FREE AUXINS AND FREE TRYPTOPHANE IN THE STRAWBERRY[1,2]

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It has been previously demonstrated (15) that the growth of the strawberry receptacle is regulated by the achenes. This control is exerted through substances which are active in the Avena test, a standard test for auxins. The exact identity of these natural auxins, however, had not been investigated. The purpose of this paper is to report further experiments on the production of auxins by strawberry achenes, on the level of free tryptophane, a generally presumed raw material in the genesis of natural indolic auxins, and on the separation of the auxins of the strawberry by paper chromatography.

MATERIAL AND METHODS

Strawberries, var. Marshall,[3] were grown in pots in the greenhouse and hand pollinated. The fruits were removed at three day intervals during a period extending from 3 to 30 days after pollination, at which time the berries were ripe. Upon picking, the fruits were immediately brought to a cold room (5° C), the achenes were separated from the receptacles, both fractions were frozen by placing in a deep-freeze, and the frozen material was lyophilized. The lyophilized samples were used for auxin and tryptophane determinations.

Auxins were extracted with peroxide-free ether (water saturated) alone, or with a mixture of one part of acetone and two parts of ether (V/V) in an ice-bath at 0° C, in the dark, for 2 to 4 hours. It is generally agreed that such a procedure yields only "free" auxin, by contrast to treatments with alkali, proteolytic enzymes, or merely prolonged incubation at room temperature (29).

"Free" tryptophane was extracted according to the procedure of Dr. J. H. M. Henderson (unpublished) which consists of two steps: (1) the precipitation of proteins by boiling ethanol for 3 minutes, followed by the evaporation of the alcohol on a steam bath; (2) the extraction of tryptophane with hot water (repeated twice). The aqueous extract thus obtained was shaken with ether at pH 4.0 to purify it from possible traces of indole and anthranilic acid which give a positive test in the bioassay used. Then, the pH of the aqueous extract was adjusted to 6.0, and its tryptophane content measured quantitatively by the Lactobacillus assay (16, 17), a procedure which (in the absence of indole and anthranilic acid) is remarkably specific for L-tryptophane, as recently confirmed by Stowe (22).

The chromatographic techniques used here for auxins were similar to those of several workers in this field. Ascending chromatography on 2 cm wide strips of Whatman No. 1 paper was chosen; the solvent was that recommended by Stowe (22), i.e., isopropanol - 28 % ammonia - water (8:1:1, V/V), and the bioassay was that used by Kefford (8). An improvement of the latter technique was achieved as follows. A search made for hulless oats to avoid the tedious task of dehusking the thousands of seeds necessary for the bioassays revealed that at least two varieties, Laurel and Brighton, were suitable. The latter variety[4] was used throughout the course of this work. It was also tested in the standard Avena test in which the var. Victory has been used for years. The var. Brighton gave a satisfactory Avena test. For the straight growth bioassay, seeds were soaked for two hours in water and, then, laid down on wet facial tissue through which roots can grow freely (which is not the case with filter paper). The seeds were placed for about 4 hours at approximately 35 cm under a 32 watt G.E. white fluorescent "Circline" light filtered through a Corning filter No. 2404, to suppress growth of the mesocotyl. After the red light treatment, the seedlings were grown in a darkroom at 25° C and 85 % relative humidity. When the coleoptiles reached about 2.5 cm in length, the first 3 mm from the tip were cut off and discarded, while the next 4 mm were saved for the assay. Four mm rather than 10 mm sections were used to eliminate cells which had already elongated and to minimize geotropic curvatures (5). Sectioning was done with the van der Weij guillotine (26) which allows a precision of $\pm$ 0.1 mm. Only one 4 mm section was cut from each coleoptile. The primary leaf was left inside the sections to simplify the procedure and avoid injuring the coleoptiles (5). The sections were randomly distributed and floated on the solutions to be assayed. Kefford's technique was refined in two ways. Firstly, only 0.5 ml of total solution was used in each dish containing the section of the chromatogram. Such an amount of solution gives as good results as 1 ml (5). Secondly, in order to avoid growth variations due to pH effects, a phosphate-citrate buffer was used to maintain the pH of the solutions at 5.0. The composition of the medium was as follows: citric acid monohydrate (1.019 gm/l), K_2HPO_4 (1.794 gm/l), and sucrose (30 gm/l). Ten 4 mm sections were placed in each dish and left in the darkroom at 25° C for about 20 hours. They were then measured either with a plastic ruler to the nearest 0.5 mm or under a binocular with an ocular micrometer to the nearest 0.1 mm. Repeated measurements have shown that the accuracy and re-

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[4] Kindly supplied by the Canadian Department of Agriculture, Central Experimental Farm, Ottawa, Canada.

producibility of this technique are satisfactory, the upper limit of the absolute error made on the mean of ten measurements being 0.25 mm with the plastic ruler and the standard deviation being ordinarily around 0.2 mm.

Results

The Free Auxin Content of Strawberry Achenes as Measured by the Avena Test: The ether extractable auxin after 2 to 4 hours at 0° C was measured quantitatively by the Avena test. From 10 to 100 mg (dry weight) of achenes were used on each extraction. The crude ether extracts were incorporated into agar blocks for the curvature test. At the same time, blocks with synthetic indole-3-acetic acid (IAA) were prepared with the two standard concentrations of 20 and 40 μg per liter. Care was taken to dilute each unknown extract to ascertain that the auxin concentration was within the proportionality range. The mean of several tests was used to establish the final figure of the auxin content.

The results of these tests are plotted in the graph shown in figure 1. Figure 1 concerns the achenes only, since no auxin can be extracted in this way

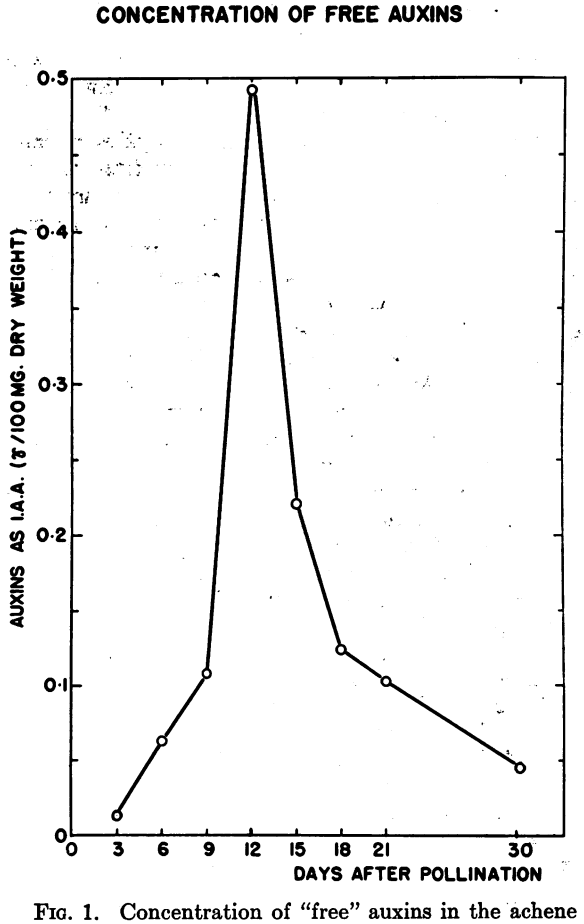

Fig. 1. Concentration of "free" auxins in the achene of the Marshall strawberry (measured by the standard Avena curvature test and expressed in IAA units).

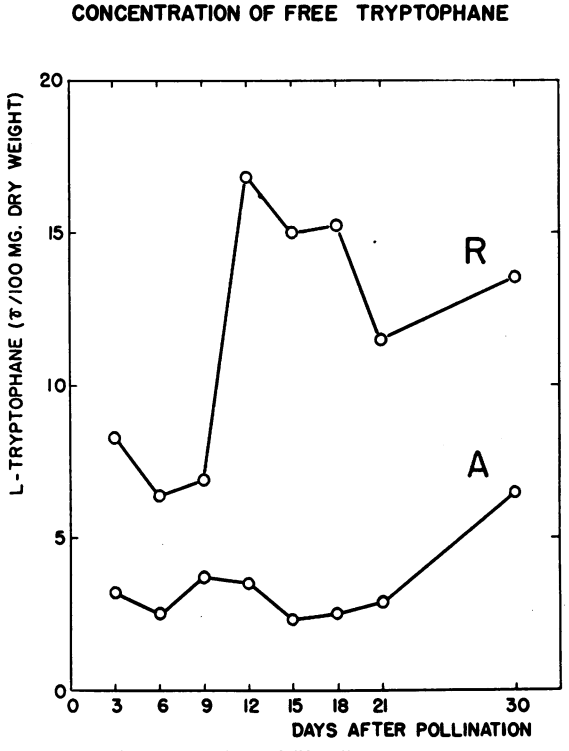

Fig. 2. Concentration of "free" L-tryptophane in the achenes (A) and the receptacles (R) of the Marshall strawberry.

from the receptacles (15). The prominent feature is the very steep peak occurring 12 days after pollination. It should be noted that the results are expressed on a concentration basis. If the total free auxin is computed per fruit, then the decline in the auxin content is less abrupt, since the weight of the achenes increases up to 30 days after pollination; but the sudden rise in the auxin level is just as steep. In the course of three days, the auxin concentration increases five fold, approaching the equivalent of 0.5 μg of IAA per 100 mg dry weight.

The Free Tryptophane Content of Strawberry Achenes and Receptacles: Preliminary determinations of free tryptophane in achenes and receptacles have been reported previously (16), but they concerned only three different stages of strawberry growth. A more complete survey has been made, using eight different samplings. The results, summarized by the graphs of figure 2, are presented on a concentration basis. At the first glance, one can see that the tryptophane level is higher in the receptacles than in the achenes. In fact, if the amount of free tryptophane per fruit is calculated, then the receptacle of a strawberry 12 days after pollination contains about 8 times more free tryptophane than all the achenes of the same fruit. In addition, it can be seen from the figure that the concentration of free tryptophane in the receptacles reaches a peak around 12 days after pollination, which is concomitant with the peak of auxin production in the achenes.

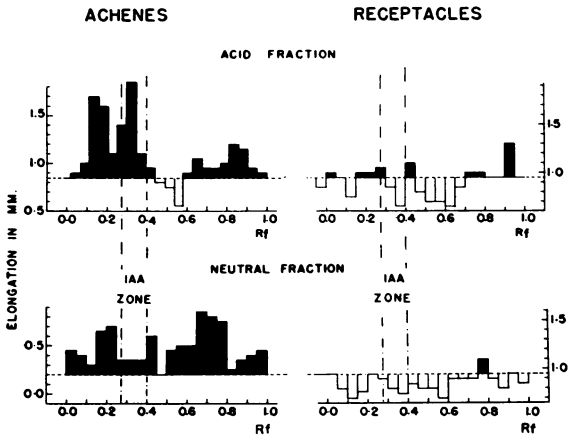

FIG. 3. Chromatographic separation of growth substances in achenes and receptacles of the Marshall strawberry, 12 days after pollination. (Note that the elongation scales do not start always at the same value. The dotted line represents the growth of the controls in each case. Black bars indicate growth promotion above growth of the controls, white bars growth inhibition. Variations in the order of 0.2 mm or less are not significant. 200–250 mg (dry wt) of material were extracted in each case.)

SEPARATION OF THE AUXINS OF THE STRAWBERRY: The Avena test which has been used both in a previous paper (15) and in the present one, to evaluate the amount of auxin in strawberry tissues, measures not so much the absolute amount of auxin as the balance between auxins and inhibitors. The negative results obtained previously with receptacle tissues may be due, perhaps, to the presence of an inhibitor masking an auxin which might, nevertheless, be there. Hence this first question: is there, in the receptacle, an inhibitor masking the presence of auxin or is there actually no auxin at all? A second problem follows: is there only one single auxin in the strawberry, or are there several?

In order to answer these questions, an attempt was made to separate the auxins and inhibitors present in the strawberry extracts by the use of paper chromatography. The results, shown on the diagrams of figure 3, show clearly that achene extracts, the neutral as well as the acid fraction, are growth promoting for coleoptiles sections as they are in the Avena test. On the other hand, extracts from receptacles show little or no growth promotion. Results of eight different chromatograms of extracts from receptacles have given erratic results with no consistent evidence for the presence of any particular auxin nor any particular inhibitor. In answer to the first question stated above, the results of the chromatograms agree with those of the Avena test and show that there is no appreciable free auxin nor inhibitor in the strawberry receptacles.

On the other hand, extracts of achenes have always been found to contain growth promoting substances. The second question as to whether there is one or several auxins in the achene extract can be easily answered. Figures 3, 4, and 5 demonstrate unquestionably that there are several auxins in the achene extract. The separation of these different auxins requires that the initial spot on the chromatogram is kept very small and that the chromatogram is cut into small sections (1 cm sections were used for a solvent front of 20 cm). It further requires that the extract has been sufficiently purified from fatty material which interfere with chromatography by not moving in the solvents used here and retaining a fraction of the auxins which are more soluble in fats than in aqueous solutions. To remove the fatty material, the technique of Jones and Riddick (7) was used. The ether extract was evaporated to dryness, then shaken with hexane and acetonitrile. Hexane removes fats while auxins of the IAA type remain in the acetonitrile. This fact was ascertained by shaking synthetic IAA, indole-3-acetonitrile (IAN) and indole-3-acetic ethyl ester (IAE) with hexane and acetonitrile: all three compounds remained in the acetonitrile fraction. After such a purification, the hexane was discarded, the acetonitrile fraction evaporated to dryness under reduced pressure, and the residue dissolved in a small volume of ether.

Another, even more effective purification technique was found to be a preliminary chromatography in glass-distilled water, the paper strips being equilibrated over the solvent for 17 hours before the actual run. When such a water chromatography is completed and the paper has been dried, the initial spot is cut off and discarded, and the rest of the chromatogram is re-extracted with ether. This extract, purified from colored material and fats, is then rechromatographed in the isopropanol-ammonia-water solvent. The total (acid + neutral) extract of 250 mg of achenes (12 days after pollination) corresponding to the diagram of figure 4, has been purified first by

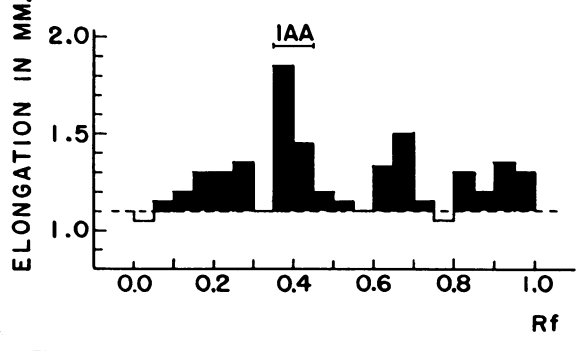

FIG. 4. Total extract of 250 mg (dry wt) of achenes (12 days after pollination) chromatographed in the isopropanol-ammonia-water solvent after purification through hexane and acetonitrile and a preliminary chromatography in distilled water. (The dotted line gives the growth of the controls. Black bars indicate growth promotion above controls. Each bar represents the mean of 10 sections. Differences under 0.2 mm are not significant.)

shaking with hexane and acetonitrile, then by preliminary chromatography in distilled water. Certain peaks are well separated.

Unfortunately, even then, certain auxins, especially near the ends of the chromatogram, are not well separated. For example, on the diagram of figure 4, it is hard to decide if the growth areas between Rfs 0.1 and 0.3, and Rfs 0.8 and 1.0 are due to one or more different substances. The differences between the growth responses are within the limits of the standard deviation from the mean. If, however, one pools the data of several different chromatograms, the picture becomes clearer. Although each individual experiment has its own errors and limitations, the successive growth peaks follow a common pattern. Figure 5 represents diagramatically the position of the various growth peaks of 11 different chromatograms of extracts of the same material (achenes, 12 days after pollination) in the same solvent. Although the extracts have been made in different ways and on different occasions (extending over a period of 5 months), one can recognize that certain peaks, even very inconspicuous ones, are found always in the same positions. They indicate the presence of several growth-promoting substances, of which the tentative list may be as follows:

Substance A: Rf around 0.1
Substance B: Rf around 0.23
Substance C: Rf around 0.35
Substance D: Rf around 0.5
Substance E: Rf around 0.7
Substance F: Rf around 0.83
Substance G: Rf around 0.95

There may be, possibly, another substance with Rf 0.6.

IDENTIFICATION OF THE AUXINS OF THE STRAWBERRY: The problem which we are facing now is the identification of the various substances which have been separated on the chromatogram.

Exposure to ultra-violet light failed to show any fluorescent compounds at the positions of the biologically active spots.

Color tests for indole compounds were more successful. One gram (dry weight) of 12-day-old achenes was extracted, and its acid fraction chromatographed. After spraying with a modified Salkowski reagent (4) diluted with an equal volume of ethanol, a definite pink spot (having the same shade of pink as synthetic IAA) appeared between Rfs 0.32 and 0.25, the center of the darkest area being located at Rf 0.29. The Rf of synthetic IAA chromatographed alone is 0.37, but it was found to be somewhat depressed by the addition of the achene extract. Another experiment, in which the extract of only 250 mg of the same material (acid fraction) was used, gave, with the Ehrlich reagent (2 % *p*-dimethylaminobenzaldehyde in 2 *N* HCl in 80 % alcohol), a purplish spot of the same color as synthetic IAA at the Rf 0.36. Thus, the Rf position and the color reactions with two different reagents agree in showing

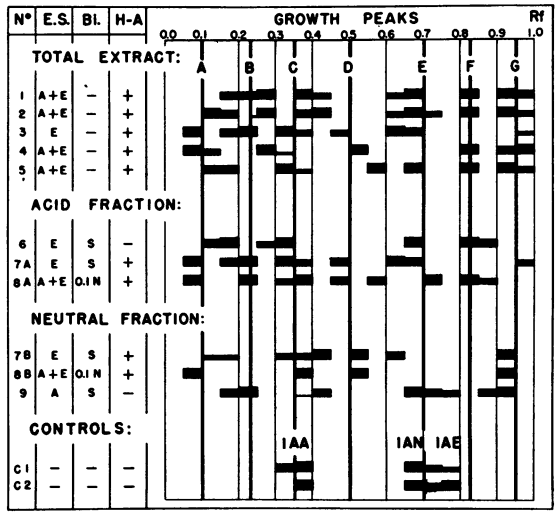

FIG. 5. The auxin spectrum in the strawberry achenes, 12 days after pollination (results of 11 different chromatograms in the isopropanol-ammonia-water solvent). *The 1st column* gives the number of the experiment: 7A and 7B mean that the same extract was used for the acid and the neutral fraction; C 1 gives the results of the chromatography of 0.1 microgram each of IAA, IAN and IAE using the bioassay; C 2 corresponds to the chromatography of IAA (2 μg), IAN (10 μg) and IAE (4 μg) in which the position of the auxins was located with the Ehrlich color reagent. *The 2nd column* gives the extracting solvent: ether (E), acetone (A). *The 3rd column* gives the concentration of the Na bicarbonate used: saturated (S) or 0.1 *N*. *The 4th column* indicates whether or not the extract has been purified by shaking with hexane and acetonitrile. *The location of the growth peaks* is represented as follows: high black rectangles show which segments of the chromatogram contain the maxima of the growth peaks; lower rectangles mark segments in which an appreciable fraction of growth (relative to the nearby peak) is located. The height of a rectangle is by no means proportional to the absolute amount of growth. The chart is only a qualitative one showing the position of the growth peaks, even the small ones.

that substance C, the most important quantatively of all the growth-promoting substances separated on the chromatogram, is IAA. From the size and the intensity of the colored spots, one could judge that the amount of IAA per gram of dry achene material was in the order of 1.5 to 2.0 micrograms. When measured by the Avena test, the total free auxin content of the same amount of material approaches the equivalent of 5 micrograms of IAA. This means that the natural IAA present in the achene extract accounts for only one-third to one-half of the growth-promoting activity. No other colored spot appeared; if other indole compounds were present, their amounts were too low to be detected colorimetrically.

In an effort to get a clue as to the identity of some of the other growth substances, chromatography

in other solvents was tried. Among about twenty different mixtures and combinations of solvents, distilled water was found to be the best, which confirms recent results by Sen and Leopold (19). Distilled water separates very well IAN and IAE which run very closely together in the isopropanol solvent. The paper strips were equilibrated overnight over the solvent as usual and double distilled water was used. Under these conditions, the Rfs which we consistently obtained were not those indicated by Sen and Leopold but the following ones: IAA (0.66), IAE (0.47), IAN (0.32). The extract of achenes 12 days after pollination, when chromatographed in water (after a preliminary chromatography in the isopropanol solvent), showed five growth peaks around the following Rfs: 0.15, 0.30, 0.45, 0.70, and 0.95. The second, third, and fourth may indicate the presence of IAN, IAE, and IAA in the extract.

Discussion

The data presented in figure 1 confirm the previous report on auxins in the strawberry (15), and substantiate the more general conclusion that the level of "free" auxins in developing fruits varies greatly from pollination to maturity. Such a variation has been reported in corn (1, 21, 30), in rye (6), in bean (14), and in the apple (10, 12). In most of these cases, a definite peak in the auxin concentration develops in the seeds at about mid-time between fertilization and maturation. The significance of this rapid rise is not completely elucidated, although it seems to be linked to events occurring in the endosperm. In the apple seed, in which Luckwill (10) described several peaks in auxin production, the same author noted a time correlation between the first and largest rise in the auxin level and the change of the endosperm from the free nuclear to the cellular stage. The second peak was concomitant with the development of a "secondary endosperm." It may be pertinent to mention here that cultures of corn endosperm in vitro produce exceptionally high amounts of IAA (1 mg per kg fresh weight), and indolepyruvic acid (22).

The role of free tryptophane as a precursor of auxins in the strawberry achene is not very clear. The concentration of this indolic amino acid in the receptacle, where apparently no auxin is generated, appears to be higher than in the achenes. Of course, the raw materials supplied by the leaves and the roots have to move through the receptacle to reach the achenes. It is possible that the tryptophane level in the achenes is low because of a constant drain on this amino acid to manufacture various compounds, among which would be the indolic auxins. Our knowledge on the genesis of these auxins is still confused. Tryptophane is probably not the immediate precursor of an auxin such as IAA. Several intermediates, including indole-pyruvic acid (IPA), recently demonstrated in corn kernels by Stowe and Thimann (23), may exist between the amino acid and the auxin.

To be able to draw a clear picture of the genesis of auxins, one should know their chemical identity. In the present work, a variety of growth-promoting substances present in the extract of 12-day-old strawberry achenes has been separated. The identification of these substances, however, has not always been possible. Only one substance, substance C, has been identified with IAA with reasonable certainty. Among the others, some are completely unknown, some have Rfs and biological properties similar to known auxins. The tentative list is as follows:

Substance A (Rf around 0.1): unknown.
Substance B (Rf around 0.23): possibly IPA.
Substance C (Rf around 0.35): IAA.
Substance D (Rf around 0.5): unknown.
Substance E (Rf around 0.7): possibly IAN with, perhaps, IAE (as indicated by the results of chromatography in water).
Substance F (Rf around 0.83): unknown.
Substance G (Rf around 0.95): unknown.

It may be seen from figure 5 that substances A, B, and C are generally found in the acid fraction, although traces of C (IAA) seem also to be present in the neutral fraction. Substance D may be found in both acid and neutral fractions. Substances E and F generally remain in the acid fraction, whereas substance G seems to move to the neutral one. The fact that E is carried from an ether solution into a bicarbonate aqueous solution does not preclude the fact that it might be IAN which, even at low concentrations, is very active on the straight growth test (3). The same phenomenon has been observed by Bennet-Clark and Kefford (2).

The presence in plant extracts of auxins different from IAA has been reported by many workers. For example, a growth-stimulating substance moving more slowly than IAA in an isopropanol-ammonia-water solvent has been reported by Kefford (8) in broad beans, Lexander (9) in wheat roots, von Denffer et al (27) in brussels sprouts, Weller et al (28) in cauliflower, Stowe and Thimann (23) in corn kernels. The latter authors identified their spot as being due to IPA, which gives a crimson color with the Salkowski reagent. Auxins moving faster than IAA in solvents similar to the isopropanol-ammonia-water one used in the present investigation have also been found in diverse materials: apple seed (11), crab-apple leaves (5), wheat roots (9), sweet corn kernels (24), cultures of Jerusalem artichoke (20), etc. The auxin in apple seeds found by Luckwill to have a Rf in the neighborhood of 0.83 has been identified by Teubner (25) as IAE, a natural auxin previously extracted from immature corn kernels by Redeman et al (18). The two substances extracted by Stowe and Thimann (24) from sweet corn and having the Rfs of 0.52 and 0.83 gave a positive Salkowski color test for indole compounds, but have not been identified. Neither have those in crab-apple leaves, wheat roots and Jerusalem artichoke cultures.

Thus, the presence in the strawberry of auxins

other than IAA is not an isolated fact. A few authors, especially Luckwill (13) and Stowe (22) have reported four different auxins in the same plant extract; the diagram given by Lexander (9) for wheat root extracts shows five growth peaks. In the present case, however, as many as seven different growth substances have been detected in one single organ. One must consider, however, that a strawberry achene is a complex of many different tissues: embryo, endosperm, nucellus, seed coat and carpellar tissue all are present 12 days after pollination. The complexity of tissues may be reflected in the variety of substances detected. One must bear in mind also that some of these various compounds may represent breakdown products of one initial substance. Finally, some of the compounds detected in the bioassay may only be auxin synergists, merely increasing the growth resulting from residual auxin present in the coleoptile tissues. With due regard to all these possible restrictions, the multiplicity of growth substances found in the present investigation may nevertheless indicate that not one, but a whole family of auxins are at work in plants, certain ones perhaps being transformed into others.

Summary

A more detailed quantitative evaluation of free auxins in strawberry achenes confirmed the existence of a very steep and narrow peak in auxin concentration around the 12th day after pollination. The level of free tryptophane in both achenes and receptacles has also been followed during the development of the fruit. Tryptophane concentration is higher in the receptacle than in the achenes. A peak in the level of free tryptophane in the receptacle occurs concomitantly with the maximum in free auxin concentration in the achenes. Chromatographic treatment of acid and neutral fractions, as well as of total extracts of both achenes and receptacles, separated up to seven different growth substances in the achene extracts. The most prominent of these substances has been indirectly identified with indole-3-acetic acid, while the exact chemical nature of the others is not yet established. No appreciable quantities of either auxins or inhibitors were detected in the ether extracts of receptacular tissues.

In the course of this work some technical improvements have been devised, especially in the purification of the plant extracts and in the bioassay.

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CHANGES IN NITROGEN FRACTIONS AND PROTEOLYTIC ENZYMES OF SOYBEAN PLANTS TREATED WITH 2,4-DICHLOROPHENOXYACETIC ACID[1,2]

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The pronounced effects of the growth regulator, 2,4-dichlorophenoxyacetic acid (2,4-D) on protein and other forms of nitrogen in plants have been noted by a considerable number of investigators. The authors have referred to some of these studies in reporting (7) a loss of protein nitrogen from the leaves and an accumulation of this nitrogen fraction in the stems and roots of soybean plants treated with 2,4-D.

Rebstock et al (11), working with red kidney bean seedlings after application of 2,4-D to the primary leaf, found greater proteolytic activity in 2,4-D treated than in control stems, but lower proteolytic activity in treated than in control leaves.

Studies of the effect of 2,4-D on certain other enzymes in plants have been summarized by Weintraub (17) and by Bonner and Bandurski (2).

In the experiment described below attempts were made to measure proteolytic activity in organs of plants in which the distribution of a number of nitrogen fractions was also studied before and after treatment of the plants with 2,4-D.

Growth of the plants in nutrient solutions from which nitrogen was omitted during and following treatment with 2,4-D made possible a study of the effects of treatment on nitrogen already absorbed and largely assimilated prior to exposure of the plants to 2,4-D.

MATERIALS AND METHODS

CULTURAL TREATMENT: Solution culture techniques described by Shive and Robbins (13) were followed in general, with nutrient solutions renewed intermittently at decreasing intervals as the plants increased in size. Lincoln soybean seedlings, after germination at 30° C and a short period of growth while held upon plastic netting, were transferred to one-gallon culture jars, three plants to each jar. The basic nutrient solution used until the plants were 32 days old and more than 14 cm tall was essentially formula I of Shive and Robbins (13) with respect to macronutrient elements, supplemented with micronutrient elements as follows: 1 ppm Fe, 0.25 ppm B, 0.5 ppm Zn, and 0.5 ppm Mn. When the plants were 32 days old, the level of Mn and Zn in the solutions was reduced to 0.125 ppm of each. At this time all $(NH_4)_2SO_4$ was omitted from the solution, which was also modified to include the following concentrations of salts: 0.002 M KH_2PO_4, 0.0005 M K_2SO_4, 0.002 M KNO_3, 0.0045 M $Ca(NO_3)_2$, and 0.002 M $MgSO_4$. The initial pH of this solution was 4.7. Later, during and following exposure to 2,4-D, the plants were grown in a minus-N nutrient solution modified from the above by the substitution of 0.0045 M $CaSO_4$ for the $Ca(NO_3)_2$, by the omission of all KNO_3, and by an increase in the concentration of K_2SO_4 to 0.0015 M.

2,4-D TREATMENT: When the plants were 39 days old and from 38 to 40 cm tall with six fully expanded trifoliate leaves, they were treated with sodium 2,4-dichlorophenoxyacetate (2,4-D). Treated plants were exposed for 24.5 to 25 hours to 5 ppm acid equivalent of 2,4-D dissolved in the above-mentioned minus-N solution adjusted to pH 4.0. Control plants were transferred to the minus-N solution at pH 4.0 without 2,4-D. Omission of nitrogen from the treatment solution prevented further absorption of nitrogen during exposure of the roots to 2,4-D and also minimized pH changes. The low pH level in the solution was used because of evidence (5, 6) that a greater response was obtained to 2,4-D dissolved in solutions of low pH.

At the end of the one-day exposure period, plants were removed from the solutions, their roots were rinsed with distilled water, and the plants were placed in fresh minus-N nutrient solution without 2,4-D. Control plants were harvested at the start of 2,4-D treatment, and treated and control plants were har-

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