	24	40¼			253¼
		20.0	14.7	10.2	2.4	0	0 #	18.6	17.2	12.5	12.0	5.4	44.0	20.3	13.1	13.7	11.5	60.0			275.6

(15a)

The heavy vertical bar denotes a storage of ca. five days in solvent at 15°C. The auxin diffusing out in this period has been added to the subsequent tabular value

0.5 cc. water added (see text)

the minimum concentration of growth substance which will cause measureable curvature of the coleoptile, the same procedure was repeated using 1.5 cc. water instead. The auxin values are given in Table IV.

2. Extraction with ether

According to van Overbeek (21), purification of the ether from peroxides is very important. The fact that a sample of ether gives a negative benzidine test, a test which is considered extremely sensitive for detecting peroxides, does not mean that it is pure enough for use in extracting auxins; such a sample may completely destroy indole acetic acid in solution (ibid.). Before being used for extraction, the ether was therefore freed of peroxide by distilling one liter of the commercial solvent over ca. one and one-half grams $FeSO_4$ and one-half gram $Ca(OH)_2$ in about twenty cc. of water (ibid.).

Method a. A repitition of chloroform method a, except that ethyl ether replaced chloroform as the solvent. The results are shown in Table V.

Method b. A sample of ground seed was oven dried to a constant weight (8 hours, 90-100°C.) and immediately placed in a Soxhlet thimble under ethyl-ether. After the forty-first Soxhlet hour, 0.5cc. water was added to the dry seed as in chloroform methods a and b. Table VI lists the auxin values of the repeated extractions.

A more ready picture and comparison of the auxin production by the above methods can be obtained when the data are

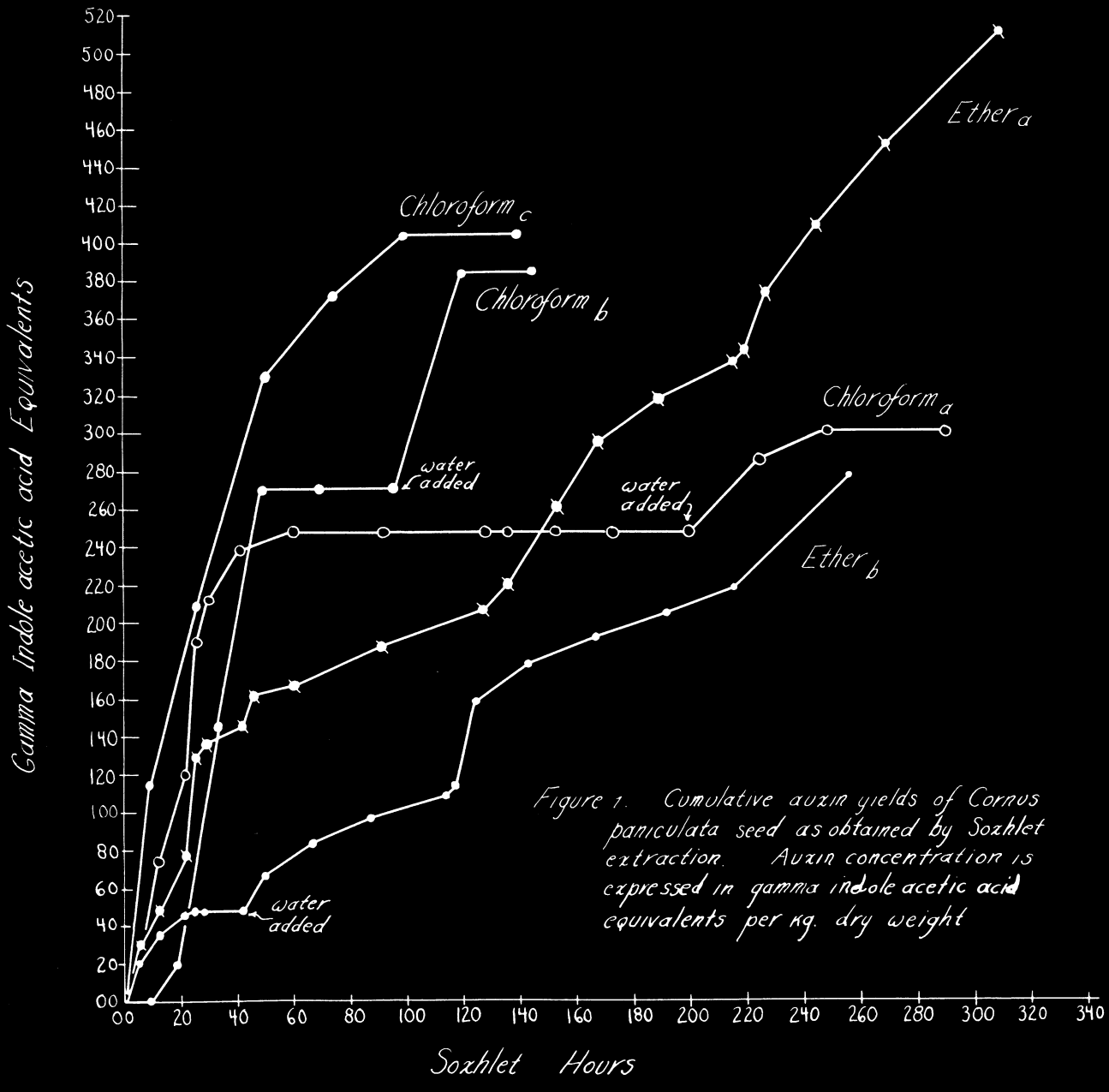


Figure 1. Cumulative auxin yields of Cornus paniculata seed as obtained by Soxhlet extraction. Auxin concentration is expressed in gamma indole acetic acid equivalents per kg. dry weight

plotted in graph form (Figure 1). Chloroform extracts the auxin more readily, or at first may facilitate precursor breakdown, than does ether. It may perhaps be that a different solubility fraction of growth substance exists between ether and chloroform. However, the extraction rate of the latter levels off, while ether, in the long run, gives a much larger yield, obtained where the extract was drop-pipetted into vials.

A higher amount of auxin was obtained at first where the chloroform extract was taken up in water, as compared to the yield

The dried Cornus seed yielded some auxin at first, but the output of growth substance soon ceased (Ether method b); when water was added to the dry seed, a continuous supply of auxin could again be obtained. The freeing of the auxin from its bound form may thus be a hydrolytic action, since water is necessary for its consumation. When dry seeds were used, no growth substance could be obtained after the available auxin was extracted. Upon the addition of water, the course of active auxin yield went on at a rate of about the same magnitude as the normal sample under ether (cf. ether curves, Fig. 1).

Additional evidence that the reaction is probably hydrolytic in nature can be adduced from Fig.1. Upon examining the slopes of auxin production of the ether curves, it can be seen that the slope trends are approximately linear. Therefore it seems permissible to conclude that, under conditions which will withdraw the auxin as it is liberated and prevent the attainment of an equilibrium (conditions we have in the presence of an organic solvent, or a growing germinant), growth substance will be produced from the storage form in seeds at a rate which can be characterized by an equation of the first degree, $y = mx+b$. In the physical chemistry of reactions, the rate of a reaction such as

$$A+B+C \ldots\ldots\longrightarrow \text{Products}$$

is given in its initial stage by

$$\text{Rate} = k \cdot C_a C_b C_c \ldots\ldots$$

i.e., the rate is proportional to the product of the concentrations of the reacting substances at the time considered (18). Let us suppose that only precurser and water are involved in the reaction under consideration. The rate of the reaction should be given by

$$\text{Rate} = k\,(C \text{ precurser})(C \text{ water})$$

In dilute solution (as we most certainly have here), the percentage change in the concentration of water as the reaction proceeds is very small. In other words, the concentration of water remains practically constant whereas that of the precursor varies, relatively, in the time of reaction. Hence the rate of the reaction is proportional to the concentration of the precursor alone, i.e.,

$$\text{Rate} = k\,(C \text{ precursor})$$

The reaction must then be of the <u>first order</u>, viz.,

$$\text{Precursor} + \text{Water} \longrightarrow \text{Auxin}$$

which can be characterized by an equation of the <u>first degree</u>, $y = mx + b$.

The same phenomenon was noted in the chloroform extractions. Evidently the cessation of auxin yield was due, at least in part, to the unavailability of water, for when water was added, an additional amount of growth substance could be obtained. (Chloroform Method a, chloroform method b.)

These results tend to explain the findings of Cholodny (7), who noted that auxin was absent from Avena seeds, but appeared as soon as the seeds had taken up some water; and those of Pratt and Albaum (24), who observed that the auxin content of Sorghum seeds increased during the pregerminative soaking.

Taking up of the extract by water and testing an aliquot is unsatisfactory when there are small amounts of auxin. An auxin concentration just sufficient to cause an Avena response will, upon dilution, be lowered below the threshold reactivity

of the coleoptile. Thus in all probability a measurable auxin yield was obtained at 142 and 138 hours in the chloroform extraction methods b and c respectively, but the dilution was sufficient to cause zero coleoptile curvatures. Pulverization of the seeds by sand, as compared to the ordinary grinding in a mortar does not seem to appreciably hasten the rate of extraction.

It is evident that the seeds of Cornus paniculata have a large amount of auxin tied in storage form. At cessation of the above experiments there was still a steady output of auxin into the ether. The storage form or precursor may perhaps occur as an ester or as part of a protein complex. This remains to be definitely determined. It is pertinent to note that the mere combining of an auxin with a polyhydroxy compound would keep the hormone from diffusing into a solvent like ether (8).

B. Extraction of Active Auxin

The preceding work shows the necessity of water for the obtaining of maximum amount of auxin. The growth hormone probably obtained from a precursor through hydrolytic action. It thus seems logical to conclude that, if the precursor is rendered immobile by the removal of available water, and this dehydrated state is maintained, extraction by an organic solvent will remove all of the active auxin present at any given time. In this light, a series of experiments were performed with the object of developing a suitable dehydration and extraction technique.

To determine the length of time and temperature that could be used for drying the seed material, tests with Crataegus crus-galli and Pinus monticola were run. About half a gram of the naked seeds were thoroughly ground in a mortar. Approximately equal proportions were placed in three weighing vials for each species and brought to constant weight by the following methods: Vacuum oven, 40°C. 85mm.; vacuum oven, 60°C. 85 mm.; drying oven, 90°C.. The samples were immediately transferred to Ehrlenmeyer flasks containing 50 cc. freshly distilled ether and left for one hour with occasional agitation. From each flask the ether was decanted, reduced in volume, and drop-pipetted into vials, where the extract was taken up in 1.5% agar. Standard Avena assay followed. In Table VII the times required to reach constant weight and the resulting auxin concentration in heteroauxin equivalents are given for each manner of drying.

It may be concluded from the values that, of the three methods tried, dehydrating in vacuo at 60°C. is preferable. As compared to the lower temperature, there seemed to be no auxin destruction, and the least time is required to attain constant weight. The samples dried at 90° showed a decrease in assayable growth substance. This loss was more marked in the C. crus-galli seed, which likewise showed a slight heat discoloration.

Ether was used for the extraction since it is separated from plant material and water with greater ease than is chloroform, and the low boiling point of the former enables a more facile

Table VII. The effect of three dehydrating methods on the auxin content of the seeds of Crataegus crus-galli and Pinus monticola. Auxin concentrations are given in gamma indole acetic acid equivalents per kilogram dry weight of seed.

Method	Hours required to reach constant wt.	dry wt. in gms.	Curv.°	Vagar cc.	gamma equivalents
Crataegus crus-galli					
Vac. 40°C.	14	0.103	5.1	0.35	22.6
Vac. 60°C.	12	0.110	5.8	0.35	24.0
Vac. 90°C.	18	0.115	3.7	0.35	14.5
Pinus monticola					
Vac. 40°C.	18	0.156	4.6	0.40	15.3
Vac. 60°C.	16	0.155	4.4	0.40	14.8
drying oven 90°C.	22	0.162	3.9	0.40	12.7

Sensitivity constant = 1.3

concentration by distillation. However, ether contains free water, ca. 7%. The question arises as to whether this water is sufficiently available to permit precursor hydrolysis. At the same time can be determined the length of time which the seed tissues should be extracted to remove the available auxin. Dry ether was obtained by placing anhydrous $CaCl_2$ in about twice its volume of freshly distilled ether and storing overnight in a cold room. The small amount of water remaining was removed by contact with metallic sodium.

Outer coats of Crataegus crus-galli and Pinus monticola seeds were removed and the kernels ground in a mortar. Two approximately equal portions of each species were brought to constant weight in vacuo at 55°C.,70mm.. They were then transferred to Soxhlet thimbles and immediately subjected to ether extraction, comparing anhydrous ether and freshly distilled ether as the solvents for each species. Calcium chloride tubes were plugged in the outlet vents of the condensers using anhydrous ether, separating the condensing vapor from air moisture. After 2,4,8, and 16 hour intervals, the solvent was removed to be reduced in volume for assay, and the Soxhlets refilled with fresh ether of the same character, i.e., either anhydrous or "wet" ether. In Table VIII is recorded the auxin yields obtained by a standard Avena test of each extraction.

It is evident from Table VIII that dehydrated ether should be used, as the "wet" solvent had sufficient water to enable auxin production, either by allowing water to be carried in the vapor-phase to the material, or by carrying down the precursor to the

Table VIII. The auxin yield, in gamma indole acetic acid equivalents per kilogram dry weight, obtained at various Soxhlet extraction intervals from the seeds of Crataegus crus-galli and Pinus monticola. Anhydrous and undried ether are compared as solvents.

Species	Solvent	Dry weight grams	Extraction period: 2 hours			4 hours			8 hours			16 hours			Totals
			Curv.°	Vagar	gamma equiv.	Curv.°	Vagar	gamma equiv.	Curv.°	Vagar	gamma equiv.	Curv.°	Vagar	gamma equiv.	
Crataegus crus-galli	Anhydrous ether	0.133	15.7	0.40	99.1	1.7	0.35	11.2	–	0.30	–	–	0.30	–	110.3
	Moist ether	0.164	16.7	0.40	85.7	3.7	0.35	16.5	3.9	0.30	15.0	–	0.30	–	117.2
Pinus monticola	Anhydrous ether	0.201	19.1	0.40	80.0	–	0.35	–	–	0.30	–	–	0.30	–	80.0
	Moist ether	0.199	16.8	0.40	71.0	4.6	0.35	17.0	11.1	0.30	35.0	–	0.30	–	123.0

Sensitivity constant = 2.1

Soxhlet flask where it was activated. Thus, with C. crus-galli, the active auxin was removed by anhydrous ether in a total of six hours of extraction; on the other hand, moist ether continued to remove auxin over a much longer period, probably due to conversion of the inactive form. The seeds of P. monticola behaved in virtually the same fashion, except that a Soxhlet extraction of only two hours with anhydrous ether served to remove all of the active auxin. After extraction with anhydrous ether, the samples were placed in flasks containing fresh dry solvent. With their tops sealed by paraffin, these flasks were stored in the dark at 15°C. An assay performed one week later showed no auxin in the ether extract.

Based on the above experiments, the following procedure was finally adopted to determine active auxin in the embryo and endosperm of seeds. The kernels were separated from their integuments after an initial cracking in a hand-vise, and the embryos were then excised from the endosperm under a dissecting microscope. Fifteen seeds of the Pinus and twenty seeds of the Crataegus are sufficient to secure adequate Avena curvature. The tissues were ground, weighed, dried in a vacuum oven at 60°C.-85mm. for 16 hours, and weighed again to give dry weight and percentage of moisture. Extraction with anhydrous ether for 6 hours followed the transference of the samples to Soxhlet thimbles. The ether was then reduced in volume, drop-pipetted into vials, and assayed by the standard Avena technique.

The method adopted for dehydrating ether is as follows:

ether, freshly distilled over $Ca(OH)_2$-$FeSO_4$, was shaken with half its volume of silica gel (dried at 500°C. for four hours) and allowed to remain in the cold room overnight. The ether was decanted and shaken with anhydrous $CaCl_2$ and again left overnight. Removed from the chloride, the solvent was completely dried over metallic sodium which, being a strong reducing agent, would also serve to inhibit the formation of peroxides. Before use, the ether was re-distilled, with the first few cc. of distillate discarded. This re-distillation was found to be necessary, as the suspension of NaOH formed in the ether over sodium made the ether useless as an extraction solvent.

C. Extraction of the Precursor Auxin

When seeds or vegative tissues are placed in an organic solvent, or periodically extracted by Soxhlet tubes, auxin continues to be extracted over a long period of time. This auxin is undoubtedly derived from the activation of a precursor. Any attempt to determine a metnod whereby the amount of precursor could be measured quantitatively is complicated by a lack of knowledge concerning the identity of the precursor. A step may be moved in this direction by ascertaining, for the seeds in question, which of the native plant hormones are present. For instance, if heteroauxin is found to be the growth substance, the precursor may be either tryptophane, tryptamine, or tyrosine.

The first method used was based upon the differential acid-alkali destruction test of Kögl, Haagen Smit and Erxleben (22). One gram of Cornus paniculata seed was ground, placed in purified

ether, and kept at 15°C. for about twenty four hours. The decanted solvent was divided into four equal parts. One part was drop-pipetted into a vial for assay in order to determine the original amount of auxin. The other three portions were placed in Ehrlenmeyer flasks and the ether removed by distillation. To one sample was added 3cc. of 5% HCl, to the second sample, 3 cc. of 0.5 N NaOH, and to the third sample 3 cc. of redistilled water. The liquids were then refluxed for 15 minutes, cooled, neutralized and extracted with ether. These ether samples were transferred to the bottom of vials and assayed by the standard technique. The destruction test was also performed on the seeds of P. monticola, and later on those of C. crus-galli. In these species, the auxin was obtained by Soxhlet extraction with ether for two hours. Otherwise the procedure was the same. The results of the above experiments are summarized in Tables IX, X, and XI.

The differential destruction test is based on the fact that heteroauxin is heat stable in alkali but is destroyed by acid, that auxin a is stable in acid but is destroyed by alkali, and that auxin b is unstable in both acid and alkali. From the data tabled, it can be seen that some of the auxin was stable in alkali, and that some was stable in acid. Assuming that the auxin in the seeds tested was one of the aforementioned three, we may conclude that in all of the species both auxin a and heteroauxin are present. It is not likely that auxin b occurs since the auxin obtained by refluxing with water was less in all cases than the sum of the acid and alkali values. Conclusions as to comparative amounts of the two auxins present cannot be drawn, since O_d analyses show the average curvatures obtained from the acid and alkali refluxes are not significantly different.

Tables IX., X., XI. The differential acid-alkali destruction test on the seeds of Cornus paniculata, Crataegus crus-galli and Pinus monticola. Auxin values are given in gamma indole acetic acid equivalents per kilogram dry weight.

Refluxed with:	weight grams	Curv.°	Sens.	Vagar cc.	gamma equivalents
HCl	0.25	2.8	1.5	0.30	5.1
NaOH	0.25	2.2	1.5	0.30	3.9
H_2O (redistilled)	0.25	3.4	1.5	0.30	6.1
not refluxed	0.25	4.1	1.5	0.40	9.8
HCl	0.25	2.4	1.9	0.60	10.7
NaOH	0.25	2.0	1.9	0.60	8.9
H_2O (redistilled)	0.25	3.5	1.9	0.60	15.8
not refluxed	0.25	4.4	1.9	0.80	26.4
HCl	0.25	1.8	2.0	0.30	4.3
NaOH	0.25	3.5	2.0	0.40	11.2
H_2O (redistilled)	0.25	3.3	2.0	0.40	10.5
not refluxed	0.25	4.2	2.0	0.60	20.0

I believe that the differential acid-alkali destruction test is open to at least one major criticism as an absolute method of differentiating growth hormones. That is, its efficacy is unquestionable if we can conceive that all plant hormones are either auxin a, auxin b, or heteroauxin. But many other compounds occurring in plant tissues, aside from the delayed response of substances like tryptophane, tryptamine and tyrosine, give growth stimulating responses. Avery and Sargent (3), utilizing straight growth of Avena as the test, found stimulatory action with asparigine, glycollic acid, lactic, malic, and mucic acids, hypoxanthine and xanthine, phenylalanine, adenine and others. The identification of the auxin would thus seem to be complicated, and Went and Thimann's generalization that "There is nothing against the view that the same auxin occurs throughout all the higher plants" (38) is open to question.

The remaining precursor experiments had one object in view---that of hastening the breakdown of the precursor to the active auxin form. To this end were tried alkali hydrolysis, enzymic hydrolysis by lipase, and oxidative deamination with permangenate; all were performed on the seeds of Cornus paniculata. In each determination, a one gram sample of the crushed seed was dried and the active auxin extracted using the previously detailed anhydrous ether technique (cf. Pg.22). Thus dried seed tissue was obtained with no active auxin; any auxin which was then evolved can be considered as arising from the storage form. The dry ether which remained in the tissue after the active auxin

extraction was removed over a water bath. The seed material was utilized immediately for the experiment.

Alkali hydrolysis

Four seed samples were taken. To one was added 15cc. of buffer (Sorenson's sodium borate-sodium hydroxide mixture) of pH 11.08; to another was added 15 cc. of redistilled water. The first three were refluxed for two hours, while the fourth was allowed to remain at room temperature for the same length of time, with an occasional agitation. The alkali was neutralized with HCl and all four solutions were thoroughly shaken with purified ether as a partition agent. Centrifuging cleared the ether which was then decanted, and the remaining water washed twice with fresh ether. The ether was reduced in volume, drop-pipetted into vials, and the extract taken up in 0.6 cc. of 1½% agar for Avena assay. Two serial dilutions of the agar were run for each treatment. Table XII gives the results of the assays in indole acetic acid.equivalents.

It can be seen that the alkali refluxes were effective enough to yield some auxin, though relatively very little when it is considered that by proper treatment, at least five hundred gamma equivalents of auxin are obtained from the seeds of C. paniculata (cf. Table V.).

Refluxing with the alkali inhibited the further formation of auxin, perhaps through the destruction of the enzymes. The ground tissue remaining in the flask which was buffered at a pH of 11.08 was washed with distilled water and stored in the cold

Table XII. The result of alkali treatments of the seeds of Cornus paniculata from which the active auxin had been removed. Auxin yields are given in gamma indole acetic acid equivalents per kilogram dry weight.

Refluxed with:	Dry weight in grams	Vagar cc.	Curv.°	Sens.	gamma equivalents
Buffer pH 9.97	1.10	0.60	4.1	2.1	4.7
		1.20	1.9		4.3
		2.40	-		-
Buffer pH 11.08	1.18	0.60	6.7	2.1	7.2
		1.20	3.2		6.8
		2.40	-		-
H_2O	0.96	0.60	-	2.1	-
		1.20	-		-
		2.40	-		-
Not refluxed but H_2O added	1.12	0.60	(0.9)*	2.1	(2.0)*
		1.20	-		-
		2.40	-		-

*values questionable due to low curvature response

room under purified ether. An assay made of this ether at the end of two weeks gave negative results.

Lipase

A lipase preparation was obtained from germinated Ricinus seeds. The seeds were hulled, the kernels frozen by dry ice and then ground in a mortar. This ground material was then extracted with ether in a Soxhlet tube for about 20 hours, dried, reground, and stored in a dessicator. A test of its enzyme activity by the method of Haley and Lyman (13) showed a fat hydrolyzing power of 14%.

About one gram of the previously extracted Cornus seed was mixed with one-tenth of a gram of the lipase powder. Five cc. of 0.023% HCl was gradually added in the mixing. With a few drops of toluol the mixture was placed in a 35°C. incubator for 24 hours. After incubation, the flasks were shaken with several changes of purified ether (each being cleared by centrifuging), the ether reduced in bolume, drop-pipetted into vials and then assayed. Two controls were run: one-tenth of a gram of the lipase powder in five cc. of the HCl, and one gram of the extracted Cornus seed in five cc. of HCl. The results of the assays are as follows: seed alone incubated with the acidified water yielded 2.3 gamma indole acetic acid equivalents, probably through natural hydrolysis. The lipase alone gave 4.9 gamma equivalents, while the lipase-seed mixture resulted in 5.5 gamma equivalents. It can therefore be concluded that the lipase had no effect.

Considering the initial experiments, it is difficult to see how oils or fatty substances can be the chief precursor bases

in the seeds experimented upon. It is true that Kögl, Erxleben, and Haagen Smit (15) obtained considerable auxin by hydrolyzing oils from seeds. Yet in ether Method a, to cite one instance, we have seeds from which presumably all of the fatty materials have been extracted---the seeds having been Soxhleted with ether for over 300 hours, excluding the time spent in ether between Soxhlet extractions (cf. Table V)---but still large amounts of auxin were produced. It seems more likely that the precursor is, or is a portion of, a protein complex. It is probably then produced by enzymic hydrolysis, either into its active state or into an amino acid or amide which is then changed to the active state. This may perhaps be the explanation of yield obtained by alkali hydrolysis, since Gortner (12) states that the strong alkali brings about a rapid and complete hydrolysis of proteins frequently accompanied by the deamination of amino acids.

After the completion of this investigation, a preliminary paper appeared by Thimann and Skoog (36) in which a greatly increased auxin yield was obtained from Lemma by subjecting it to the action of a proteolytic enzyme, chymotrypsin. The authors conclude that the auxin in Lemma is bound to a protein from which it is liberated by hydrolysis. This further substantiates the above view.

As previously mentioned (page 7), the amino acid and amine, tryptophane and tryptamine, have been considered as models of the auxin precursor by Thimann and Dolk (35); they explained their further increased auxin yields in Rhizopus cultures, which was

found to be proportional to the extent of aeration, by the oxidative deamination of tryptophane to heteroauxin by way of the keto-acid. Tryptamine would behave in almost the same fashion, being converted by oxidation to a deaminized alcohol. Further oxidation would yield heteroauxin by way of the acetaldehyde. An experiment was therefore performed endeavoring to oxidize the breakdown products of the protein from seeds by potassium permanganate.

Oxidation by $KMnO_4$

To hydrolyze the proteins of the seed to amino acids, alkali hydrolysis was used, since, according to Gortner (12), the amino acids like tryptophane and tyrosine are destroyed by acid hydrolysis but are relatively stable to the alkali. A hundred mg. of dry Cornus seed, free of active auxin, was placed in a Pyrex test tube and 2 cc. of 20% NaOH added. The tube was then refluxed over a water bath for 15 hours, cooled, brought to about 10 cc. in volume with water, and neutralized with HCl till the pink of phenophthalein disappeared. Approximately one-eighth gram of permanganate was added, the mixture shaken and allowed to stand till the permangenate became slightly brown. The solution was then partitioned with several changes of purified ether, each change being decanted after centrifuging. Following reduction in volume, the ether was drop-pipetted to a vial and then assayed.

No Avena activity could be obtained from the extract. It is possible that potassium permanganate is not a sufficiently strong oxidant to carry the amino acid to the alcohol or alpha-hydroxy acid. This suggests the use of nitrous acid (Van Slyke reaction) as the first step, which could then be followed by

either permanganate or lead tetra-acetate.

D. The Auxin in Seeds During the Period of Afterripening

It can be recalled that this investigation purposed to determine the active and precursor auxin content of embryo-dormant seed endosperms and embryos before, during, and after a period of afterripening by stratification. The preliminary research on methods only succeeded in developing a procedure for the quantitative estimate of active auxin. Since the time alloted for this problem was drawing to a close, a tentative series of experiments were run on the effect of stratification on the active auxin content of Pinus monticola seeds.

Small packets of cheesecloth containing about twenty seeds were immersed for a few minutes in a 10% calcium hypochlorite solution, washed in running water, and then stratified in moist, sterile sand. The container was kept in a dark cold-room at ca. 0-5°C.. At one and three weeks a packet was removed, the integuments cracked from the seeds, and the embryos excised from the endosperms under a dissecting microscope. The determination of the active auxin followed the technique detailed on page 22. Table XIII gives the active auxin content in gamma indole acetic acid equivalents obtained from the embryos and endosperms. It can be seen that the embryo content stayed about the same, while the endosperm tissues showed a rise in the third week. However, no conclusions can be drawn from these limited data on the effects of cold storage upon the auxin content.

Table XIII. The active auxin content of Pinus monticola seed embryos and endosperms at 0,1, and 3 weeks of stratification. The auxin content is expressed in gamma indole acetic acid equivalents per kilogram dry weight.

Weeks of Stratification	Embryo					Endosperm				
	Dry weight grams	Curv.°	Sensitivity	Vagar cc.	Gamma equiv.	Dry weight grams	Curv.°	Sensitivity	Vagar cc.	Gamma equiv.
0	0.0171	8.1	1.5	0.30	208	0.149	14.9	1.5	0.40	59.1
1	0.0168	5.9	2.0	0.30	212	0.150	10.3	2.0	0.45	61.7
3	0.0180	6.8	1.8	0.30	204	0.149	13.1	1.8	0.50	78.6

SUMMARY

1. Seed dormancy, auxins, and a possible corretation between auxins and dormancy have been discussed.

2. Utilizing Soxhlet extractors over extended periods of time, a number of extraction methods were compared using the seeds of Cornus paniculata. In the main, various modifications in the use of chloroform and ether as solvents were tried. Preliminary experiments showed alcohol, water, and petroleum ether to be not as efficient as the former two solvents, either in ease of handling or in amount of auxin obtained.

3. It was discovered that water is necessary for the production of auxin from the storage form. A dehydrated state of the seed tissue precludes the formation of auxin. As long as water is present, the precursor will provide active auxin over extended periods of time. An enzymic hydrolysis is postulated as the mechanism of auxin formation.

4. A method was developed whereby the total active auxin in seeds could be quantitatively determined. The seeds are ground and dried in a vacuum oven at 60°C.-85mm. pressure for 16 hours. Thereupon the active auxin is extracted by Soxhletting for 6 hours with anhydrous ether. The method is critical insofar as great care must be taken to insure complete dehydration throughout the extraction.

5. Based on the differential acid-alkali destruction test,

auxin a and heteroauxin were identified in Cornus paniculata, Crataegus crus-galli, and Pinus monticola.

6. In an effort to hasten the precursor breakdown in C. paniculata, experiments were carried out on alkali hydrolysis, enzymatic hydrolysis by lipase, and oxidative deamination by potassium permanganate. The first seemed of some effect, while the latter two yielded negative results.

7. A tentative series of determinations of active auxin content during a three week afterripening period was made of the endosperms and embryos of Pinus monticola. Endosperm tissues showed a rise in active auxin at the third week. However, no conclusions can be drawn from the limited data.

Appendix

The Standard Avena Coleoptile Method

Oats of the pure line "Siegeshafer" are dehusked, soaked in water for two hours, and laid out on wet filter paper in a Petri dish. This is placed in a dark room which is kept at 25°C., 85% humidity, and free from any phototropically active light. The following day the seeds are planted in individual glass holders and placed with the roots in water, twelve plants in a tray. Two days later when the coleoptiles are about three centimeters long, they are ready to be used for tests. The plants are decapitated, about 2 mm of the tips being removed. Approximately 2½ hours later, a second decapitation is made of about 2 mm. The primary leaves are then pulled loose from the base but left within the coleoptile sheath. About 30 minutes are allowed to elapse, so that the upper portions of the coleoptiles become largely free from hormone, and so that any individual plants that might show curvatures due to handling or natural tension can be detected and removed. At this time small agar blocks of standard size containing the hormone solution to be tested are applied to the cut surface on one side of the coleoptiles. The foliage leaf helps to support the agar block and maintain it in a vertical plane. After 90 minutes of application, the plants are photographed as shadow pictures on bromide paper. The curvatures produced by the unilateral application of the hormone can then be measured by a goniometer from the pictures. For a single test the mean value of the curvatures of one row of 12 test plants is used. The standard error of the mean ranges from 0.8 to 1.2 degrees curvature.

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