Changes in Ascorbic Acid Metabolism Associated With Auxin-Induced Growth [1]

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Much attention has been directed recently to an interaction of auxin with certain oxidation-reduction systems of the cell in an attempt to explain the action of auxin (*2, 17, 20, 22, 24, 25, 26*). In general, good correlations have been found between the enhanced rate of tissue growth as induced by auxin and the effects of auxin on the reduction state of the ascorbic acid and sulfhydryl systems of these tissues. The major portion of that work (which was carried out by the Milan group) was conducted on excised pea stem tissue. In the present investigation the effect of several known auxins and related compounds on the concentration and reduction level of ascorbic acid in intact cucumber plants has been studied. The results of these studies show that growth promotion is associated with an increase in ascorbic acid and a more reduced state, and that growth inhibition is associated with a decrease in ascorbic acid and a more oxidized system.

Materials & Methods

Cucumber seeds (variety Improved Long Green) were germinated in moist sand; the seedlings were transplanted into a sandy loam soil (2 plants/4 inch pot) when the first leaf was about one-tenth expanded. The plants were grown in the greenhouse before being transferred into a constant environment room (80 C, 60 % relative humidity, & a day-length of 16 hr at a light intensity of ca. 600 ft-c) 1 to 3 days prior to treatment. When the second leaf was about one-half to two-thirds expanded, a solution of the designated chemical containing 0.05 % Vatsol-OT was sprayed onto the upper leaf surface to run off. All chemicals were applied as the potassium salt at pH 5.5. At the appropriate time three plants of each treatment were harvested in duplicate. Leaf tissue, 3 g, was taken from each sample for ascorbic acid analyses. The stem tissue samples consisted of the section taken between one-half inch above the soil surface and the cotyledonary node, including the petiole of leaf one.

For ascorbic acid and dehydroascorbic acid analyses the tissue was homogenized in 3.5 % ice-cold metaphosphoric acid in an ice-jacketed Virtis-45 homogenizer for 0.5 minutes (approx. 3 g of stem tissue/20 ml of solution & 3 g of leaf tissue/40 ml of solution). The extract was filtered through glass wool; the filtrate was then centrifuged for 10 minutes at 3,000 × *g*. The supernatant solution was then used for quantitative determination of ascorbic acid and dehydroascorbic acid by the method of Hughes (13).

In experiments where enzymatic activity of tissue homogenates was to be measured, the tissue was sampled in the same manner as for ascorbic acid analysis. For assays of ascorbic acid oxidase 5-g samples of stem tissue were homogenized in ice-cold 0.05 M potassium phosphate buffer (pH 5.8) containing 2×10^{-3} M ethylenediaminetetraacetate (EDTA). The homogenate was filtered through glass wool, and aliquots of the filtrate were added directly to Warburg flasks containing 10 mg of ascorbic acid (pH 5.8). Standard Warburg respirometer procedures were followed in measuring oxygen uptake over three 10-minute intervals at 25 C.

For determining glucose-6-phosphate dehydrogenase activity the samples of plant tissue were homogenized in ice-cold 2.5×10^{-3} M tris chloride buffer (pH 7.5). The homogenate was then filtered through glass wool and appropriate aliquots used in the assay. The assay mixture consisted of 10 μmoles TPN, 10 μmoles glucose-6-phosphate, 30 μmoles $MgCl_2$, 100 μmoles tris chloride buffer at pH 7.5, and 0.5 ml of homogenate in a total volume of 3 ml. The assay was run for 5 minutes at 30 C; TPNH production was measured by absorption increase at 340 mμ in a Beckman DU Spectrophotometer. The activity was completely dependent upon added TPN and 75 to 80 % dependent upon added glucose-6-phosphate. Production of TPNH probably was the result of both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities since both enzymes produce TPNH in the reaction sequence from glucose-6-phosphate to pentose phosphates.

Protein analyses were made by the method of Lowery et al. (19).

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[3] The abbreviations used are: IAA, indole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; TPN, triphosphopyridine nucleotide; 2,4,6-T, 2,4,6-trichlorophenoxyacetic acid; 2,4,5-TP, 2,4,5-trichlorophenoxy-α-propionic acid.

Results

In the experiments reported here the relationship of the relative activity of several auxins and related compounds to alterations in ascorbic acid metabolism was assessed. IAA, 2,4-D and 2,4,5-T were tested at 5×10^{-5}, 5×10^{-4}, 2.5×10^{-3}, and 5×10^{-3} M concentrations. The effectiveness of these compounds in altering the growth pattern of the cucumber plant and in producing changes in ascorbic acid was compared because the cucumber plant is able to break down these three compounds at differential rates (33 & unpublished observations). In effect the concentrations of 2,4,5-T, 2,4-D, and IAA required to produce the same overall morphological and growth response are in the order of 1:10:100, respectively. The growth aberrations produced by treatment of plants with any one of the auxins (IAA, 2,4-D, & 2,4,5-T) were those which have previously been described (6, 9, 10, 32). In general, stem growth, although abnormal, was promoted (table I). This growth consisted of lateral expansion or swelling during the first 24 hours after treatment followed by proliferation of the stem tissue. After about three days, stems of plants treated with 5×10^{-4} to 5×10^{-3} M 2,4-D (or any concentration of 2,4,5-T used) failed to show any additional increase in fresh weight. This cessation of growth possibly results from the fact that the proliferative growth which had already taken place in the stem tissue blocked translocation of materials from the leaves into the stem tissue (unpublished observations of A. S. Crafts—personal communication). In contrast to the promotion of stem growth (table I), leaf growth and apical development of treated plants were inhibited (table I). The effects of IAA and 2,4,5-T on growth were similar to 2,4-D except that the chemicals differed in their relative activities as described above, i.e. 2,4,5-T>2,4-D>IAA. All plants treated with IAA, except at 5×10^{-3} M, and those treated with 5×10^{-5} M 2,4-D showed a marked recovery during the course of the 5-day experiments. Recovery as used here refers to renewed leaf growth and apical development and lack of pronounced proliferation in the stem tissue.

Table I

Effect of 2,4-D on Fresh Weight of Different Parts of Cucumber Plants*

Treatment time hr	Concentration 2,4-D			
	0	5×10^{-5} M	5×10^{-4} M	5×10^{-3} M
	*Stem I***			
6	1.9	2.3	2.3	2.3
12	2.1	2.6	2.8	2.7
24	2.5	2.9	3.2	3.2
48	3.2	4.2	4.5	4.5
72	4.3	5.3	5.3	5.0
96	5.0	5.5	5.3	5.3
120	5.1	6.2	5.0	4.8
	*Stem II****			
6	...	...	...	...
12	...	...	...	...
24	0.1	0.1	0.1	0.1
48	0.2	0.2	0.1	0.1
72	0.4	0.5	0.2	0.1
96	0.9	1.1	0.2	0.1
120	1.6	1.5	0.4	0.2
	Leaves†			
6	1.1	0.9	1.1	1.2
12	1.2	1.0	1.1	1.3
24	1.5	1.2	1.1	1.2
48	2.1	1.7	1.1	1.2
72	2.2	1.8	1.2	1.2
96	3.4	2.4	1.4	1.3
120	3.9	2.9	1.7	1.3

* Values are g fresh weight/sample and represent averages of 12 individual samples.
** Stem tissue, including petioles, up to node 3.
*** Stem tissue above node 3, including petioles.
† Total leaf weight exclusive of leaf number 1.

In addition to IAA, 2,4-D, and 2,4,5-T, other known auxins and a series of chlorinated phenoxyacetic acids were tested for activity in altering ascorbic acid metabolism. The 2,4,6-T analog of 2,4-D was inactive, the *o*-Cl substituted compound was only weakly active, and the *p*-Cl substituted compound was intermediate between the *o*-Cl compound and 2,4-D in producing the growth responses described above. The α-propionic acid derivative of 2,4,5-T (2,4,5-TP) was as effective as 2,4,5-T in altering the growth pattern of the cucumber plant.

The concentration of ascorbic acid increased markedly in stem tissue of plants which were treated with auxin (figs 1, 2, 3, & 4). The concentration of ascorbic acid reported in figures 1, 2, 3, and 4 compares to a concentration of 60 ± 7 μg/g fresh weight in untreated stem tissue. The magnitude and duration of the increase in ascorbic acid was dependent upon the auxin used and upon the concentration of that auxin. A small increase in ascorbic acid in the stem usually was measurable within two or three hours after treatment. Resumption of somewhat normal growth, as defined above, was associated with a decline in stem ascorbic acid to a level comparable to that of control tissue. Stem tissue which was induced to proliferate by addition of auxin maintained an abnormally high concentration of ascorbic acid throughout the 5-day period.

In contrast to the auxin-induced increase in stem ascorbic acid, the concentration of ascorbic acid in leaf tissue decreased in response to added growth regulator (tables IIa, IIb, & IIc). The decrease in ascorbic acid concentration was associated with inhibited leaf growth. The data in tables IIa, IIb, and IIc show that the ascorbic acid content of untreated leaf tissue decreased during the course of the experiments. This decrease in ascorbic acid was associated with a lower light intensity in the growth chambers than in the greenhouse where the plants were grown prior to the start of the experiments (unpublished observations). Since the effect of light intensity on ascorbic acid concentration was not recognized until most of the experimental work had been done, plants were not kept in the growth chambers under low light intensity (approx. 600 ft-c) for

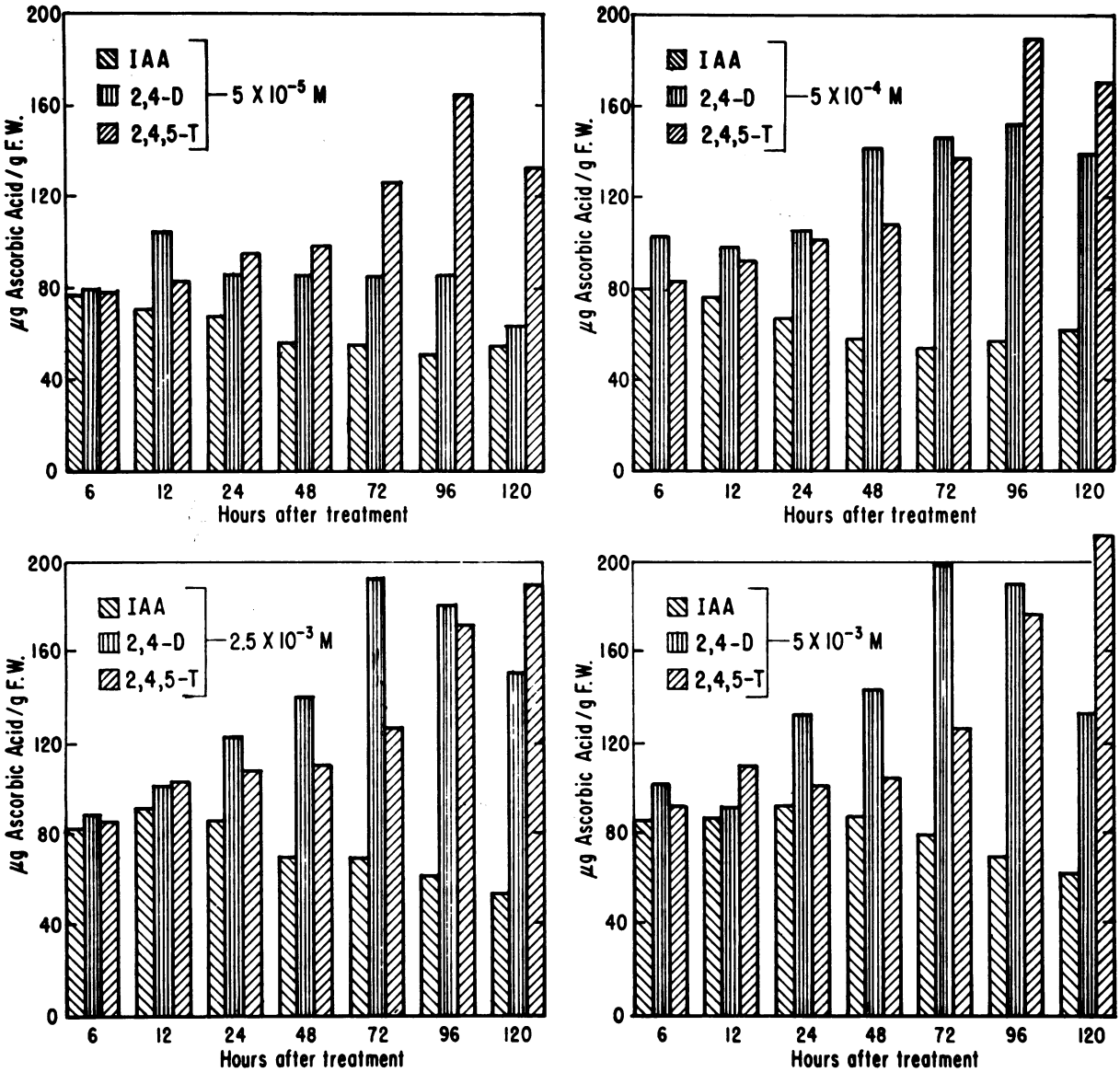

Fig. 1–4. Alterations of ascorbic acid concentration in cucumber stem tissue in response to IAA, 2,4-D and 2,4,5-T. (Control ascorbic acid content was 60 ± 7 µg/g fresh weight.)

Fig. 1 (*top left*). Response to 5 × 10^{-5} M chemical.

Fig. 2 (*top right*). Response to 5 × 10^{-4} M chemical.

Fig. 3 (*bottom left*). Response to 2.5 × 10^{-3} M chemical.

Fig. 4 (*bottom right*). Response to 5 × 10^{-3} M chemical

Table IIa

Effect of IAA on Concentration of Ascorbic Acid in Leaves of Cucumber Plants*

Conc (M)	μg Ascorbic acid/g fr wt						
	6**	12**	24**	48**	72**	96**	120**
0	835	835	670	600	560	...	560
5×10^{-5}	805	800	775	570	560	...	580
5×10^{-4}	780	695	660	620	570	...	540
2.5×10^{-3}	760	640	505	540	540	...	520
5×10^{-3}	770	525	490	560	470	...	360

* Values reported in tables IIa, IIb, and IIc are averages from three different samples in a single experiment. Each experiment was repeated at least once.
** Hours after treatment.

Table IIb

Effect of 2,4-D on Concentration of Ascorbic Acid in Leaves of Cucumber Plants

Conc (M)	μg Ascorbic acid/g fr wt						
	6*	12*	24*	48*	72*	96*	120*
0	1,490	1,250	1,300	980	870	830	775
5×10^{-5}	1,040	1,110	1,130	910	805	820	840
5×10^{-4}	1,210	1,000	950	860	635	620	550
2.5×10^{-3}	1,070	1,000	990	860	650	575	575
5×10^{-3}	1,180	980	960	780	630	550	515

* Hours after treatment.

Table IIc

Effect of 2,4,5-T on Concentration of Ascorbic Acid in Leaves of Cucumber Plants

Conc (M)	μg Ascorbic acid/g fr wt						
	6*	12*	24*	48*	72*	96*	120*
0	970	1,125	880	670	660	620	550
5×10^{-5}	850	890	650	530	570	480	390
5×10^{-4}	775	850	630	500	420	250	280
2.5×10^{-3}	850	875	650	345	315	260	260
5×10^{-3}	800	760	590	280	255	220	230

* Hours after treatment.

Table III

Effect of 2,4,5-T* on Concentration of Ascorbic & Dehydroascorbic Acids of Cucumber Plants

Tissue	Treatment	24**		48**		72**	
		Ascorbic acid	Dehydro-ascorbic acid	Ascorbic acid	Dehydro-ascorbic acid	Ascorbic acid	Dehydro-ascorbic acid
		μg/g fr wt		μg/g fr wt		μg/g fr wt	
Stems	None	59	15	60	19	58	23
	2,4,5-T	128	4	120	6	107	15
Leaves	None	1050	40	685	40	715	30
	2,4,5-T	695	80	445	85	325	135

* Tested at a concentration of 2.5×10^{-3} M.
** Number of hours after treatment that analyses were made.

the same length of time before the start of each experiment. It is believed that this accounts for the major difference between the initial level of ascorbic acid in leaf tissue in the different experiments reported.

Since Marrè et al. (25) had indicated that the ratio of reduced to oxidized ascorbic acid was a critical factor in auxin-induced growth changes, analyses were made in some experiments for the dehydroascorbic acid content of leaf and stem tissue. The data reported in table III show the concentration of ascorbic acid and dehydroascorbic acid in leaf and stem tissue from plants treated with 2,4,5-T. In stem tissue where auxin induced an abnormally high accumulation of ascorbic acid, the concentration of dehydroascorbic acid was consistently lower than in untreated stem tissue. On the other hand, the concentration of dehydroascorbic acid was consistently higher in treated leaf tissue where the concentration of ascorbic acid was lower than in untreated control tissue.

Table IV

Relative Effectiveness of Several Known Auxins on Ascorbic Acid Concentration of Cucumber Plants*

Compound	μg Ascorbic acid/g fr wt			
	Stems		Leaves	
	24**	72**	24**	72**
None	61	45	975	610
3-Indoleacetic acid	107	73	780	510
3-Indolepropionic acid	96	...	755	...
1-Naphthaleneacetic acid	100	116	715	430
2,4-Dichlorophenoxyacetic acid	110	153	630	425
2,4,5-Trichlorophenoxyacetic acid	110	145	710	350

* All compounds tested at a concentration of 2.5×10^{-3} M

** 24 and 72 refer to hours after treatment.

The data presented in tables IV and V demonstrate that the response of the ascorbic acid system was produced only by those chemicals which are active auxins. The relative effectiveness of several known auxins in inducing the ascorbic acid responses is shown by the data in table IV. The magnitude of the change in the concentration of ascorbic acid was comparable to the relative activities of the auxins tested. Where a series of phenoxy acids was tested, only those compounds showing auxin activity were effective in causing an increase in stem ascorbic acid and a decrease in leaf ascorbic acid (table V). A good correlation exists between the magnitude of the ascorbic acid changes and the known auxin activity of these phenoxy compounds (18).

Preliminary experiments were done to check on possible changes in ascorbic acid oxidase and glucose-6-phosphate dehydrogenase activities in the stem tissue. Treatment of the cucumber plant with 2,4-D

Table V

Alteration of Ascorbic Acid Concentration of Cucumber Plants by a Series of Phenoxyacetic Acids*

Compound tested	μg Ascorbic acid/g fr wt			
	Stems		Leaves	
	24**	72**	24**	72**
None	70	72	1,080	930
2,4,6-Trichlorophenoxyacetic acid (2,4,6-T)	70	77	1,090	1,040
o-Chlorophenoxyacetic acid	66	74	940	860
p-Chlorophenoxyacetic acid	94	96	845	805
2,4-Dichlorophenoxyacetic acid (2,4-D)	115	104	735	670
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	113	129	610	400
2,4,5-Trichlorophenoxy-α-propionic acid (2,4,5-TP)	108	111	780	530

* All compounds tested at 5×10^{-3} M.

** 24 and 72 refers to time after treatment in hours.

resulted in a marked reduction of ascorbic acid oxidase activity in stem tissue (table VI). The reduction in this activity correlated with the increase in ascorbic acid noted in this tissue (figs 1, 2, & 4). It should be mentioned, however, that there appears to be little or no effect of 2,4-D on ascorbic acid oxidase activity of leaf tissue where there is a decrease in ascorbic acid.

Table VI

Ascorbic Acid Oxidase Activity of Stem Homogenates of Control & 2,4-D-Treated Cucumber Plants

Conc (M)	μl O_2/hr/g fr wt				
	6*	12*	24*	48*	72*
0	1,755	1,640	1,840	1,700	1,720
5×10^{-5}	1,740	1,230	1,325	1,550	1,640
5×10^{-4}	1,655	1,170	990	1,115	1,480
5×10^{-3}	1,455	1,025	915	660	1,200

* Numbers refer to hours after treatment.

The results which were obtained in the assay of glucose-6-phosphate dehydrogenase between 6 and 72 hours after treatment are shown in table VII. The activity of this enzyme continued to increase up to 72 hours after treatment. Production of TPNH not only was greatly enhanced when based on fresh weight but also when based on total protein. There is a net synthesis of protein in the stem tissue after treatment with 2,4-D (table VII); a 50% increase in this activity on a protein basis, however, indicates either a preferential synthesis or an activation of this enzyme. Although the activity of glucose-6-phosphate dehydrogenase was not enhanced when measured within 6 hours after treatment, the enzyme may have shown a greater activity in the intact plant. The observations of Freed et al. (11) would in fact

Table VII

Effect of 2,4-D* on Glucose-6-phosphate Dehydrogenase Activity Measured in Homogenates of Cucumber Stem Tissue

Hours after treatment	Units activity**/g fr wt	mg Protein/g fr wt	Units activity/ mg protein
6	24.6	0.80	30.8
12	31.2	0.85	36.8
24	41.0	1.03	40.0
48	57.0	1.24	46.0
72	69.0	1.42	48.6
Control***	24.5±1.3	0.78±0.02	31.5±2.1

* 2,4-D tested at a concentration of 2.5×10^{-3} M.

** One unit of activity is given as that amount of enzyme which was required to produce unit optical density increase at 340 mμ per hour.

*** Values reported for activity in control tissue are averages for five experiments showing maximum deviation obtained between experiments. One experimental value was obtained at each time interval noted in the table.

suggest that an interaction of 2,4-D with this and other dehydrogenases can result in enhanced activity when the auxin is applied in appropriate concentrations. Because of very high absorption of leaf homogenates in the near ultra violet, leaf samples were not assayed for glucose-6-phosphate dehydrogenase activity. Other work is in progress on the sulfhydryl status of the auxin-treated cucumber plants as it relates to altered enzyme activities.

Discussion

The data presented above lend support to the view of Tonzig and Marrè (34) that a rather direct control by auxin of the oxidation-reduction state of the ascorbic acid system could be important in mediating the final physiological effects of the hormone. With the concentrations of auxin used in these experiments apical development and leaf growth were inhibited while considerable expansion and proliferation were induced in the stem tissue. These growth responses appear to be dependent upon, or associated with, shifts in the ascorbic acid system: growth inhibition correlating with decreased ascorbic acid concentration and a more oxidized system and growth promotion with increased ascorbic acid concentration and a more reduced system. At present, however, there is no proof for a direct interaction of auxin with the ascorbic acid system in the regulation of growth. There is, however, considerable evidence for such an interaction.

When excised tissue is incubated in a medium containing ascorbic acid, there is normally a marked inhibition of growth (17, 21). This inhibition is correlated with the accumulation of dehydroascorbic acid in the tissue (21, 22, 34). In addition, the ratio of reduced to oxidized glutathione is markedly decreased. This phenomenon may be of considerable importance since the reduction state of the glutathione system appears to be important in growth regulation (24, 26). Moreover, when auxin is fed to excised tissue in growth promotive concentrations there is an associated shift of the ascorbic acid and glutathione systems toward a more reduced state (25). Sulfite is synergistic with auxin in promoting both growth and glutathione reduction (2). In inhibitory concentrations auxin causes a decrease in the ascorbic acid to dehydroascorbic acid ratio (25). Treatment of etiolated soybean seedlings with 2,4-D also results in an increase in ascorbic acid, soluble sulfhydryl, and protein sulfhydryl in the hypocotyl tissue (17). These changes showed a good correlation with the growth response produced by 2,4-D. Chinoy et al. (6) have also reported interactions of IAA and ascorbic acid in growth regulation. These workers believe the oxidation-reduction state of the ascorbic acid system to be important in the conversion of vegetative growth into reproductive growth (7). The work of Prochazka et al. (29, 30, 31) with auxin and ascorbic acid is especially interesting. These workers have characterized a compound (termed ascorbigen) which on acid hydrolysis liberates ascorbic acid and on alkaline hydrolysis IAA.

There is little evidence as to how auxin acts in causing alterations in the oxidation-reduction state of the cell, especially the ascorbic acid and sulfhydryl systems. The data of Marrè and Arrigoni (20) and of Wagenknecht et al. (36) support the thesis that the auxin-induced inhibition of ascorbic acid oxidase may account, at least in part, for the accumulation of ascorbic acid in the stem tissue. Certainly the data reported in table VI would be consistent with this view. As suggested by Marrè and Arrigoni (27), a decrease in leaf ascorbic acid could result from the inhibition of monodehydroascorbic reductase by high concentrations of auxin. Humphrey et al. have reported a stimulation of the pentose phosphate pathway activity in response to added auxin (3, 14,15, 16). Since there are known enzyme systems which utilize TPNH (which is produced by pentose pathway enzymes) to reduce oxidized glutathione and in turn dehydroascorbic acid (35), an enhanced production of TPNH in the stem tissue could lead to a more reduced state of the glutathione and ascorbic acid systems. The data reported in table VII show that the activity of at least one TPNH generating enzyme system is enhanced in the stem tissue of auxin-treated plants. An increase in reduced pyridine nucleotides as well as ascorbic acid and sulfhydryl in soybean seedlings treated with 2,4-D was reported by Key and Wold (17). An auxin-induced increase in TPNH in pea stem sections was also reported by Marrè (28). Whatever the exact mechanism may be it appears that enzymes involved in the alternate oxidation and reduction of the ascorbic acid system in association with pyridine nucleotides and glutathione will be involved.

The present work shows that ascorbic acid oxidase activity was lowered by auxin treatment in tissues where ascorbic acid accumulated. On the other hand

glucose-6-phosphate dehydrogenase activity (as measured by TPNH production) was greatly enhanced in the same tissue. It should also be pointed out that many other enzymes show altered activity in plants which were previously treated with auxin (4, 37, 38). It seems quite possible that shifts in the oxidation-reduction state of the cell, especially in relation to protein sulfur, may play an important role in the altered activity of so many enzymes. Certainly the activity of some enzymes is dependent upon maintenance of sulfhydryl groups and others upon disulfide groups (5). The observations of Freed et al. (11) show that a direct interaction of the auxin molecule with certain enzymes leads to enhanced activity at low concentrations and reduced activity at higher concentrations. It may be that both a direct interaction of the auxin molecule with enzymes and changes in the reduction state of sulfur in enzyme molecules are responsible for altered activity of so many enzymes in auxin-treated plants.

There is insufficient evidence, as previously pointed out by Galston and Purves (12), to differentiate between A, an effect of auxin on growth leading to changes in the reduction state of the cell and B, an effect of auxin on the reduction state of the cell initiating the growth response. However, the intriguing possibility that a causal relationship actually exists points clearly to the need for more research in this area.

Summary

Ascorbic acid accumulated in stems of cucumber plants which had been treated with auxin. The same treatments with auxin which produced an increase in stem ascorbic acid caused a decrease in leaf ascorbic acid. At the same time the dehydroascorbic acid concentration decreased in treated stem tissue and increased in treated leaf tissue. Thus, growth promotion of the stem tissue induced by auxin was associated with an increase in ascorbic acid and a more reduced system while growth inhibition of the leaf tissue was associated with a decrease in ascorbic acid and a more oxidized system. The effectiveness of several auxins and related compounds in producing these changes in ascorbic acid paralleled the known auxin activity of the compounds tested.

Auxin treatments which caused ascorbic acid to accumulate in the stem tissue resulted in a decrease in ascorbic acid oxidase activity of stem tissue homogenates and an increase in glucose-6-phosphate dehydrogenase activity. The relationship of the oxidation-reduction state of the ascorbic acid system to altered enzymatic activities was discussed.

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