OBSERVATIONS ON THE MECHANISM OF AUXIN FORMATION IN PLANT TISSUES

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(WITH TWO FIGURES)

Received April 10, 1948

Auxin physiologists often have attempted to establish correlations between the amount of auxin in a plant tissue and the manifestation of auxin activity such as growth, rooting, parthenocarpy, bud inhibition, etc. With Avena coleoptiles and tomato stem tips, to use specific examples, it is possible to demonstrate a correlation between growth and the amount of auxin which can be diffused from the tissue into agar. Many tissues produce too little diffusible auxin or are too cumbersome to lend themselves to this type of auxin analysis and resort must be made to more convenient methods of auxin extraction. Ether extraction has been used extensively in correlation studies, particularly since ether was considered to remove the free auxin. It soon became evident, however, that auxin was liberated during the extraction and that many months of successive ether extractions were required before auxin production disappeared. While there often appeared to be a correlation between the amount of auxin obtained in the first ether extraction and the growth process under consideration, the correlation usually disappeared as the extraction process was prolonged.

THIMANN and SKOOG (8) made an extensive study of the continued production of auxin during the extraction of fresh, green tissues with ether and came to the following conclusions: 1) Many successive ether extractions spaced over several months were required to extract all of the potentially ether soluble auxin contained in Lemna tissue. 2) Water was necessary for auxin production during ether extraction since oven-dried tissue failed to give off auxin when extracted with anhydrous ether. 3) Drying not only prevented auxin production but appeared to fix added auxin in some form not extractable with ether. 4) Addition of proteolytic enzymes to the tissue prior to ether extraction greatly increased the total amount of auxin which could be extracted but did not shorten the time appreciably nor the number of extractions required for total auxin extraction. They concluded that the continued production of auxin in ether was caused by the slow, enzymatic liberation of auxin from an inactive form, presumably a protein.

GUSTAFSON (4) has developed a method for the extraction of "free" auxin, or auxin which is present in the tissue as such but does not arise by liberation during the extraction process. The tissue is frozen, ground and then boiled with water before extraction with ether. Boiling prevents the continued production of auxin encountered in previous methods. While

the method has proven quite satisfactory for some tissues, in others boiling evidently liberates some material which inhibits the action of auxin in the Avena test (**9**).

Recent work has shown that spinach leaves contain an enzyme which rapidly converts tryptophan to auxin (**10**). Because of the existence of such an enzyme, it is possible that one of the reasons for the slow production of auxin during ether extraction is the failure to inactivate this enzyme prior to extraction. The present investigation is therefore an attempt to associate the auxin production process with some of the known properties of the tryptophan-auxin converting enzymes and to examine the possibility of inactivating the enzymes by less drastic means than used previously thus enabling one to measure "free" auxin with confidence. Fertilized tobacco ovaries are advantageous for this kind of study because large amounts of auxin can be diffused into agar from them (**6**) and thus free auxin and diffusible auxin can be compared. Such tissues can be obtained in reasonably large quantities in contrast to other tissue such as Avena or corn coleoptiles from which auxin can be obtained by diffusion.

Methods and materials

PREPARATION OF PLANT MATERIALS.—Since the principal aim of this work was to investigate the nature of free auxin and its origin in plant tissues, it was necessary that the free auxin should be immobilized completely at the time of sampling and maintained in an immobilized condition during subsequent extraction procedures. Hence, fertilized tobacco ovaries were dissected from the flowers 80–100 hours after pollination and plunged immediately into liquid air for rapid freezing. They were then dried *in vacuo* in the frozen state in a Campbell-Pressman lyophil apparatus (**2**), a method closely similar to that used by LINK, EGGERS and MOULTON (**5**) for the preparation of plant material for auxin analysis. When dry, the ovaries were ground to 40 mesh in a Wiley mill and then stored *in vacuo* over P_2O_5 in darkness until used as tissue for auxin analysis or as enzyme preparations.

ETHER EXTRACTION.—Weighed samples of dried ovarian tissue were placed in 150-ml. Erlenmeyer flasks and covered with 75 ml. of freshly-distilled, peroxide-free ether, unless otherwise indicated. Such ether has a water content of about 5%. The samples were occasionally shaken. At the end of the extraction period the ether was decanted from the plant tissue, the sample was washed twice with small portions of fresh ether, and the combined ether extracts were reduced in volume to a few milliliters which were then quantitatively transferred to melted agar which was used for Avena assay.

AVENA DETERMINATION.—The methods used in this investigation were exactly as described previously (**10**). Auxin was applied to individual oat coleoptiles in 8.76 $mm.^3$ agar blocks. Results compared on the basis of curvatures were either obtained from the same Avena experiment or the necessary sensitivity values are given.

Experimental results

DIFFUSION AND EXTRACTION OF AUXIN FROM FERTILIZED TOBACCO OVARIES.— As previously demonstrated (**6**) large quantities of auxin can be diffused out of fertilized tobacco ovaries into agar. Eighty-five hours after pollination, twelve ovaries were excised from the flowers and the basal cut surfaces were placed on 8.76 mm.^{3} agar blocks for four hours. When these blocks were tested for auxin activity, an average of 21.2° curvature was produced as shown in table I. The amount of diffusible auxin was compared with the

TABLE I

COMPARISON OF THE AMOUNTS OF AUXIN OBTAINED FROM TOBACCO OVARIES BY VARIOUS METHODS

METHOD	MG. DRY WEIGHT	NO. OF OVARIES	CURVATURE	ML. OF AGAR	SENSITIVITY 50 γ IAA/LITER	TOTAL CURVATURE PER OVARY
Fresh ovary diffused on agar for 4 hours	4	1	21.2 ± 1.8	8.76×10^{-3}	11.8	21.2
Lyophilized tissue extracted 1 hour at 23° C. with dry ether	20	5	0.0	0.4	11.8	<5.0*
Lyophilized tissue extracted 1 hour at 0° C. with wet ether	20	5	0.0	0.4	11.8	<5.0*
Lyophilized tissue extracted 1 hour at 23° C. with wet ether	20	5	6.3 ± 0.6	0.4	14.3	47.6
Lyophilized tissue extracted 12 hours at 23° C. with wet ether	20	5	17.8 ± 1.7	0.4	10.6	181
Lyophilized tissue incubated 3 hours with 1.0 mg. of tryptophan	10	2.5	9.4 ± 1.0	3.2	8.3	1953

* Minimum sensitivity of Avena test.

amount of auxin which could be extracted from the tissue by different ether treatments. The free auxin was immobilized by freezing in liquid air and the tissue was dried in the lyophil apparatus before ether extraction. As the data in table I indicate, no auxin was obtained from five ovaries by extraction, either with anhydrous ether for one hour at 23° C. or with freshly-distilled, "wet" ether when the tissue and solvent were kept at 0° C. for one hour. If the tissue and solvent with water were maintained at 23° C. however, auxin causing 6.3° curvature was obtained in one hour, and in 12 hours the auxin obtained was sufficient to produce 17.8° curvature. When the total amounts of auxin obtained per ovary were compared, it was found that the amount of auxin extracted in one hour at 23° C. actually exceeded the amount obtained by diffusion from the fresh ovary in four hours.

On the basis of the above data, it is evident that rapid auxin production occurs during ether extraction of lyophilized tobacco ovaries provided the

tissue is rewetted and a suitable temperature of extraction is used. The fact that the production of auxin could be prevented completely by keeping the tissue and solvent cold or by keeping them dry suggests at once that the process is enzymatic. It was of interest therefore to see if the production of auxin could be increased in the presence of excess tryptophan. That this is true is shown in table I. Lyophilized tobacco ovary tissue was incubated for three hours at 28° C. with 1.0 mg. of tryptophan in 3.0 ml. of phosphate buffer, pH 7.0. At the end of this period the digest was centrifuged free of cellular constituents, the supernatant liquid was acidified, and the auxin was extracted with ether (**10**). When tested by Avena assay, curvature equivalent to 1953° per ovary was obtained. Other experiments not presented here

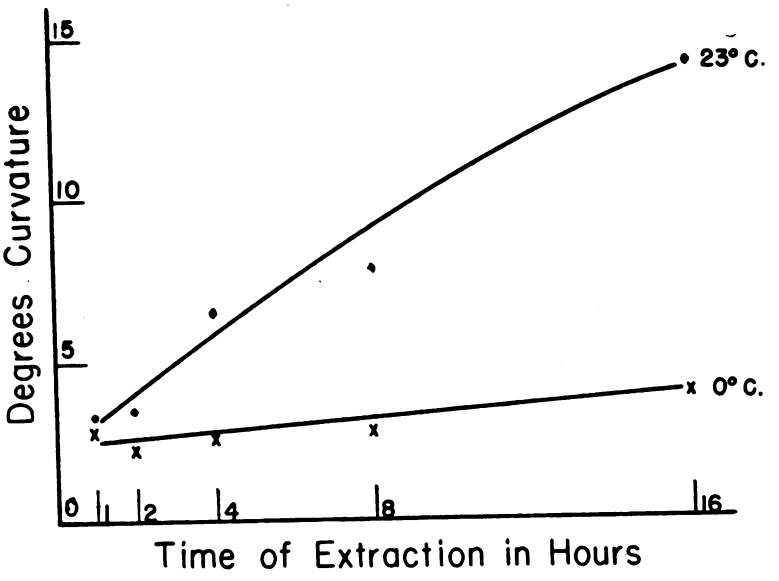

FIG. 1. The amount of auxin produced by wet ether extraction as effected by time and temperature of extraction. Twenty mg. samples of lyophilized tobacco ovaries.

have shown that boiling the tissue prior to adding tryptophan prevents the production of auxin. It can be concluded that tobacco ovaries contain a very active tryptophan-auxin converting enzyme which is not destroyed by the lyophilization procedure.

Because of the short time intervals involved in all of these experiments, it is exceedingly doubtful that the hydrolysis of auxin-protein complexes could account for the auxin produced. As a matter of fact, very drastic treatment such as heating for prolonged periods in the presence of alkali, or long incubation with proteolytic enzymes is necessary to release auxin from its bound form in spinach leaves (**11**). Another explanation of rapid auxin production must be considered and the following experiments have been designed to show that auxin production during ether extraction is a consequence of the enzymatic conversion of tryptophan to auxin.

TABLE II

RECOVERY OF AUXIN ADDED TO 10-MG. SAMPLES OF LYOPHILIZED TOBACCO OVARY TISSUE BY EXTRACTION WITH WET ETHER AT 0° C.

PERIOD OF EXTRACTION AT 0° C.	IAA ADDED IN DEGREES CURVATURE	IAA RECOVERED IN DEGREES CURVATURE	RECOVERY %
2 hours	5.1 ± 0.9	5.1 ± 1.0	100
3 hours	12.4 ± 1.1	10.3 ± 0.9	83

THE EFFECT OF TEMPERATURE ON THE EXTRACTION OF AUXIN.—The data presented graphically in figure 1 show that the production of auxin in lyophilized tobacco ovaries can be reduced to a very low value for periods up to 16 hours when the ether and tissue are kept at 0° C. In marked contrast to this result there is a continuous production of auxin during 16 hours when the extraction is conducted at 23° C. in the presence of water. Twenty milligram samples of tissue were extracted with freshly distilled ether for various lengths of time. Since a temperature of 0° C. will partially dry the ether by causing some water to separate, all of the ether used in this experiment was maintained overnight at 0° C. and then decanted from the small amount of water which separated by the cold treatment before the ether was used for auxin extraction.

It might be thought that the failure in obtaining auxin at the lower temperature was due to the insolubility of auxin in ether at 0° C. The data in table II show, however, that when small amounts of indole acetic acid

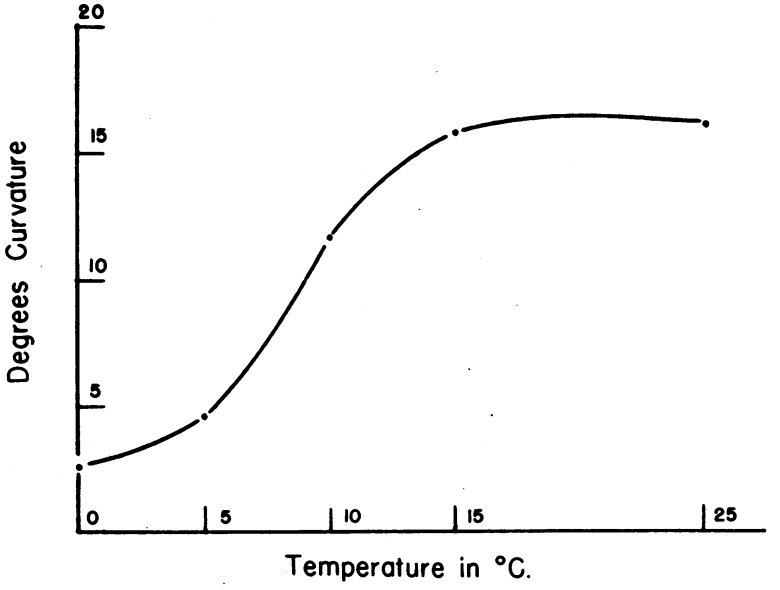

FIG. 2. The amount of auxin produced by wet ether extraction at different temperatures. Twenty mg. samples of lyophilized tobacco ovaries extracted 16 hours.

(IAA) were added to the tobacco tissue prior to extraction with ether at 0° C., the added auxin was quantitatively recovered by ether extraction.

The data plotted in figure 2 confirm the enzymatic nature of the auxin production process. Samples of tobacco ovary tissue weighing 20 mg. were extracted with ether for 16 hours at 0, 5, 10, 15 and 25° C. When the amount of auxin produced is plotted against temperature of extraction, the resulting curve approximates the data for typical enzymatic processes (**7**). For each 5° rise in temperature to 15° C. the amount of auxin produced is nearly doubled. At 15° C. an optimum production is attained. The low boiling point of the ether prevented an extension of this curve to higher temperatures at which it might be anticipated the auxin production would fall due to heat inactivation of the enzyme.

As a consequence of these temperature relationships it is possible to conclude that auxin production during ether extraction of lyophilized tissues resembles an enzymatically activated process.

THE EFFECT OF CYANIDE ON THE FORMATION OF AUXIN DURING ETHER EXTRACTION.—Small amounts of cyanide completely block the enzymatic conversion of tryptophan to auxin by spinach leaf breis (**10**). Since tobacco ovaries were also found to contain an active tryptophan-auxin converting enzyme, it was of interest to see if the production of auxin could be blocked when the tissue was extracted with ether in the presence of cyanide. There

TABLE III

EFFECT OF CYANIDE ON THE FORMATION OF AUXIN IN WET ETHER. 20-MG. SAMPLES OF LYOPHILIZED TOBACCO OVARY TISSUE EXTRACTED 16 HOURS AT 23° C. WITH ETHER IN THE PRESENCE OR ABSENCE OF CYANIDE. SODIUM CYANIDE DISSOLVED IN WATER AT SUCH A CONCENTRATION THAT 0.5 ML. WAS ADDED IMMEDIATELY BEFORE ETHER EXTRACTION

	MG. NaCN ADDED	IAA ADDED IN DEGREES CURVATURE	TOTAL AUXIN EXTRACTED IN DEGREES CURVATURE	APPARENT % INHIBITION OF AUXIN PRODUCTION	% INHIBITION OF THE AVENA ASSAY
Composite #1	0.0	0	16.0 ± 1.4	0	.....
" "	1.0	0	3.5 ± 0.6	78	.....
" "	1.0	17.5 ± 1.3	11.0 ± 1.2	.....	57
Composite #2	0.0	0	9.5 ± 0.8	0	.....
" "	0.5	0	4.2 ± 0.5	56	.....
" "	0.5	12.9 ± 0.8	11.8 ± 1.1	.....	41
" "	0.25	0	4.8 ± 0.7	50	.....
" "	0.25	12.9 ± 0.8	15.8 ± 1.4	.....	15

are certain difficulties attached to the use of cyanide in this type of experiment which need to be mentioned. Both HCN and NaCN are soluble in ether. While it is a simple matter to get rid of HCN by evaporation since it boils at a temperature below the boiling point of ether, this is not the case with NaCN and there is always the danger that this poison will be carried over into the agar used for the Avena test and thus prevent the coleoptiles from reacting to the auxin. The pH of the plant tissues rewetted with water

from the ether is about 6.3 and it is to be expected that both HCN and NaCN will be present in the ether extracts at this hydrogen-ion concentration. To ensure that a reduction in auxin production was a result of inactivation of the enzyme rather than the inhibition of the Avena assay, IAA was added in the presence of cyanide to all of the tissues involved so that it is possible to estimate the degree of inhibition of the enzymatic production of auxin during ether extraction.

Two different lots of lyophilized tobacco ovaries were used in these experiments. Samples of tissue weighing 20 mg. each were extracted for 16 hours at 23° C. The data are presented in table III. The auxin obtained from the tissue in the absence of cyanide produced 16.0° curvature. In the presence of 1.0 mg. of NaCN auxin production was reduced to 3.5° curvature. Since the apparent auxin production was inhibited 78% by this amount of

TABLE IV

EXTRACTION OF AUXIN FROM VARIOUS PLANT TISSUES BY ETHER. EXTRACTION PERIOD OF 16 HOURS

TISSUE	MG. DRY WEIGHT	TEMPERATURE DEGREES C.	ML. OF AGAR	CURVATURE
Avena coleoptile tips	10	0	0.25	7.4 ± 0.5
" " "	10	23	0.25	13.2 ± 0.9
Tomato stem tips	20	0	0.25	3.2 ± 0.7
" " "	20	23	0.25	9.2 ± 0.8
Carrot root	20	0	0.30	0
" "	20	23	0.30	2.5 ± 0.8
Corn coleoptile tips	10	0	0.30	6.3 ± 0.3
" " "	10	23	0.30	8.8 ± 0.6

cyanide and the Avena assay was inhibited only 57%, it is evident that an enzymatic blocking or inactivation had occurred in the auxin production process. When the experiment was repeated with a second lot of ovaries and smaller amounts of cyanide it was more evident that the cyanide prevented the formation of auxin. The extraction of 20 mg. of tissue without cyanide yielded sufficient auxin to produce 9.5° curvature but in the presence of 0.5 mg. or 0.25 mg. of NaCN the yield of auxin was reduced 50%. With the higher concentration of cyanide there was 41% inhibition of the Avena assay but with the lower concentration the inhibition was only 15%. The effect of cyanide on the production of auxin during ether extraction bears a striking resemblance to the effect of cyanide on the tryptophan-auxin converting enzyme of spinach leaves. This fact, coupled with the rapidity of the formation process as a function of the temperature of extraction, strongly suggests that auxin production results from the enzymatic conversion of tryptophan.

ETHER EXTRACTION OF OTHER LYOPHILIZED PLANT TISSUES.—That rapid auxin production during ether extraction is not limited to tobacco ovaries is shown by the data in table IV. Etiolated corn and Avena coleoptile tip

tissue, green tomato stem tip tissue, and carrot root tissue were prepared in exactly the same way as the tobacco ovary tissue. Although the levels of free auxin, as indicated by the 0° C. extraction, were much higher for the etiolated tissues, in all cases more auxin was produced in a 16-hour ether extraction period at 23° C. than in an identical period at 0° C. It is probable that the same mechanism operative in tobacco ovaries also accounts for the production of auxin in the other tissues.

Discussion

On the basis of these experiments which show that rapid auxin production in lyophilized tissues is probably due to the enzymatic conversion of tryptophan to auxin, it is possible to suggest a method for the determination of free auxin with confidence that this is actually the auxin state being determined. Obviously, the tryptophan-auxin enzyme must be inactivated at the time of sampling and kept inactivated during the extraction with ether. The preferable method is rapid freezing of the tissue followed by drying in the lyophil apparatus and ether extraction conducted at 0° C. for periods up to 16 hours. This treatment causes the least possible change in the tissue. Boiling the tissue in order to inactivate the enzyme is unsatisfactory because of the danger of liberating inhibitors of the Avena test (**9**) and, as will be shown in a future publication, at least 15 minutes at 100° C. is necessary to inactivate completely the tryptophan-auxin converting enzyme in Avena coleoptiles.

These results help to explain the difficulties encountered previously in auxin extraction, and also substantiate a previous hypothesis (**10**) that tryptophan is a principal precursor of auxin in plants. There is no experimental evidence which suggests that plants contain enzymes capable of liberating auxin rapidly from the various bound-auxin complexes so far investigated (**1, 11**). Free tryptophan in the presence of the tryptophan-auxin converting enzyme now demonstrated in many diverse plant species, is converted to auxin, and in this sense the tryptophan-converting enzyme must necessarily be responsible for the formation of auxin.

Summary

1. Lyophilized tobacco ovaries produce auxin during ether extraction in the presence of water at 23° C. but not at 0° C. The process is shown to be enzymatic.

2. Tobacco ovaries contain an active tryptophan-auxin converting enzyme. Production of auxin during ether extraction resembles the enzymatic conversion of tryptophan to auxin as both processes are blocked by cyanide.

3. Several other plant tissues appear to produce auxin by the same mechanism found in tobacco ovaries.

4. A simple method for the extraction of free auxin is proposed.

5. The evidence presented supports the view that tryptophan is a principal precursor of auxin in plants.

This work was partly supported by a grant from the American Cancer Society. We are greatly indebted to Miss Martha Kent for assistance with the Avena tests.

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