The Isolation of 3-Indole Acetic Acid from Immature Corn

Thesis

by

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Introduction

Introductory facts concerning auxins

The concept that plants contain special substances which move about within the plant and serve to correlate and integrate the functions of the organism is not new.

The ideas brought forth by Sachs near the end of the last century accounted for the known phenomena occurring in plants and even today are not outdated. His basic postulate was that differences between plant organs must be due to differences in chemical composition, and that these differences must be present even before the earliest visible stages of organ development. He pointed out that these differences might be extremely minute and perhaps not detectable by ordinary micro-chemical methods. He also considered the possible importances of optical isomerism of compounds taking part in reactions *in vivo*. He assumed that there were substances elaborated in the plant which travel in definite directions and which bring about special responses, for example, the formation of flowers or roots.

Darwin, about the same time, showed that the stimuli of gravity and light were perceived by only the tips of seedlings and concluded that some influence was transmitted from the tip to the lower parts

of the plant, which then reacted with a characteristic movement.

The material nature of this influence was demonstrated by early experiments of Boysen Jensen. He found that Avena seedlings deprived of their tips showed no response to light, but that by replacing the cut-off tips on the wounds the sensitivity to light was restored. Paál repeated these experiments and found that the substance responsible for this behavior could pass through a layer of gelatin but not cocoa butter, mica, or platinum.

Later Went succeeded in removing the active substance from plants by allowing it to diffuse out of oat coleoptile tips into agar blocks. This substance, which has been shown to be necessary for the process of cell elongation in Avena, is a member of the class of phytohormones known as auxins.

The actions of auxins in plants explain not only effects due to increase in length, such as photo- and geotropism, but also a variety of other phenomena, such as inhibition of lateral buds by terminal buds, root formation, and cambial growth.

A number of bioassays for auxins have been devised, and it is with these as tools that subsequent isolation and chemical work has been made possible.

Probably the most widely used of these bioassays is the standard Avena test as described by Went (43). Briefly, the procedure involved is to cut off the tip of an oat coleoptile and to place a small agar block containing an unknown amount of auxin on one side of the cut surface of the coleoptile. The auxin in the agar block diffuses out into the oat seedling and stimulates growth on only the side on which the block is placed. This results in a curvature of the plant away from the agar block, which curvature is proportional, within limits, to the auxin concentration.

A modification of the standard Avena test has been made by Skoog (33). The endosperm is removed from the test plant and the plant held in a cotton plug. This test is sensitive to much lower auxin concentrations than the standard Avena test since the seed acts as a source of auxin for the coleoptile and lessens its sensitivity to any added auxin.

Isolation and chemical work on auxins

The Avena test formed the basis for the classical researches of Kögl, Haagen-Smit, and Erxleben in isolating auxins a and b, and 3-indoleacetic acid from natural sources (14,16,18). In their work the Avena Einheit (AE) was taken as a unit of curvature-producing activity and was defined as the amount of auxin necessary to give a ten degree curvature in the Avena test, using an agar block of $2mm^3$ in volume.

After an examination of the various possible sources of substances active in the Avena test, human urine was concluded to be the raw material of choice and was found to contain about 10^8 AE per liter. Other sources investigated included the tips of maize and oat plants, cultures of Rhizopus, various yeasts, and distillery slops.(14)

The acid fraction from 200 l. of pregnancy urine was subjected to a large number of purification steps, the activity of each fraction being followed by the Avena test. The procedures used included fractionation between solvents, extraction of inactive material with various solvents, precipitation with lead ion and calcium ion, lactonization of auxins a and b with acid methanol, and distillation of the active substances in high vacuum. The fraction distilling at 125-130 C. in

high vacuum was found to be the most active and crystallized on standing. Recrystallization gave 6.9 mg. of a product which melted at 173 C. and the activity of which was 30×10^6 AE per mg. This substance proved to be the lactone of an acid (auxin a) which melted at 192 C. The activity of auxin a was found to be 50×10^6 AE per mg.

The isolation of auxin a and its lactone was repeated successfully on mixed urine and various plant materials. The first plant material subjected to the isolation procedures was corn oil, and here a new substance, auxin b, was obtained along with auxin a. Auxin b melts at 183 C. with decomposition and has the same biological activity as auxin a. Malt, like corn oil, yielded both auxin a and b.

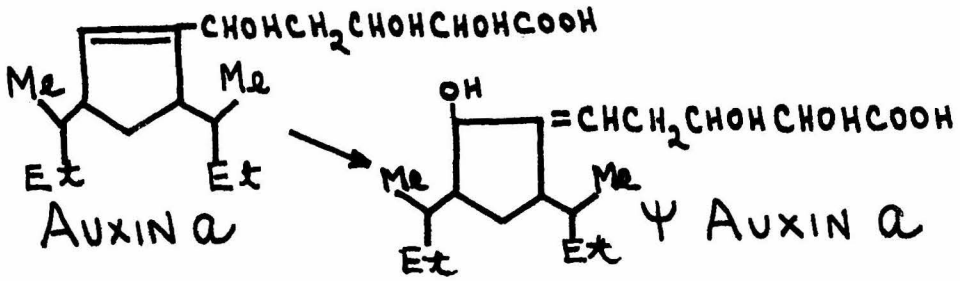
In the course of the search for rich sources of the auxins, 36 kinds of malt were investigated. Most of them contained 0 to 540 gammas of auxin a plus auxin b per kg. One sample was found, however, which contained 10 to 13 mg. of auxin per kg., but in samples subsequently investigated only much lower concentrations have been found.

Two samples of malt examined by Bergren (7) were about 4 and 8% as potent respectively as the very rich source above.

The best sample of corn oil located by Kögl contained 700 gammas of auxin per kg., but this too was an unusually rich sample. The reasons for the extraordinarily high auxin contents of the materials used by Kögl are not known.

During the course of about 3 years a total of about 700 mg. of auxin a and b was obtained from various sources. With only a part of this small amount of material the structure of both compounds was elucidated in a series of brilliant researches (17,21) although neither compound has yet been synthesized.

A remarkable characteristic of both auxin a and b is their spontaneous inactivation by internal molecular rearrangement (15,22). This reaction involves the shift of a double bond and a hydroxyl group to give the biologically inactive pseudo-auxin.



The fact that the above rearrangement is practically complete in two months greatly increases the difficulty of isolating the auxins from even very rich sources.

In working up further large amounts of human urine it was discovered that an auxin was present which had different solubilities and chemical properties than either auxin a or b (18). The isolation procedures were modified somewhat and finally a crystalline substance was obtained which after several recrystallizations melted at 165 C. and had a biological activity of about 20×10^6 AE per mg. What was more surprising still was that the elementary analysis showed the presence of nitrogen. The compound was finally proven to be 3-indoleacetic acid, a substance known perhaps sixty years previously and the presence of which in human urine had already been established.

In 1934 Kögl and Kostermans succeeded in isolating the same substance from yeast (20), and in 1935 Thimann succeeded in obtaining a small amount of 3-indoleacetic acid from cultures of Rhizopus (40).

The discovery that 3-indoleacetic acid occurs naturally and has an activity of the same order of magnitude in the Avena test as that of auxin a or b presented a difficult and critical problem. Is its biological activity a mere coincidence of no importance in nature, or does it perhaps play the role of growth hormone along with auxin a and b? The complete

answer to this question even today is not known with certainty, but an immense amount of work has been done and considerable evidence amassed on this point.

The history of this problem goes back to the experiments of Went who, in 1928, measured the diffusion constant and calculated the molecular weight for the auxin in oat plants and determined it to be 376. Kögl and his co-workers repeated this work for the tips of young corn plants and obtained a value which indicated the presence of auxin a or b (19). It is of interest to note that their value for the molecular weight of crystalline auxin a as determined by the diffusion method was 376, the same value as that obtained by Went approximately six years before. Further characterization of the auxin in young corn plants was obtained by differertial destruction tests in acid and alkali, and these likewise indicated that auxin a was predominant (19).*

The following technique is employed in the determination of the diffusion constant. A small agar block is prepared containing an amount of the auxin the diffusion constant of which is desired. Three other agar

* The differential acid-alkali destruction test relies on the fact that auxin a is stable in hot acid but labile in hot alkali; 3-indoleacetic acid is stable in hot alkali and labile in hot acid; auxin b is destroyed by either medium.

blocks the same size as the first are stacked on top of the first and diffusion allowed to take place for a measured time. Then all four agar blocks are assayed for their auxin contents. It is a matter of greatest importance to avoid air bubbles between the agar blocks and also to maintain a constant temperature and a humidity high enough to prevent the agar blocks from drying.

The auxin contents obtained for the four agar blocks are now multiplied by a factor calculated to make the sum of the relative amounts equal 1000. Knowing these four relative concentrations, the thickness of the agar blocks, and the time for which diffusion took place, the diffusion constant can be determined from the following diffusion tables, Stähler, (35), Arndt (1).

$X=\frac{h^2}{4Dt}$	Number of Layer				$X=\frac{h^2}{4Dt}$	Number of Layer			
	1	2	3	4		1	2	3	4
0.040	241	247	254	259	0.360	21	106	320	552
0.048	233	243	257	267	0.410	16	94	317	575
0.058	222	238	262	278	0.462	11	82	311	596
0.068	209	233	267	291	0.518	8	71	305	616
0.078	194	227	274	307	0.578	5	62	299	634
0.090	177	219	280	323	0.640	4	53	292	651
0.102	161	212	287	340	0.706	2	46	285	667
0.116	144	205	293	358	0.774	2	39	277	681
0.130	128	197	299	375	0.846	1	34	271	695
0.144	114	190	305	392	0.922	1	29	264	707
0.160	100	182	310	409	1.000	-	25	257	718
0.194	76	166	317	441	1.082	-	21	250	729
0.230	56	150	322	472	1.166	-	18	243	739
0.270	41	135	324	500	1.254	-	15	236	748
0.314	30	120	323	527	1.346	-	13	230	757
—					1.440	-	11	224	765

In the function

$$X = \frac{h^2}{4Dt}$$

h = the thickness of the agar blocks in cm.

t = the time of diffusion in days

D = the diffusion constant based on time in days

By interpolation each of the four relative concentrations gives a value of X and consequently of D. If p is the difference between the two relative concentration values in the table, between which interpolation was made, then the final value of D is calculated as follows:

$$D = \frac{D_1 p_1 + D_2 p_2 + D_3 p_3 + D_4 p_4}{p_1 + p_2 + p_3 + p_4}$$

since the significance of a value of D increases with increasing differences between the two concentration values.

In the case of auxins, if four quite different values of D are obtained with a definite trend from one agar block to the next, one of the following situations is indicated:

a. An inhibitor is present which diffuses at a rate different from that of the auxin.

b. More than one auxin is present.

c. Factors of both a and b are at work.

The change of D with change in temperature is expressed by the following equation:

$$\frac{D_2}{D_1} = 1 + a(T_2 - T_1)$$

The coefficient a itself is a function of D and can be evaluated approximately for aqueous solutions from the following table, taken from Taylor, Treatise on Physical Chemistry.

D	a
2.4	0.018
2.0 - 1.8	0.020
1.6 - 1.4	0.022
1.2 - 1.1	0.025
0.8 - 0.7	0.029
0.4 - 0.3	0.035
0.2 - 0.1	0.040

The following empirical relationship between the diffusion constant and the molecular weight has been found to hold good for a variety of compounds of molecular weight up to a range of about 500, with an expected error of 10 - 20% in the predicted molecular weight.

$$D\sqrt{M} = \text{constant}$$

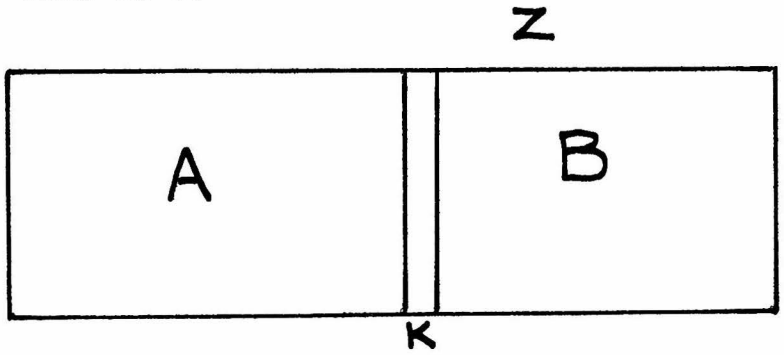
The value of the above constant was determined by Oholm (26) and for a variety of compounds was given an average value of 7.0 for aqueous solutions at 20 C.

Since the diffusion constant usually increases almost linearly with dilution in the region of 1.0 to

o.1 molar, it is necessary to extrapolate to infinite dilution to obtain D in the above equation.

It is an interesting and pertinent fact that the diffusion constant at a given temperature is about the same in most gels, such as agar, as in water.

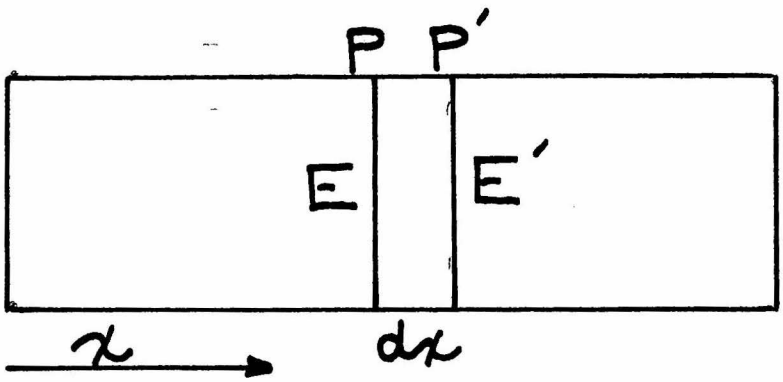
The derivation of the diffusion constant has been carried out by Einstein (8) with only meagre assumptions. Consider a cylinder Z filled with a solution and divided into compartments A and B by a movable semi-permeable membrane K, the concentration of solute in A being greater than in B.



Now in order to prevent K from moving, a force must be applied in the direction of A, or if no force is applied then K will move in the direction of B until the concentrations in A and B are equal. This simple consideration illustrates the identity of the forces causing osmotic pressure and those responsible for diffusion.

Consider now a cylinder of 1 cm^2 cross section with no membrane, but filled with a solution in which

diffusion is taking place because of a concentration gradient.



At the plane E call the osmotic pressure P, and at E', a distance dx from E, the pressure P' (P > P'). Then the force K acting on the solute molecules in dx cc. of solution can be expressed:

$$K = \frac{P - P'}{dx} = \frac{-dP}{dx} \qquad (1)$$

But the osmotic pressure due to n moles of solute per dx cc. is

$$P = n R T \qquad (2)$$

and

$$K = - R T \frac{dn}{dx} \qquad (3)$$

combining (1) and (2). Now if a force K due to osmotic pressure acts on one molecule then v, the velocity, is

$$v = \frac{k}{F} \qquad (4)$$

where F is the friction function, depending on the size

and shape of the solute molecule and the viscosity of the medium. If in dx cc. there are n moles of solute, there are Nn molecules where N is Avogadro's number, and if the above force K is divided up between Nn molecules, the velocity of the molecules is

$$v = \frac{1}{Nn} \quad \frac{K}{F} \tag{5}$$

or combining (3) and (5)

$$vn = -\frac{R\ T}{N\ F} \cdot \frac{dn}{dx} \tag{6}$$

The diffusion constant D is defined by Fick's first law of diffusion, assuming a cross section of 1 cm^2:

$$\frac{dN'}{dt} = -D \frac{dn}{dx} \tag{7}$$

where N' is the number of moles of solute and t is the time, but

$$-\frac{dN'}{dt} = vn \tag{8}$$

so that

$$D = \frac{R\ T}{N\ F} \tag{9}$$

Stokes law gives the friction function for molecules large in comparison to those of the solvent and spherical in shape. In this case

$$F = 6\pi\eta r$$

where η is the viscosity of the medium and r the radius of the diffusing molecule.

Since m, the mass of a spherical molecule, is

$$m = \frac{4}{3} \pi r^3 d$$

where d is the apparent density in solution, we would expect the following to hold true for the diffusion of spherical molecules:

$$D \sqrt[3]{M} = \text{constant}$$

The fact that $D \sqrt{M}$ constant holds better than the above relationship for a variety of substances investigated means simply that as a rule their molecules are not approximate spheres in solution. No doubt a statistical treatment of chain-like and disc-like molecules would result in friction functions which could satisfactorily describe their diffusion behavior.

The diffusion technique was further employed by Heyn in 1935 (13) who investigated auxins from several sources. He found that the auxin in Phycomyces cultures had a molecular weight close to that of 3-indoleacetic acid, while the auxin from Avena coleoptiles, Vicia faba roots, and regenerated Avena tips gave values close to that of auxin a.

Thimann in 1935 (40) published his results on the isolation of the auxin found in cultures of Rhizopus suinus. This compound has the same acid-base stability as 3-indoleacetic acid and both compounds distill in

high vacuum (molecular still) at about 100 C. A small amount of the crystalline substance was obtained and melting point determinations were used to establish identity with 3-indoleacetic acid. Thimann noted in 1933 (38) that the auxin production of Rhizopus cultures was correlated with the constituents of the medium. Certain peptones resulted in very large auxin production, while others yielded almost none at all. It has been shown that 3-indoleacetic acid arising in cultures of micro-organisms is produced by the oxidative deamination of tryptophane so that the effects observed by Thimann are due simply to the tryptophane content of the peptone used.

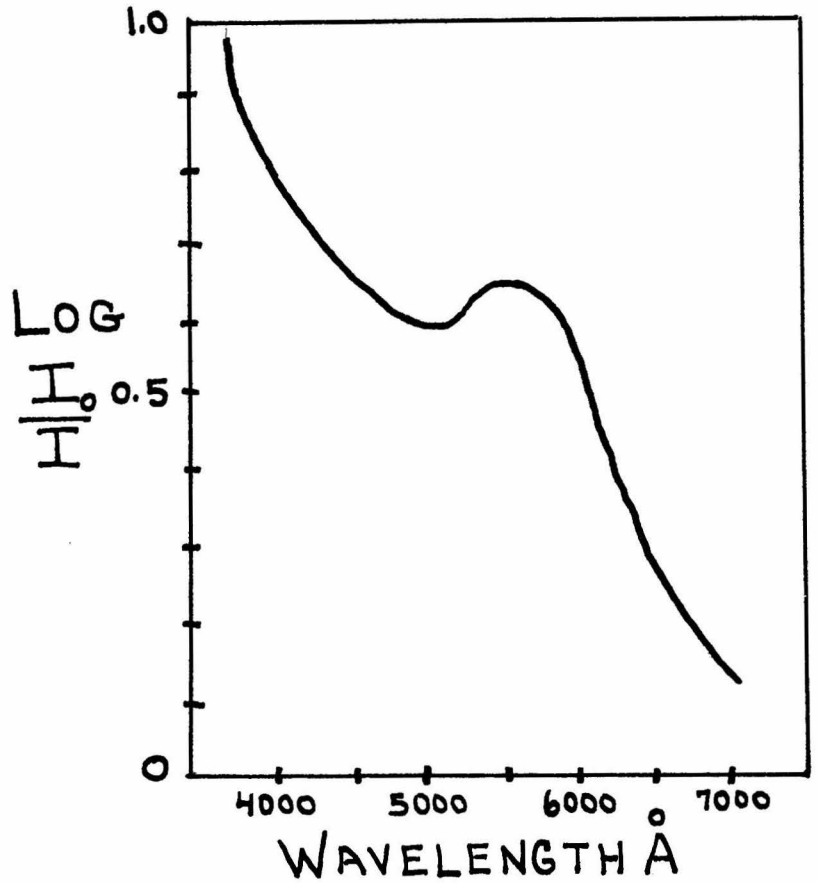
In 1936 an important phenomenon was discovered by van Overbeek (27). He found that if crystalline auxin a (obtained from Kögl) is applied to Avena plants in the dark, a greater curvature is obtained than if the plants are in the light. This effect is not shown, however, by 3-indoleacetic acid. Moreover, a distilled water extract of corn meal yields an auxin solution which behaves like auxin a in this respect and not like 3-indoleacetic acid. This characteristic light sensitivity of auxin a in vivo affords an additional tool for the characterization of unknown auxins but as yet has not been generally applied.

Van Overbeek and Bonner (30) using the acid-alkali destruction test investigated the auxin from pea roots and concluded that auxin a was probably present.

An examination of the auxins in the algae Macrocystis and Bryopsis and in Elodea, a higher plant, employing both diffusion and acid-alkali tests (van Overbeek 31) showed that the auxin present has the characteristics of 3-indoleacetic acid.

Lefévre (25) applied the ferric chloride and color reactions for indole derivatives to various plant extracts and made subsequent absorption spectra measurements. He concluded that 3-indoleacetic acid, or a near relative, is present in numerous higher plants. It must be recognized, however, that color reactions carried out on mixtures of unknown composition and complexity, such as the expressed juice of a plant, can easily lead to erroneous conclusions.

Nevertheless, the spectrophotometric analysis of the pigments produced in color reactions should afford a powerful tool in the identification of unknown or new compounds. The following, for example, is a plot of the absorption of a mixture of 10 cc. of 0.001 molar 3-indoleacetic acid and 0.1 cc. of 0.1 molar ferric chloride in 1 molar hydrochloric acid about one half hour after mixing. (The absorption of this reaction mixture is not constant with time.)



Absorption spectrum of indole acetic acid plus ferric chloride.

It is thus conceivable that information of this sort concerning the absorption maxima and minima of the products of color reactions might give information about the structural details of the reactants.

Goodwin in 1939 (9) employed the diffusion method on corn meal extracts. He concluded that certain inhibitors of auxin were present, which when removed gave diffusion constants near those of auxin a and b.

In 1941, Haagen-Smit and co-workers (11) reported the first isolation of 3-indoleacetic acid from a higher

plant source. Corn meal was treated with dilute sodium hydroxide solution and hydrolysis allowed to take place. The extract was fractionated and one of the potent concentrates was crystallized. 3-indoleacetic acid was the chief product but also a small amount of pseudo-auxin a was obtained. It was concluded that the latter substance arose from the rearrangement of auxin a during the isolation procedures.

Wildman and Gordon (44) in their work on the enzymatic hydrolysis of spinach proteins concluded that an auxin is liberated which has a lower molecular weight than that of 3-indoleacetic acid, the liberated auxin being characterized by the diffusion technique. The same authors (10) studied the action of alkali on tryptophane, casein, and gelatin with respect to auxin liberation. Under the most favorable conditions found for the liberation of auxin from tryptophane approximately 0.008% of the tryptophane appeared as auxin after hydrolysis. Casein and gelatin were found also to liberate small amounts of an auxin on treatment with alkali.

Avery in 1941 (3) published his results of treating dormant maize endosperm with various solvents and borate buffers at pH 9-10 and 100 C. Extraction with several solvents successively or with water alone removes only

about one tenth of the available auxin (2). This constitutes the so-called free auxin. The remainder exists in a bound form liberated by alkaline hydrolysis, and in dormant corn amounts to the equivalent of 7×10^{-5} gm. of 3-indoleacetic acid per gram of corn. This is essentially confirmation of the results obtained previously by Haagen-Smit (11). Avery found that the auxin in corn has the acid-alkali stability of 3-indoleacetic acid.

In a subsequent paper (4) Avery applied the same treatment to wheat grains with a most interesting result. The total auxin from dry wheat was equivalent to 0.5×10^{-6} to 3×10^{-6} gm. of 3-indoleacetic acid per gram of wheat. On heating this auxin with 1.0 N. sodium hydroxide, marked destruction takes place, and he concludes that 70% or more of the auxin present is a compound or compounds different from 3-indoleacetic acid.

In continuing work on the auxin in dormant corn, Berger (6) purified an "auxin precursor" which on alkaline hydrolysis gives as one of the products about 3.7% of 3-indoleacetic acid. The latter was purified further and crystallized but was concluded to contain 10 to 30% of an additional as yet unidentified substance.

Both Avery (5) and Wittwer (45) have studied the changes in auxin concentration in immature corn kernels

during the period following fertilization. Avery found that for "Country Gentleman" corn the peak in total auxin content occurred about ten days after pollination. At this stage the total is equivalent to about 0.3 to 0.5 mg. of 3-indoleacetic acid per gram of dry weight, and of this total only about 6% is free, that is, extractable by water or organic solvents. At this stage the kernels are about 35 to 40% dry weight, so that on a fresh weight basis one gram of kernels contains the equivalent of 1.2×10^{-4} to 2.0×10^{-4} gm. of 3-indoleacetic acid of which only 7.2×10^{-6} to 1.2×10^{-5} gm. is present in an unbound form.

The results of Wittwer indicate a similar peak in the free auxin concentration about 10 to 15 days after pollination. Moreover, his results bring out the interesting fact that unfertilized ovules do not contain any demonstrable amount of auxin even at a time when the fertilized ones contain comparatively large concentrations.

As the development of the kernels proceeds, both the total auxin and free auxin content decrease rapidly to the value for dormant corn, that is, a total auxin content equivalent to about 7.4×10^{-5} gm. of 3-indoleacetic acid per gram of corn. Of this total roughly

5% is in an uncombined form readily extractable by water.

Bergren (7) found that ether extraction of whole corn yielded the equivalent of 2 to 7×10^{-7} gm. of 3-indoleacetic acid per gm. of corn.

This is in contrast to the case of rye studied by Hatcher (12). Up to the attainment of maximum auxin concentration, all the auxin exists in a free form. Thereafter the free auxin disappears and the bound form increases in amount. In the dormant stage, as in corn, about 5% exists still in the free form.

Summary of Information about Characterization of Auxins

Source	Auxin found	Method of Characterization	Ref.
Avena tips	Auxin of MW, 376	Diffusion	43
Pregnancy urine	Auxin a	Isolation	14
Corn Oil	Auxin a and b	"	16
Malt	"	"	"
Human urine	Auxin a and IAA	"	18
Yeast	IAA	"	20
Corn tips	Auxin a	Diffusion	19
Corn tips	"	Acid-alkali	19
Phycomyces	IAA	Diffusion	13
Avena coleoptiles	Auxin a	"	"
Vicia faba roots	"	"	"
Regenerated Avena tips	"	"	"
Rhizopus	IAA	Isolation	40
Corn meal	Auxin a	Light Inactivation	27
Macrocystis	IAA	Diffusion and acid-alkali	31
Pea roots	Auxin a	Acid-alkali	30
Corn meal	Auxin a or b	Diffusion	9
Bryopsis	IAA	Diffusion and acid-alkali	31
Elodea	"	Diffusion and acid-alkali	"
Corn meal	Pseudo auxin a and IAA	Isolation	11
Corn meal	IAA	Acid-alkali	3
Wheat grains	70% not IAA	"	4
Spinach proteins	Auxin of lower MW than IAA	Diffusion	44
Corn kernels	IAA	Isolation	6

Evaluation of the methods used to characterize auxins

Of all the methods employed to establish the identity of an auxin the method of isolation is by far the safest and most likely to lead to unequivocal conclusions. Although there exists the possibility of alteration of substances during isolation, this is certainly a minor objection when compared with the disadvantages of the other methods employed.

The determination of diffusion constants is theoretically a good method but subject to the approximations already noted. Inasmuch as inhibitors are probably not uncommon in plant extracts, (24,36) cognizance must be made of this factor in interpreting diffusion data.

The determination of light sensitivity, it seems, should afford an excellent method to distinguish between auxin a and 3-indoleacetic acid, but because it has not been extensively applied its advantages and disadvantages are not known.

The differential acid-alkali destruction test has been widely used and although there is no specific evidence to show that its results on crude extracts might be unreliable, they should be interpreted with caution.

Comparison of the biological effects of 3-indoleacetic acid and other auxins

(1) Effect on cell elongation.

The action of crystalline auxin a and of 3-indoleacetic acid on cell elongation in Avena in investigations thus far undertaken has proved to be qualitatively the same. The activities in the Avena test as determined by Kögl and his co-workers are for auxin a, 50×10^6 AE per mg.(14), and for 3-indoleacetic acid, about 20×10^6 AE per mg. (18). The velocity of transport in the Avena coleoptile is slightly different for auxin a and 3-indoleacetic acid, auxin a being transported in the cut coleoptile at a rate of 10 to 12 mm. per hour, or about 10% faster than 3-indoleacetic acid (Went 43, p. 90).

(2) Effect on roots.

The action of crystalline auxin a and 3-indole acetic acid on the roots of Avena was studied by Kogl and co-workers (19). Concentrations of auxin a down to 10^{-7} gm. per liter showed an inhibitory effect on the growth of roots. The same effect is shown by 3-indoleacetic acid although at very low concentrations, that is about 10^{-9} molar, a stimulation of root growth is observed.

Thimann and Koepfli (41) have demonstrated that pure 3-indoleacetic acid is fully active in the stimu-

lation of root initiation and growth.

However, a difference in the action of auxin a and 3-indoleacetic acid has been observed on root formation, (Went 43, p. 195). In these experiments pea cuttings were treated with the two auxins separately and together. If very small concentrations of auxin a are added to optimal concentrations of 3-indoleacetic acid, the number of roots formed is far greater than the number formed by 3-indoleacetic acid alone. However, the addition of small amounts of 3-indoleacetic acid to optimal concentrations of auxin a does not show this effect.

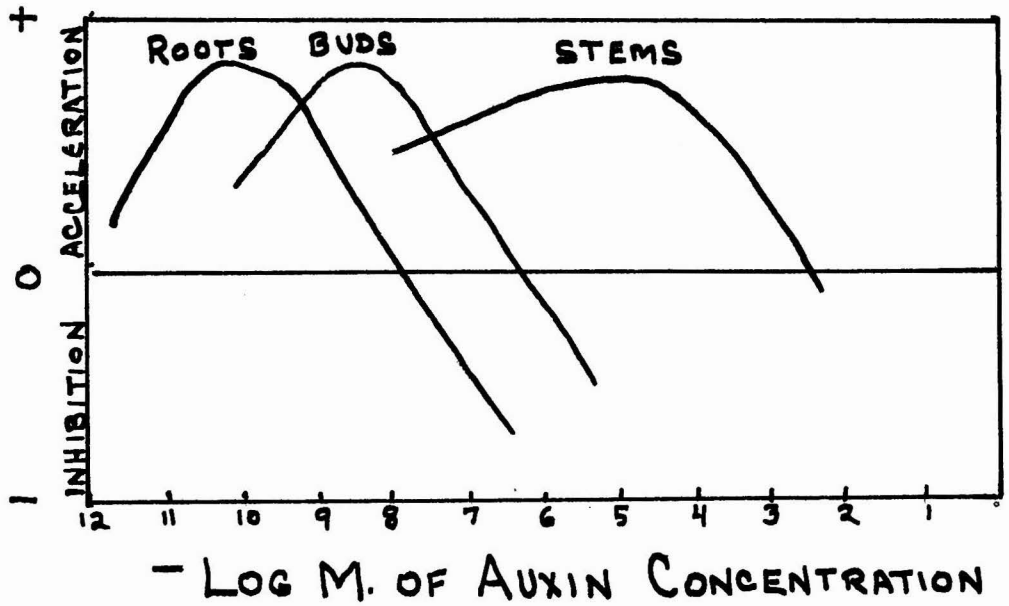
(3) Effect on bud development.

It is a widely known fact that the lateral buds of plants are inhibited by the terminal bud. If the terminal bud be removed, the lateral buds begin to develop almost immediately. This, of course, is the theoretical basis of pruning trees and shrubs.

These effects were investigated by Thimann and Skoog (39) using young plants of Vicia faba. They found that young terminal buds produced large quantities of auxin and that this production falls off in older buds. Moreover, normal lateral buds produce almost no auxin, but if the terminal bud of a shoot is removed, the laterals begin to develop and give off auxin which acts then

to inhibit the other lateral buds. The inhibitory effect of the normal terminal bud can be reproduced by putting in its place an agar block containing 3-indoleacetic acid.

As in the case of roots, low concentrations of 3-indoleacetic acid exert a stimulating effect upon the growth of buds. These relationships are illustrated in the following plot taken from Thimann (42), the bud and stem curves being partly hypothetical.



Inhibition and growth promotion of different organs as a function of auxin concentration. The abscissae for the bud and stem curves are only approximate.

In subsequent work Skoog and Thimann (32) compared the action of crystalline auxin b with that of 3-indoleacetic acid on bud inhibition. The auxin b was obtained from Professor F. Kögl. Solutions of the auxin to be tested were placed in small wax cups molded around the stem of a decapitated seedling and the effects observed after a number of days. These workers concluded that in respect to bud inhibition the action of auxin b and 3-indoleacetic acid was indistinguishable in kind.

(4) Effect on cambial growth.

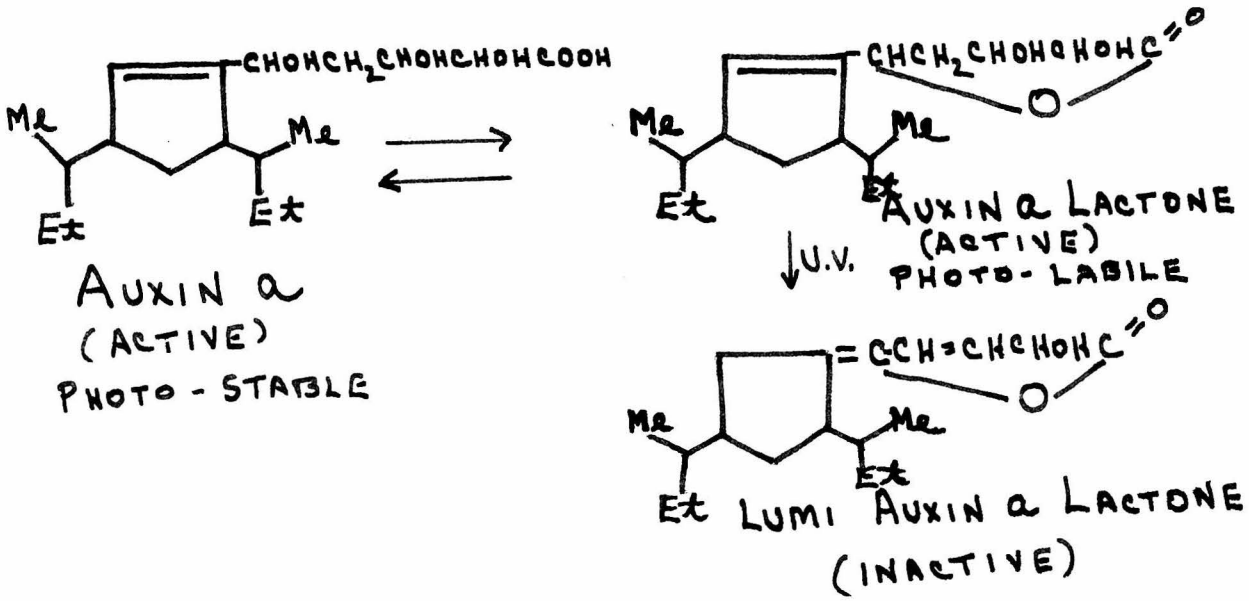
Snow (34) studied the action of crystalline auxin a (presented by Kögl) and 3-indoleacetic acid on cambial growth and found that their effects on this tissue were identical. While the lateral buds of a stem are inhibited by the auxin liberated by a terminal bud, the activity of the cambium layer is stimulated.

(5) Effect on phototropism.

The physiological effects of auxin a and 3-indoleacetic acid thus far discussed are different in only very minor respects. The physiological differences are much greater, at least in Avena plants, when the effects of light are considered. The experiments of van Overbeek (27.28.29) first detected this important physiological

distinction between the two auxins. He showed that Avena curvatures produced by auxin a were up to two times as great in the dark as in the light, while those of 3-indoleacetic acid were the same in either case.

The same problem was studied by Koningsberger and Verkaaik (23) using the chemical results of Kögl as their starting point. The latter (22) found that, in making ultra-violet absorption measurements on auxin b solutions, an enol form could be detected by the ferric chloride test after a time, but auxin b itself gave no color reaction with this reagent. Moreover, solutions of auxin a lactone rapidly lost their biological activity on exposure to ultra-violet radiation. These changes were formulated as follows:



Kögl (22) mentions the possibility that the above photochemical inactivation of auxin a and b might conceivably be of importance in the normal responses of plants to light.

Koningsberger and Verkaaik first repeated the work of van Overbeek and obtained fully similar results using a highly purified preparation supplied by Kögl. They, however, added a very important and enlightening experiment. First, deseeded and decapitated Avena plants were prepared; these have been shown to be practically free of auxin and moreover do not form more auxin during the test period. Now on one set of such plants were placed agar blocks containing auxin a and on another 3-indoleacetic acid. Diffusion was allowed to take place, and then the plants illuminated on one side only. After a period the test plants were photographed. The result obtained was that expected from theory. Seedlings given auxin a showed a curvature (7 to 8 degrees), but those given 3-indoleacetic acid showed no significant curvature (less than 1 degree). It was postulated that the observed phototropic curvature was due to the in vivo inactivation of auxin a lactone. It was later found in Kögl's laboratory that solutions of auxin a lactone are inactivated by visible wavelengths in the presence of carotene.

Results were obtained by Stewart and Went (37) which apparently contradicted those of Koningsberger. They found first that exposure of Avena coleoptiles to light decreased the amount of auxin which could be extracted by ether. This is in harmony with the findings of van Overbeek and Koningsberger. However, they were unable to find any destruction of an auxin prepared from corn meal on allowing it to be transported through illuminated coleoptile sections. Stewart and Went conclude that this is due to light sensitivity of auxin when it is bound to some constituent of the cell and to light stability of free-moving auxin. It is perhaps possible that this auxin which was prepared from corn meal contained 3-indoleacetic acid although the available information indicated that the "free" auxin of corn meal is auxin a or b (9,27).

To summarize in general what has been said about auxins and their comparative physiological properties, we see that the differences, for example, between auxin a and 3-indoleacetic acid are not merely quantitative in nature. This is an inescapable result of their differences in structure and, as investigation proceeds and more information accumulates in this field, it is to be expected that further differences in biological activity will be discovered.

The foregoing review of the literature brings us to the real purpose of this problem.

The "bound" or combined auxin of dormant corn kernels has been conclusively identified by isolation by both Haagen-Smit (11) and Berger (6). However, the "free" portion (that readily available by water extraction) has not been isolated for either immature or dormant kernels. It is with this fraction in immature kernels that the present work is concerned.

This "free" portion is of special interest and significance for the following reasons:

(1) It is present in a rapidly growing tissue.

(2) It can be extracted under very mild conditions, i.e., with water or organic solvents.

(3) There is evidently some connection between auxin production and the changes which occur subsequent to fertilization. Whether or not this relationship is causal is at present not known.

Experimental Section

Bioassay

The auxin concentrations throughout this work were determined by the standard Avena test, as described by Went (43). The interval between application of the agar blocks ($2mm^3$ in volume) and photographing of the test plants was 110 minutes in all cases. The following procedure was adopted in evaluating the experimental data. The angles of curvature were measured and those between 0 and 2 degrees discounted unless the mean curvature was under 5 degrees. This is justified on the basis that errors in technique can result in approximately a 0 degree curvature even though a large concentration of auxin is present. The arithmetic mean of the remaining curvatures was calculated and those means within the proportionality range taken as proportional to the auxin concentration.

In each test, the Avena seedlings were calibrated by applying blocks soaked in solutions of known concentrations of 3-indoleacetic acid. One mg. of 3-indoleacetic acid, Merck, diluted up to 3 l. ($1:3x10^6$) usually gave curvatures of about 15 degrees. Dilutions were made with a medium composed of distilled water containing 0.005 moles of potassium chloride and 0.003 moles of acetic acid per liter.

Description of Starting Material

The crude extract used in this isolation of 3-indoleacetic acid was prepared by Dr. S. H. Wittwer at the University of Missouri. Approximately 100 kg. of fresh corn kernels in the milk stage, that is about 15 days after fertilization, was extracted by placing it in 95% ethanol. About 80 kg. of kernels was extracted for two weeks and the remainder about 24 hours. The alcoholic solution was concentrated under reduced pressure to 3700 cc. and the syrupy extract shipped to The California Institute of Technology via Air Express. About two days were spent in transit but on arrival there was no noticeable evidence of fermentation or spoilage. The total activity of this crude extract was equivalent to 1.48 gm. of 3-indole-acetic acid (Appendix Sept. 8.). The residual corn now once extracted was sent to us by Railway Express and stored at 4 degrees C. The liquid still held in this residue by capillarity was about 50 to 60% ethanol.

Chemical fractionation

(1) Preparation of the crude acid fraction.

The crude syrupy extract was acidified to a pH of 3.0 to 3.5 by adding 230 cc. of concentrated HCl.

The resulting mixture was divided into two equal parts to facilitate handling and each part extracted in a separatory funnel with on 2 liter portion and two 1 liter portions of Et_2O. All ether used was purified by distillation over a mixture of $FeSO_4$, excess $Ca(OH)_2$, and H_2O. The ether solution was dried over anhydrous Na_2SO_4 and concentrated by evaporation under reduced pressure. It contained 26.2 gm. of solids, equivalent in auxin activity to 0.186 gm. of IAA (Appendix Sept. 8.).

The concentrated ether solution (735 cc.) was extracted in a separatory with three 370 cc. portions of saturated $NaHCO_3$ in water. The bicarbonate layer was acidified with about 110 cc. of concentrated HCl and extracted with three 1 liter portions of Et_2O. On evaporation of the latter ether solution under reduced pressure, 4.81 gm. of solid remained. This fraction was equivalent to about 0.48 gm. of IAA and is composed chiefly of the free organic acids (Appendix Sept.11.). This sudden rise in activity from 0.186 gm. to 0.48 gm. of IAA indicates that the bicarbonate fractionation removes an inhibitor or inhibitors. Probably then too the original material contained more than 1.48 gm. of IAA.

Since the original crude extract still contained a large amount of auxin activity (Appendix Sept. 8.),

it was further extracted with Et_2O. A total of 6 liters in three portions was used, and the aqueous phase was then almost free of auxin (Appendix Sept. 13.).

As before, the ether solution was concentrated under reduced pressure and extracted with saturated $NaHCO_3$ solution. A total of 1350 cc. of bicarbonate (seven portions) was used; the bicarbonate was acidified and the solution extracted with four 500 cc. and one 250 cc. portions of Et_2O. The ether was evaporated under reduced pressure and the residue combined with the previous portion of organic acids. The resulting fraction weighed 10.52 gm. and contained an amount of auxin equivalent in activity to 1.09 gm. IAA (Appendix Sept. 18). This is equal to about 74% of the activity in the original extract. This fraction which forms the basis for the succeeding steps of the isolation will hereinafter be referred to as the crude acid fraction.

(2) Tentative identification of the auxin present.

At this juncture it was decided to determine how much auxin remained in the extracted corn kernels and to attempt to characterize the auxin or auxins in the extracted kernels and in the crude acid fraction.

A representative sample of the extracted kernels was ground up in a mortar and extracted with water at

room temperature (1 gm. of moist kernels per 15 cc. of water). The bioassay showed that the 100 kg. of kernels contained an amount of auxin equivalent to 1.2 gm. of IAA (Appendix Sept. 11). Thus the concentration of auxin in the fresh kernels as determined by extraction with ethanol plus water was equivalent to 2.7×10^{-5} gm. IAA per gm. of fresh corn.

Characterization of the auxin present was accomplished by the use of the differential acid-alkali destruction test, as described in the introductory section. When this test was applied it was found that the auxin in the crude acid fraction and in the residual corn kernels behaved like IAA.

The following experiment will illustrate the technique used and the results obtained. 53 mg. of the crude acid fraction was dissolved in 0.48 cc. of 96% EtOH and four 0.1 cc. portions of the solution withdrawn. These portions were treated variously, as follows:

(a) 1.0 cc of 0.1 N. KOH was added and the mixture heated on a boiling water bath for 3.5 hours.

(b) 1.0 cc. of 5% HCl was added and heated as above.

(c) 1.0 cc. of pH 7 phosphate buffer was added and heated as in (a).

(d) 1.0 cc. of water was added and the mixture put in the cold room as a control.

After the heating period the mixtures were neutralized and dilutions made. The following curvatures were obtained from an Avena test of the products.

Treatment	Dilutions (based on weight of crude acid fraction)					
	1:2.5 $\times 10^4$	1:5 $\times 10^4$	1:1.25 $\times 10^5$	1:2.5 $\times 10^5$	1:6.25 $\times 10^5$	1:1.25 $\times 10^6$
KOH		> 12		12		0
HCl	14		0		0	
pH 7 buffer		> 10		10		0
Control		> 10		10		0

This shows that approximately 86% of the activity was lost by treatment with hot acid and none by treatment with hot pH 7 buffer or 0.1 N. KOH Treatment with 1.0 N. KOH likewise resulted in no destruction (Appendix Sept. 20).

Synthetic IAA was likewise subjected to the above tests, and it was found that hot 5% HCl destroys 40 to 50% of the activity in 3½ hours at 98 C. whereas 1 N. KOH gives no measureable destruction under the same conditions (Appendix Sept. 20).

An active extract made from a representative sample of the residual corn kernels was found likewise to be stable toward base but labile in acid (Appendix Sept. 27). The reason for the differences observed in the percentage destruction of pure IAA and the auxin from corn is not known. The acid-alkali tests on the crude acid fraction were completed 13 days after the alcoholic extract was received from the University of Missouri, those on the residual corn kernels 7 days later. The above results indicate that the chief component of the auxin in immature corn kernels behaves toward acid and alkali like IAA. From this information it was evident that the steps of the isolation procedure should be patterned after those for IAA from urine rather than those for the isolation of either auxin a or b.

The small decreases in activity of the crude acid fraction with time are difficult to interpret. Thus, on Sept. 18, it was found to contain the equivalent of 10.4% IAA, on October 2, 9.7%, and on October 6, 9.0%. These differences are quite probably within the range of experimental error.

(3) Fractionation of the crude acid fraction

Approximately half (5.02 gm.) of the crude acid fraction containing the equivalent of about 10.4% IAA was dissolved in 100 cc. of 60% EtOH and the resulting

solution extracted in a separatory funnel with twelve 50 cc. portions of purified benzene. The benzene layer was then extracted with six 50 cc. portions of 50% MeOH and with six 50 cc. portions of water. The water and methanol extracts were combined and the methanol evaporated under reduced pressure. The remaining aqueous solution was extracted with purified Et_2O, two 100 cc. portions, and three 50 cc. portions, and the resulting ether solution dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue weighed 1.25 gm. and contained the equivalent of 0.26 gm. IAA (Appendix October 13). This fraction will be referred to as the benzene-methanol fraction. The activity here is only about fifty percent of that present in the 5.02 gm. of crude acid fraction, but the concentration of auxin is nearly twice as great.

At this point an attempt was made to crystallize the benzene-methanol fraction. 0.87 gm. was dissolved in 20 cc of hot $CHCl_3$: the solution was filtered and allowed to cool. A few crystals appeared but they were obviously not well formed and quite impure. Inasmuch as no more raw material could be obtained it was essential to get as high yields as possible in the crystallization procedures and thus it was necessary to further fractionate the benzene-methanol fraction by other means.

The above 0.87 gm. of active material, after removal of the chloroform, was extracted on a water bath with five 5 cc. portions of petroleum ether (40 to 60 degrees) at its boiling point. The residue was then further extracted with five 5 cc. portions of high boiling petroleum ether at its boiling point (100 to 130 degrees). A crude crystalline material deposited out of the high-boiling petroleum ether on cooling to room temperature. This deposit was filtered off, redissolved in xylene, and the solution crystallized in a few hours in the cold room. This product amounted to only a couple of mg. but was recrystallized later along with other highly purified fractions.

The main bulk of material having been extracted with petroleum ether was treated with five 15 cc. portions of hot xylene. A residue (83 mg.) remained which proved to be inactive in the Avena test (Appendix Nov. 8). The xylene solution was evaporated under reduced pressure and gave a residue of 505 mg. This residue was extracted on the water-bath with twelve 5 cc. portions, three 10 cc. portions, and two 25 cc. portions of boiling cyclohexane. About 317 mg. of material remained undissolved. The cyclohexane extracts deposited a crude crystalline material on standing in the

cold room, and this proved to contain the equivalent of about 30% IAA (Appendix Nov. 8). This material was recrystallized from xylene and chloroform and later combined with other fractions for the final recrystallizations.

The material remaining after the cyclohexane treatment was now re-extracted with five 10 cc. portions of hot xylene. Only 13 mg. of residue remained, and as the xylene insoluble material was previously shown to be inactive in the Avena test this residue was discarded. The xylene solution was evaporated and the residue (328 mg.) assayed and found to contain the equivalent of about 21% IAA (Appendix Nov. 8); thus its total content was about 69 mg. of the pure substance. This fraction solidified partly in the cold but the crystals were still contaminated by large amounts of other substances.

The following step designed to remove various contaminating impurities consists of a precipitation with lead ion in dilute acid under which conditions IAA remains in solution. The 328 mg. of IAA was dissolved in 10 cc. of 96% EtOH, and 1 cc. of saturated neutral lead acetate was added slowly with stirring, whereupon a dense precipitate formed. The mixture was

filtered, the precipitate washed with a small volume of EtOH and the filtrate and washings combined. The moist precipitate was immediately dissolved in a mixture of 1 cc. of 1 N. HCl and 20 cc. of 1 N. AcOH. The resulting solution was extracted with four 10 cc. portions of Et_2O and the ether phase evaporated under reduced pressure. The residue (123 mg.) proved to contain the equivalent of 38% IAA (Appendix Nov. 13).

The filtrate from the lead precipitation was concentrated in vacuo to remove the EtOH, and a mixture of 10 cc. of water and 1 cc. of 1 N. AcOH added to the residue. The solution was extracted with five 10 cc. portions of Et_2O and the ether layer evaporated as before. The resulting fraction (118 mg.) containing the equivalent of 33% IAA (Appendix Nov. 13) was dissolved in a small amount of $CHCl_3$, the mixture filtered and placed in the cold room. Crystallization occurred in a short time and resulted in 24 mg. of crude crystalline material. This was recrystallized once from $CHCl_3$ and the product reserved for the final pooling of all the active crystalline fractions.

Inasmuch as IAA itself does not yield a precipitate with lead acetate in weakly acid medium, the precipitation was repeated on the fraction which was insoluble in the first treatment. This fraction (123 mg.)

was dissolved in EtOH and saturated neutral lead acetate added. The precipitate was dissolved in dilute AcOH and, along with the filtrate, extracted as before. This time the insoluble fraction, after removal of the lead ion, weighed 84 mg. The filtrate afforded 27 mg. of a crystalline material which had the solubility behavior of IAA and gave a brilliant red color with a mixture of $FeCl_3$ and HCl. This fraction was recrystallized from $CHCl_3$ and combined with the other crystalline fractions. Evidently the material which was soluble in the second precipitation was merely adsorbed on the precipitate during the first treatment.

The crystalline material obtained from the petroleum ether and cyclohexane extractions and from the two lead acetate precipitations was combined and dissolved in $CHCl_3$. This crude crystalline material amounted to about 70 mg. from which 49 mg. of thin flat plates was obtained on recrystallization. This substance sintered on the hot stage at 163 C. and melted to a reddish liquid at 165 to 169.5 C. Recrystallized IAA, Merck, sintered at 167 C. and melted at 168 to 170.5 C. under the same conditions.

The 49 mg. of crystals was recrystallized from $CHCl_3$ and dried overnight over P_2O_5 <u>in vacuo</u>. The resulting crystalline compound (43 mg.) sintered at 164 C.

and melted from 165 to 169.4 C. on the hot stage, whereas the recrystallized synthetic product sintered at 164 C. and melted from 167 to 170 C. An intimate mixture of the two sintered at 164 C. and melted at 166 to 170 C. (hot stage).

Capillary melting points were made simultaneously on the material from corn and on recrystallized synthetic IAA. The former sintered at 165 C. and melted at 165.7 C. to 166.5 C. The synthetic compound sintered at 165 and melted at 165.5 to 166.5 C. (all m.p.'s uncorrected). An intimate mixture of the two crystalline materials sintered at 164.5 and melted at 165.5 C. while a simultaneously observed capillary of recrystallized synthetic IAA sintered at 164.5 and melted at 165 C.

Carbon, hydrogen, and nitrogen micro-analyses of the crystalline material gave the following results:

	Calc. for $C_{10}H_9O_2N$	Found
Carbon	68.54%	68.99%
Hydrogen	5.18%	5.17%
Nitrogen	8.00%	8.06%

As an additional check on the identity of IAA and the substance obtained from corn, the two were assayed simultaneously in the Avena test. The following curvatures were obtained:

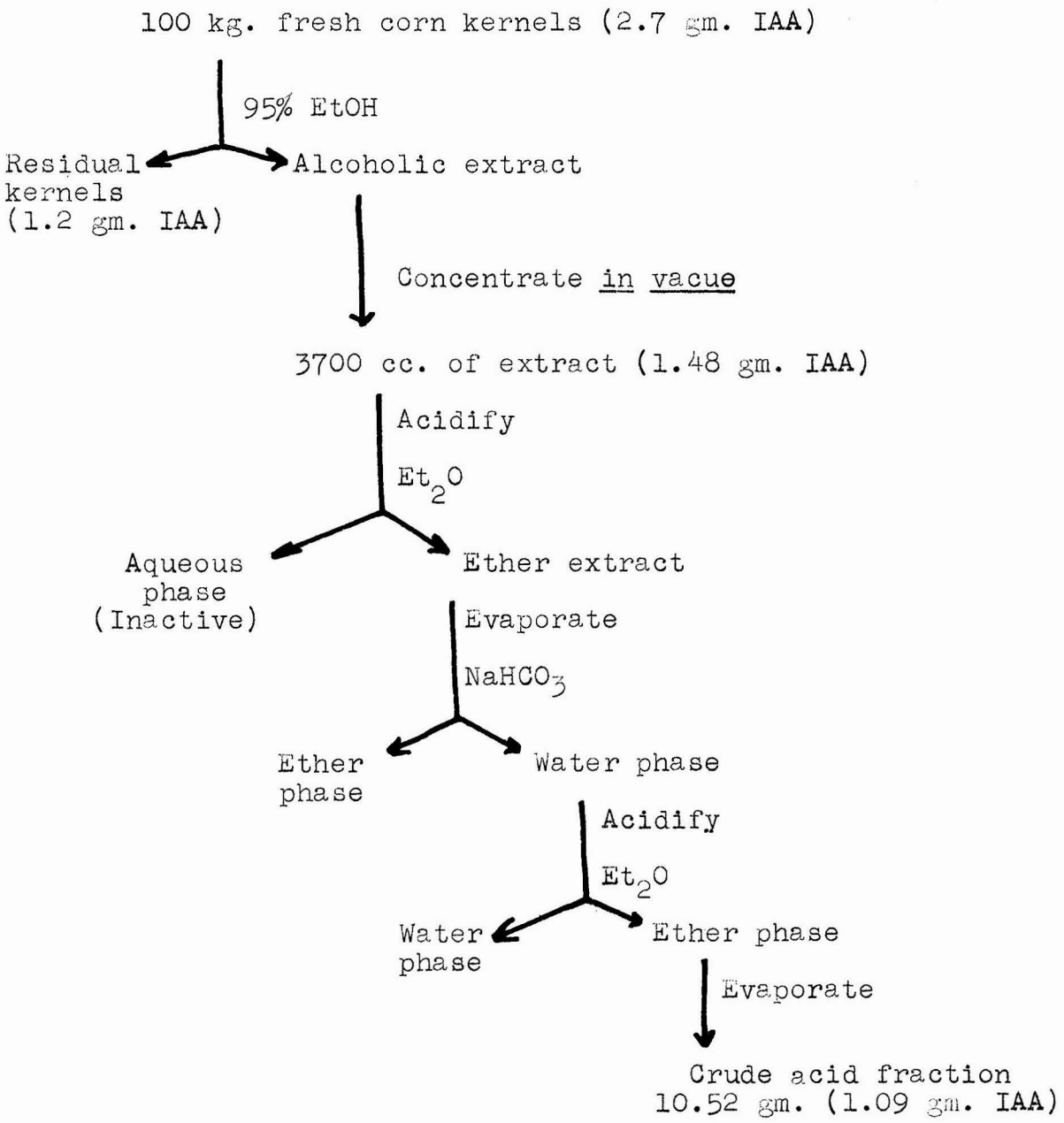
Dilution	Crystals from corn	Synthetic IAA Merck
1: $3x10^6$	25 degrees	19 degrees
1: $3x10^6$	23 "	14 "
1: $9x10^6$	11 "	7 "
1: $27x10^6$	3 "	4 "

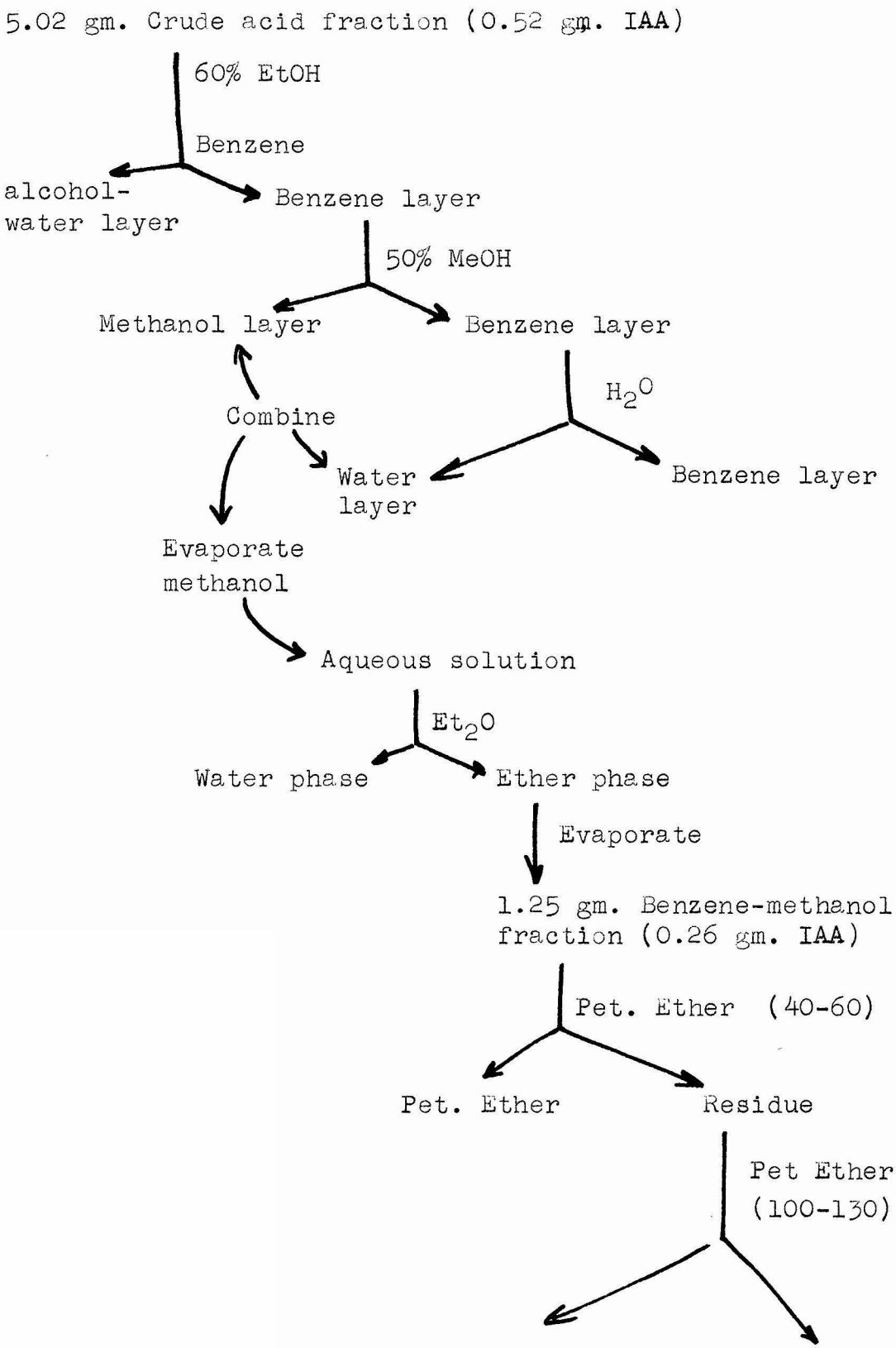
This discrepancy in biological activity may be due to the fact that the synthetic IAA used in the Avena test was not recrystallized but used directly from the Merck container. All dilutions for the above test were made on the same day.

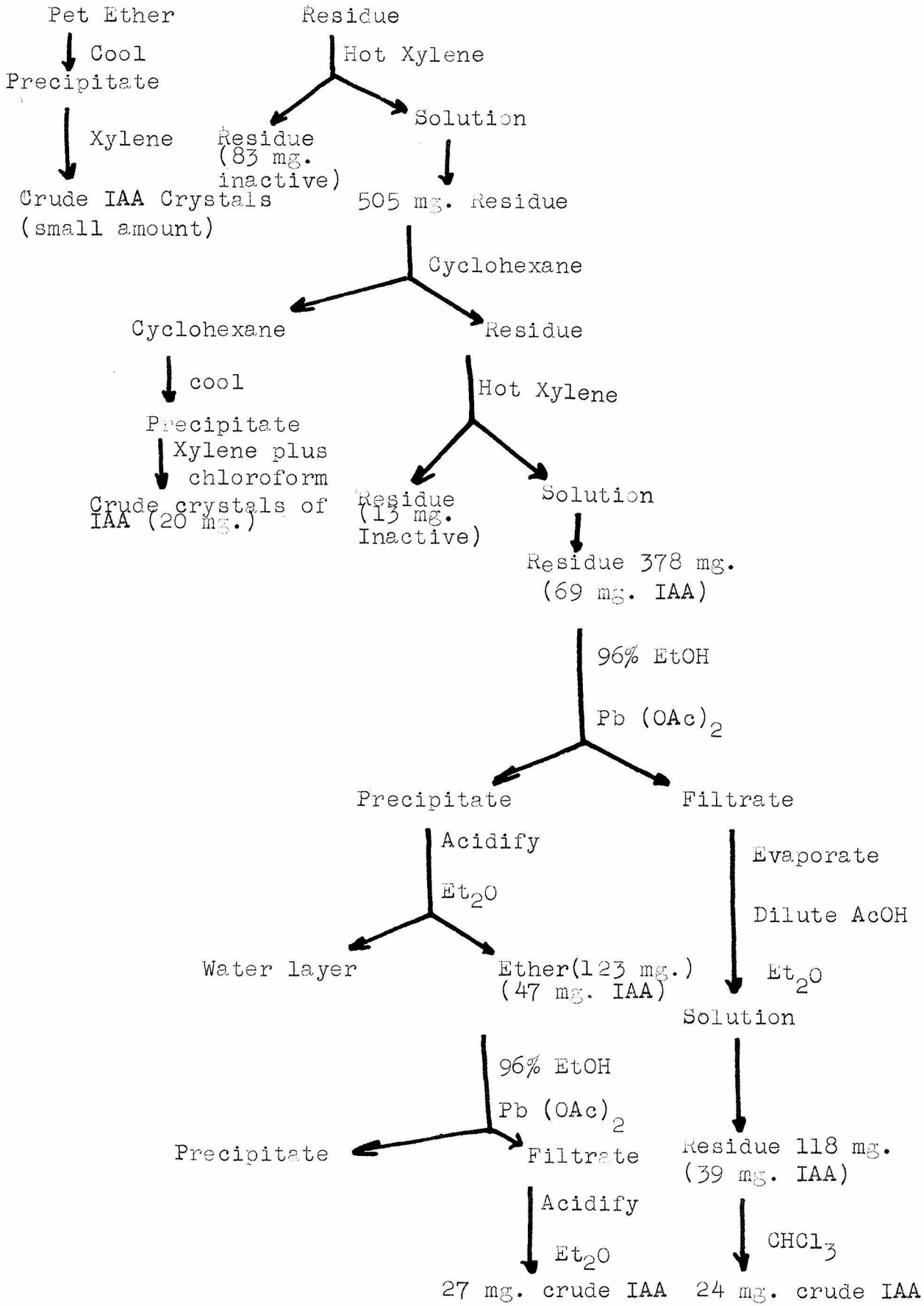
Thus the information afforded by melting point, mixed melting point, quantitative analysis, and bio-assay shows that the auxin isolated from corn kernels is 3-indoleacetic acid. The overall yield of IAA making allowance for the fact that only a part of each active fraction was used for further purification is about 9%.

(4) Summary of the fractionation scheme.

The following will serve to summarize the isolation procedure which has been described in detail above.




5.02 gm. Crude acid fraction (0.52 gm. IAA)
60% EtOH
Benzene
alcohol-
water layer
Benzene layer
50% MeOH
Methanol layer
Benzene layer
H_2O
Combine
Water
layer
Benzene layer
Evaporate
methanol
Aqueous solution
Et_2O
Water phase
Ether phase
Evaporate
1.25 gm. Benzene-methanol
fraction (0.26 gm. IAA)
Pet. Ether (40-60)
Pet. Ether
Residue
Pet Ether
(100-130)


Pet Ether
Cool
Precipitate
Xylene
Crude IAA Crystals
(small amount)
Residue
Hot Xylene
Residue
(83 mg.
inactive)
Solution
505 mg. Residue
Cyclohexane
Cyclohexane
Residue
cool
Precipitate
Xylene plus
chloroform
Crude crystals of
IAA (20 mg.)
Hot Xylene
Residue
(13 mg.
Inactive)
Solution
Residue 378 mg.
(69 mg. IAA)
96% EtOH
Pb (OAc)2
Precipitate
Filtrate
Acidify
Et2O
Water layer
Ether(123 mg.)
(47 mg. IAA)
Evaporate
Dilute AcOH
Et2O
Solution
96% EtOH
Pb (OAc)2
Precipitate
Filtrate
Residue 118 mg.
(39 mg. IAA)
Acidify
Et2O
CHCl3
27 mg. crude IAA
24 mg. crude IAA

Appendix

Appendix.

Date and Material Being Tested	Dilution	Mean Curvature Degrees	Percentage IAA
Sept. 8, 1944			
IAA Standard	$1:9x10^6$	7	
	$1:27x10^6$	0	
Original Crude Extract (3700 cc.)	1:100	<20	
	1:500	22	
	1:2500	10	0.04%
Et_2O soluble fraction of crude extract (26.2 gm)	$1:10^4$	14	
	$1:5x10^4$	9	0.71%
	$1:25x10^4$	Trace	
Original crude extract after extraction with Et_2O	1:10	8	
	1:50	8	
	1:250	16	0.006%
Sept. 11, 1944			
IAA Standard	$1:9x10^6$	0	
	$1:27x10^6$	0	
Original crude extract (also tested Sept 8, taken to be 0.04% IAA	1:500	14	0.04%
	1:2500	Trace	
	1:12,500	0	
Crude acid fraction (first part equal 4.81 gm.)	$1:5x10^4$	>10	
	$1:25x10^4$	7	10%
	$1:125x10^4$	0	
Residual corn kernels (about 100kg.)	1:15	14	0.0012%
	1:75	0	
	1:225	0	
Sept 13, 1944			
IAA Standard	$1:9x10^6$	3	
	$1:27x10^6$	0	
Residual crude extract after 2 extns with Et_2O	1:500	0	Inactive
	1:2500	0	
	1:12500		

Date and Material Being Tested	Dilution	Mean Curvature Degrees	%-age IAA
Sept 18, 1944			
IAA Standard	$1:3x10^6$	8	
	$1:9x10^6$	< 8	
Crude acid fraction (10.52 gm)	$1:5x10^4$	> 10	10.4%
	$1:25x10^4$	10	
	$1:125x10^4$	0	
Sept. 20, 1944			
IAA Standard	$1:3x10^6$	8	
	$1:9x10^6$	0	
Crude acid fraction heated with 1 N. KOH	$1:5x10^4$	14	10%
	$1:25x10^4$	10	
	$1:125x10^4$	0	
Crude acid fraction control (untreated)	$1:5x10^4$	12	8%
	$1:25x10^4$	8	
	$1:125x10^4$	0	
Synthetic IAA heated with 5% HCl	$1:25x10^4$	9	52%
	$1:125x10^4$	10	
	$1:625x10^4$	trace	
Synthetic IAA heated with 1 N. KOH	$1:50x10^4$	12	100%
	$1:250x10^4$	8	
	$1:1250x10^4$	0	
Sept 27, 1944			
IAA Standard	$1:3x10^6$	14	
	$1:9x10^6$	5	
Active extract from residual corn kernels (1 gm. = 1060 gm kernels.	$1:25x10^4$	8	4.4%
	$1:125x10^4$	0	
	$1:625x10^4$	0	
Control (Untreated)			
Same extract as above heated with 5% HCl	$1:25x10^4$	0	Inactive
	$1:125x10^4$	0	
	$1:625x10^4$	0	
Same extract as above heated with 1 N. KOH	$1:25x10^4$	10	5.5%
	$1:125x10^4$	0	
	$1:625x10^4$	0	

Date and Material Being Tested	Dilution	Mean Curvature Degrees	%-age IAA
Oct 2, 1944			
IAA Standard	$1:3x10^6$	6	
	$1:9x10^6$	0	
Crude acid fraction	$1:10^4$	22	
	$1:5x10^4$	20	
	$1:25x10^4$	7	9.7%
Oct 6, 1944			
IAA Standard	$1:3x10^6$	13	
	$1:3x10^6$	13	
	$1:9x10^5$	8	
	$1:9x10^6$	10	
Crude acid fraction	$1:10^4$	ca 10	
	$1:5x10^4$	ca 10	
	$1:25x10^4$	14	9.0%
	$1:125x10^4$	trace	
Oct 13, 1944			
IAA Standard	$1:3x10^6$	13	
	$1:3x10^6$	14	
	$1:9x10^6$	4	
Benzene-methanol fraction (1.25 gm.)	$1:10^6$	8	20.5%
	$1:3x10^6$	trace	
Nov 8, 1944			
IAA Stnadard	$1:3x10^6$	19	
	$1:9x10^6$	11	
Xylene insoluble fraction (83mg.)	$1:5x10^4$	0	Inactive
	$1:25x10^4$	0	
	$1:10^6$	0	
Crystalline material from cyclohexane extracts	$1:5x10^4$	Max. angle	
	$1:25x10^4$	"	
	$1:10^6$	19	33%
Xylene soluble fraction (328 mg)	$1:50x10^4$	Max. amgle	
	$1:10^6$	17	
	$1:3x10^6$	7	21%

Date and Material Being Tested	Dilution	Mean Curvature Degrees	%-age found
Nov 13, 1944			
IAA Standard	$1:3\times10^6$	26	
	$1:9\times10^6$	7	
	$1:27\times10^6$	4	
Ppt. from first Pb pptn (123 mg.)	$1:10^6$	>10	
	$1:3\times10^6$	8	38%
	$1:9\times10^6$	0	
Filtrate from first Pb pptn. (118 mg.)	$1:10^6$	14	
	$1:3\times10^6$	7	33%
	$1:9\times10^6$	trace	

Conclusion

Actually there were two prime objectives in view in undertaking this present work.

The most immediate of these was to conclusively identify an auxin which occurs in a readily water-soluble, biologically active form in immature corn kernels. This has been accomplished by the isolation and identification just described. Thus the total available data concerning the distribution and identity of auxin in the corn plant is as follows:

Part of Plant	Auxin Present	Method of Characterization
Tip of young plant	auxin a	Diffusion Acid-alkali
Immature kernels (free portion)	IAA	Isolation
Corn Meal (free portion)	auxin a	Light inactivation
	auxin a or b	Diffusion
Dormant kernels or corn meal (bound portion)	IAA and Auxin a / IAA	Isolation
		Isolation and Acid-alkali
Corn oil	Auxin a and b	Isolation

From this information the reader may wonder why IAA did not appear in the course of Kögl's work on corn oil or why the isolations from mature corn or corn meal did not yield auxin a and b.

In the latter work already discussed evidence was found for the existence of auxin a in corn meal by the

isolation of a small amount of a substance which had the correct melting point for pseudo-auxin a.

In the work of Kögl one of the obvious points in the isolation procedures to examine for clues for the presence of IAA is the lactonization step. This was carried out in hot 1.5% HCl in MeOH and here it might be expected that any IAA present would be largely destroyed although the stability of IAA under these donditions is not known with certainty. In the first isolation from corn oil, for example, the active fraction before lactonization contained 55 mg. of auxin and afterwards apparently 28 mg. from which 7.4 mg. of crystalline auxin b was finally obtained. This apparent decrease in activity during lactonization does not occur with the concentrates from urine which did not yield auxin b.and can be satisfactorily explained by the formation of auxin b dimethyl acetal lactone which is much less active than auxin b.

In a second isolation from corn oil containing presumably 70 mg. of auxin, 15 mg. of crystalline auxin a and 15 mg. of auxin b were obtained. This is a very excellent yield even if all the auxin in the starting material were auxin a and auxin b.

In the purification of auxin from malt the activity practically disappeared on lactonization but reap-

peared almost entirely on distillation. The purification steps employed in all of Kögl's isolations were such that probably an appreciable amount of IAA would be carried along with auxin a and b at least to the lactonization stage if it had been present in the starting material.

These considerations are complicated by the possible presence of inhibitory substances. In the work of Kögl there was evidence for these only in the case of corn oil. An isolation was begun with 16 kg. of corn oil containing 700 gammas of auxin per kg. This was extracted with H_2O and the water extracted with $Et_2\phi$. The resulting fraction contained 130 mg. of auxin, an apparent 10 fold increase in auxin content.

Similar evidence of an inhibitor in immature corn kernels has already been pointed out; it is evidently non-acidic since it was not present in the crude acid fraction.

It is interesting to note that both the malt and the corn oil used by Kögl were probably rather fresh samples or else the auxin would have already disappeared by rearrangement. Moreover, the auxin in both these materials was probably present in a free form since it was extracted by simple water extraction.

The possibility that the immature corn used in the present isolation contained auxin a and/or b is not conclusively ruled out by the foregoing data, but the evidence indicates that if present at all, probably they are present in only low concentrations.

About 70 kg. of the corn used in this isolation was harvested on Aug. 15, 1944, and about 30 kg. on Sept. 1, 1944. The crude acid fraction was first characterized by the acid-alkali test on Sept. 18, presumably before any auxin a or b could have completely rearranged to the inactive pseudo-auxin.

The fact that no destruction occurred in alkali indicates one of the following situations:

(1) Auxin a and/or b are present but are stabilized by some other component of the mixture.

(2) Auxin a and/or b are present along with an inhibitor, which itself is an acid and which is destroyed by alkali in an amount biologically equivalent to the auxin destroyed.

(3) Auxin a and/or b are not present in appreciable concentrations.

To the author the last is by far the most likely conclusion.

The second objective of this work was to attempt to apply the information gained experimentally to shed additional light on the identity of the native plant growth hormone. As we have just seen in the case of corn, this problem is very complex. About the most we can say in a general way is that if auxin a or b or 3-indoleacetic acid are found in a locality of a plant where the corresponding compound has been proven to exert a specific effect, then the auxin found is probably responsible for this effect in the intact plant. In the case of the corn plant, 3-indoleacetic acid has been found in immature kernels, a rapidly metabolizing tissue. Inasmuch as the role, if any, of the growth hormone in maturing seeds has not been investigated, the significance of 3-indoleacetic acid in corn kernels is at present not known, and we must await further physiological findings to be able to correctly interpret these data.

Summary

Summary

The following summarizes the main results obtained in the foregoing work.

(1) An auxin occurring in a water-soluble and biologically active form in the kernels of immature corn has been proven to be 3-indoleacetic acid by isolation and comparison with an authentic sample of 3-indoleacetic acid.

(2) The concentration of auxin in these corn kernels was found to be equivalent to 2.7×10^{-5} gm. 3-indoleacetic acid per gram of fresh corn.

(3) The validity of the acid-alkali test has been confirmed for a mixture containing about 10% 3-indoleacetic acid.

(4) An isolation procedure for 3-indoleacetic acid (based on the results of previous workers) has been devised. This procedure possibly can be applied to a variety of plant materials with only minor alterations.

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