Effect of Auxin on Ascorbic Oxidase Activity in Tobacco Pith Cells.* (18538)

ELDON H. NEWCOMB. (Introduced by W. H. McShan)
From the Department of Botany, University of Wisconsin, Madison, Wis.

Much experimental work has been performed and many theories proposed in attempts to explain the mechanism of action of the plant growth hormone, 3-indoleacetic acid, or auxin, in causing cell enlargement and other growth phenomena. The subject has been carefully reviewed by Audus(1), and special aspects of the problem have been discussed by several contributors to a recent volume of review articles(2). It has been suggested that auxin causes cell enlargement by increasing the elastic or plastic extensibility of the primary wall, promoting active intussusception of new cellulose micelles into the wall, controlling cell permeability by regulating boundary potential, stimulating or reorienting respiration, causing active (*i.e.*, non-osmotic) water and solute uptake, decreasing protoplasmic viscosity, and by a number of other actions. The opinion is now widely held that the growth effects of auxin are probably due to its effect on a specific phase of cellular metabolism, quite possibly on a respiratory process. In the investigation reported here it has been found that auxin causes a striking increase in the ascorbic oxidase activity of cultured parenchyma cells of tobacco stem preceding and during their enlargement. Evidence is also presented that this enzyme is structurally associated with the cell wall. The culture of sections of pith from tobacco stems was initiated by Skoog and Tsui(3), who observed that the cells of this tissue did not enlarge appreciably on White's medium, but enlarged greatly on addition of indoleacetic acid (IAA) to the medium. Jablonski and Skoog(4) have since studied the responses of these cells to concentrations of IAA up to 20 mg/l, and have found that enlargement is slight at 0.02 mg, marked at 2.0 mg, and inhibited at 20.0 mg/l. Enlargement occurs earlier at 2.0 mg/l than at the lower concentrations tested. Careful anatomical examination has shown that no cell divisions occur in such cultures.

Since the pith consists of a single cell type in which the hormone at physiological concentrations elicits pronounced cell enlargement uncomplicated by cell division, it appeared to be a particularly favorable material for the investigation of the specific effects of auxin on the metabolic processes involved in cell enlargement.

Materials and methods. Sections of pith approximately 1.5 x 4 x 10 mm, cut from young internodes of tobacco (Wisconsin No. 38) grown in the greenhouse, were weighed in sterile vials and cultured on a modified White's nutrient medium as described for tobacco stem callus(5). The sections were grown in 125 ml Erlenmeyer flasks with 50 ml of medium and 4 sections per flask. One-half of the cultures received indoleacetic acid at a concentration of 3.5 mg/l (2 x 10^{-5}M), the remainder without auxin serving as controls. Six flasks with and 6 without auxin were used in each assay: 2 from each group for dry weight determinations, 2 for respiration, and 2 for the enzyme assays. Fresh weights were determined on a Roller-Smith

* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation administered by the University Research Committee. Appreciation is expressed to Professor Folke Skoog for making available to the writer the wide variety of tissue cultures which have been developed in his program of growth studies. Access to these cultures led to the selection of the pith tissue for the present investigation. It is a pleasure to thank Mr. John R. Jablonski for preparing the cultures used. Thanks are also expressed to Professors Folke Skoog and Paul J. Allen for comments on the manuscript, and to the latter for performing 2 of the assays.

1. Audus, L. J., *Biol. Rev.*, 1949, v24, 51.
2. Ann. Rev. Plant Physiol., v1, Ann. Rev., Inc., Stanford, 1950.
3. Skoog, F., and Tsui, C., unpublished.
4. Jablonski, J. R., and Skoog, F., to be published.
5. Newcomb, E. H., *Am. J. Botany*, 1950, v37, 264.

TABLE I. Effects of 3.5 mg/l IAA on Respiration and Ascorbic Oxidase Activity of Tobacco Pith Sections Grown *in Vitro*. Experiment 2.

Age of culture (days)	Respiration (μl O_2 uptake/g fresh wt/hr)		Ascorbic oxidase activity (μl O_2 uptake/g fresh wt/hr)		Q_{O_2} (ascorbate)	
	Control	IAA	Control	IAA	Control	IAA
0	31.4		369		6.5	
2	33.2	26.2	504	638	11.8	17.2
3	35.4	41.8	473	703	13.1	18.1
4	33.2	47.5	453	998	12.8	20.4
5	30.3	44.1	476	1703	12.6	45.1
6	34.8	42.6	468	2054	10.2	59.5
8	36.9	53.7	442	1860	7.9	54.3
10	42.5	55.2	428	1980	9.8	59.3
13	34.5	42.4	955	2394	19.5	78.2
16	45.1	34.6	1065	2284	21.2	73.4

torsion balance after the sections were carefully surface-dried with filter paper. Since the sections were not surface-dried for the first weighing and the surface liquid for 8 sections amounted to 80-100 mg, the initial weights set upper limits only. Oxygen consumption was determined using conventional Warburg equipment and technics. The intact sections were suspended in 2.0 ml of M/20 phosphate buffer at pH 6.0 containing 0.02 M sucrose, and their oxygen uptake was followed for 1 hour at 25°C.

For the oxidase assays, the sections were suspended in 4.0 ml of cold M/20 phosphate buffer at pH 6.0 and homogenized for 1.5 min. in a Potter-Elvehjem glass homogenizer chilled in ice water. Centrifugations were performed at 2°C in an International refrigerated centrifuge. For ascorbic oxidase assays, 1.0 ml aliquots of homogenate and 0.5 ml of 0.12 M ascorbate adjusted to pH 6.0 with NaOH were added to the central compartments of the vessels and the oxygen uptake at 25°C recorded for duplicate vessels at 10-minute intervals. Since there is no CO_2 production by the system, KOH was omitted from the insets. The ascorbate and the substrates in assays for other enzymes were prepared in M/20 phosphate of appropriate pH. It was ascertained that there is no endogenous oxygen consumption by the homogenates, and no measurable autoxidation of ascorbate in the absence of homogenate at the pH employed. Boiling the homogenate for 2 minutes destroys virtually all of the activity toward ascorbate. As has been reported previously(6), the rate of reaction is found to be independent of the ascorbate concentration between wide limits and directly proportional to the enzyme concentration.

Assays for tyrosinase employed 1.0 ml of the above homogenate and 0.5 ml of 0.06 M catechol neutralized to pH 6.0. In assaying for cytochrome oxidase, 1.0 ml of homogenate prepared in M/20 phosphate buffer at pH 7.0, 0.5 ml of 0.06 M p-phenylenediamine neutralized to pH 7.0, and 0.5 ml of 1×10^{-4} cytochrome c were used. Correction was made for oxidation of p-phenylenediamine in the absence of cytochrome c.

Results. The pith cells respond to auxin beginning within 2-3 days by an abrupt rise in ascorbic oxidase activity accompanied by a lesser respiratory increase, and subsequently by gains in fresh and dry weight.

The ascorbic oxidase activity responds strikingly to an exogenous supply of auxin (Table I, Fig. 1). The increase is evident by the 2nd day and quite rapid until the 6th, after which it appears to be more gradual. Thus in Exp. 1, the activity rose 1000% on a fresh weight basis and 1900% on a dry weight basis in the first 6 days on auxin, while it rose only 29% and 37%, respectively, in the controls. Assays beyond the 16th day indicate a gradual decline in the activity. The rise in activity of ascorbic oxidase begins be-

6. Hopkins, F. G., and Morgan, E. J., *Biochem. J.*, 1936, v30, 1446.

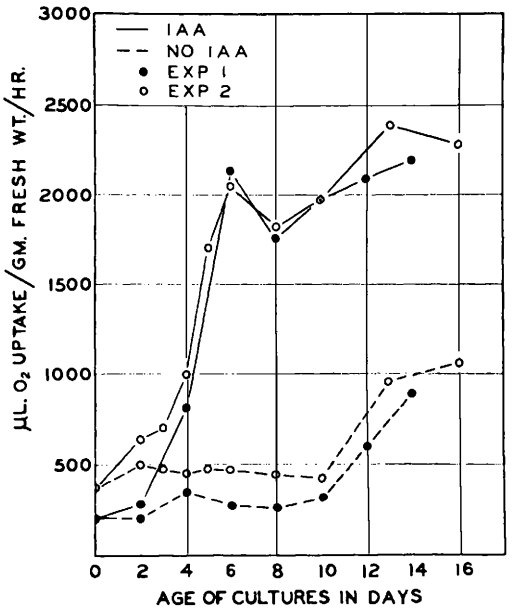

FIG. 1.

Effect of 3.5 mg/l indoleacetic acid on ascorbic oxidase activity of tobacco pith sections cultured *in vitro*. Curves based on oxygen consumption by homogenates on ascorbate.

fore any increase in respiration or weight can be detected, is most rapid either prior to or concomitant with the most rapid respiratory and weight increases, and reaches a peak after the respiratory rate has declined (Table I). The controls, on the other hand, show a constant, relatively low activity until after the 10th day, when there is a sharp rise which by the 16th day reaches a level shown by the auxin-grown tissue on the 4th day. Subsequent to the 16th day, however, the control tissue turns brown and decreases in oxidase and respiratory activity and in fresh and dry weight. The rise in the controls is being investigated further.

The respiratory rate of the pith sections on auxin shows an initial drop (Table I, 2nd day), followed by an increase until the 10th day, when a peak 76% above the initial value is reached. Thus respiration increases during the period of greatest increase in oxidase activity and gain in fresh weight. Unlike the latter two properties, it declines beyond the 10th day. The control tissue shows a lower respiratory rate which gradually increases to 46% above the initial value within 16 days.

The sections grown on auxin are not visibly different from the controls until the 4th day of culture, when they are more translucent and are bowed, with the concave face toward the agar. By the 5th day they are obviously enlarged and by the 7th or 8th, are fissured on the upper surface where groups of cells have separated. After about 2 weeks many of the individual cells become separated from their neighbors. The cells increase in size rapidly until the 10th or 12th day, after which there is little further enlargement.

The fresh and dry weight data for one experiment presented in Table II indicate the extent of growth which occurs. While there is a small amount of growth in the controls, that in the sections on auxin occurs earlier and is much greater. Jablonski and Skoog (4), using an improved method of cutting the pith sections from the plants, have reduced the variability due to tissue injury and have obtained even greater differences between control sections and those supplied with auxin.

Fractionation of the pith homogenates by differential centrifugation followed by assay and microscopic examination of the fractions indicates that the ascorbic oxidase remains attached to the wall fragments. Although the large, thin-walled cells are completely broken by a short period of homogenization, the sheets of wall material are not greatly dispersed in the Potter homogenizer. If the homogenate is then centrifuged at low speed, most of the activity is found in this sedimented wall material. For example, pith grown on IAA for 4 days was homogenized for 2 min., and the unfractionated homogenate was found to consume 1,021 μl O_2/g fresh wt/hr. The sediment equivalent to 1 g of whole homogenate, obtained by centrifuging at 500 x g for 5 min, consumed 901 μl O_2, and the equivalent supernatant, 114 μl. The recovery in the 2 fractions was 99.4% of the activity of whole homogenate, while the sediment showed 89% of the combined activity of the 2 fractions. The thermolability of the sedimented activity was shown by boiling the sediment for 2 min., after which the uptake

TABLE II. Effects of 3.5 mg/l IAA on Weights of Tobacco Pith Sections Grown *in Vitro*. Figures are values for 8 sections from 2 flasks. Experiment 2.

Age of cultures (days)	Fresh wt (mg)				Dry wt (mg)		Change in dry wt (mg) †		% dry wt	
	Control		IAA		Control	IAA	Control	IAA	Control	IAA
0		714			27.2				3.81	
2	(679)*	621	(658)*	584	26.6	21.7	— 0.6	— 5.5	4.28	3.72
3	(722)	606	(719)	663	21.8	25.7	— 5.4	— 1.5	3.60	3.88
4	(756)	641	(590)	606	22.6	29.7	— 4.6	+ 2.5	3.53	4.90
5	(614)	540	(716)	827	20.4	31.3	— 6.8	+ 4.1	3.78	3.78
6	(686)	668	(737)	785	30.7	27.1	+ 3.5	— 0.1	4.60	3.45
8	(690)	725	(678)	1223	41.7	42.0	+14.5	+14.8	5.60	3.43
10	(764)	657	(741)	953	28.6	35.2	+ 1.4	+ 8.0	4.36	3.34
13	(689)	665	(685)	1298	32.7	39.7	+ 5.5	+12.5	4.91	3.06
16	(762)	769	(753)	1248	38.5	38.8	+11.3	+11.6	5.01	3.11

* Figures in parentheses are the weights of the same 8 sections at time of planting, determined without surface-drying the sections.

† Change from average value for 8 sections of 27.2 mg based on weights of 32 sections at beginning of experiment.

was only 39 μl for the equivalent of 1 g of whole homogenate.

Even prolonged homogenization, *e.g.*, for as long as 12 min., displaces very little of the activity from the sediment into the supernatant. When the Waring Blendor is employed, however, considerably more of the activity is found in the supernatant, due presumably to a more drastic action in dispersing the cell wall and adhering cytoplasm.

The oxidase activity does not reside in plastids or nuclei. Chloroplasts are absent, and starch grains and other plastids are few. Most of these can be broken by homogenization with displacement of the fragments into the supernatant, but no appreciable fraction of the oxidase activity is transferred into the supernatant. The nuclei are large, and it has been determined that they are broken during the homogenization and that very little Feulgen-positive material is found in the sediment.† Nor can the activity be ascribed to agglutinated mitochondria which might sediment with wall material, since mitochondria centrifuged from the supernatant are without activity. Homogenates prepared in distilled water and in 0.25 M sucrose solution show a localization of activity similar to that found for homogenates prepared in phosphate buffer. The localization of the activity suggests that the enzyme may be in cytoplasm intimately associated with and perhaps interpenetrating the cell wall.

Intact cells oxidize ascorbate at a rate virtually equal to that of a homogenate of such cells. This was demonstrated with cells which had become completely separated from one another during enlargement. For example, cells 46 days old, respiring at a rate of 23 μl/g fresh wt/hr, consumed 809 μl O_2/g fresh wt/hr on ascorbate, while a homogenate of similar cells consumed 805 μl. Whether this rather surprising activity of the intact cells indicates rapid penetration of the ascorbate or localization of the enzyme at the cytoplasmic surface, where it is readily reached, has not yet been ascertained. Since ascorbate at pH 6.0 is largely dissociated ($pK_1 = 4.17$), its rapid penetration into the cell seems unlikely.

The oxygen consumed by intact cells supplied with a measured amount of ascorbate does not exceed that expected for oxidation of all the ascorbate to dehydroascorbate. In one experiment, 555 mg of cells with an initial oxygen consumption of 18 μl/hr were employed. On addition of 0.5 ml of 0.03 M ascorbate, they consumed 170 μl O_2 in 35 min., by which time the oxygen uptake had dropped to the rate before the addition of ascorbate. Corrected for respiration, the uptake on ascorbate was 160 μl, or 95% of the 168 μl expected for complete oxidation to

† The author expresses his appreciation to Mr. P. K. Nelson for examining the sediment for nuclear material.

dehydroascorbate. This indicates that there is no appreciable concomitant reduction of the dehydroascorbate, since this would be followed by re-oxidation of the ascorbate and a greater oxygen uptake than calculated. Nevertheless, in homogenates of this material, dehydroascorbate is rapidly reduced upon addition of the sulphydryl form of glutathione. The question whether an ascorbic reductase is responsible for this reaction and whether this system is similar to the one studied by Hopkins and co-workers(7-8) is being investigated.

Since auxin in high concentration inhibits plant growth, including that of the pith tissue (4), the effect of a high concentration (17.5 mg 1) in the medium on the respiration and ascorbic oxidase activity of pith cells was determined. No growth was apparent by the 5th day, and the fresh wt (449 mg for 8 sections) was below the original fresh wt (492 mg). While the respiratory rate was not noticeably decreased (O_2 uptake of 29.4 μl/g fresh wt/hr compared with 30.3 μl for the control), the ascorbic oxidase activity was strongly decreased, being only 81 μl/g fresh wt/hr compared with 476 μl for the control. Thus if the stimulation of this enzyme by auxin is important for cell growth, then its inhibition by auxin in high concentration may be the basis for the inhibition of growth by auxin.

It is an interesting fact that auxin does not stimulate the activity of ascorbic oxidase when added directly to homogenates. This was determined both on pith taken directly from tobacco plants and on that which had grown on the control medium for several days. Failure to obtain *in vitro* stimulation of this oxidase with auxin has been reported previously by Wagenknecht(9), who employed homogenates of leaves and roots of yellow wax beans.

Assays for cytochrome oxidase and tyrosinase in the pith indicate no marked response of these enzymes to auxin. Low levels of cytochrome oxidase were found in both the controls and in pith grown on auxin. Tyrosinase activity could not be demonstrated in young control or treated cultures, but after about 2 weeks it was found in both, and in increasing amounts as the tissues aged.

Discussion. While ascorbic oxidase has been reported from a number of higher plants (10,11), it may be of much more general occurrence than is apparent from the literature. The writer has observed that vigorous activity toward ascorbate is characteristic of homogenates of actively growing material, including young fern fronds, young leaves of tobacco, bean, wheat, corn, grape and onion, root tips of corn and flax seedlings, and callus tissue cultures of several species. On the other hand, the slow-growing branches of *Psilotum* have low activity, and the storage leaves of onion, none. The high ascorbic oxidase activity characteristic of young grape leaves and galls decreases markedly with age(12). Furthermore, young galls, in which the cells are enlarging greatly, have much higher activity than the contiguous leaf tissue. It appears from these facts that high ascorbic oxidase activity, like high auxin content, is associated with rapid growth. It is of particular interest in this connection that tobacco, sunflower, marigold and carrot callus tissues, which synthesize sufficient auxin to maintain a continuous high rate of growth on media without added auxin, are found to have vigorous ascorbic oxidase activity.

In all homogenates which have been fractionated by centrifugation, including those of wheat and grape leaves, corn root tips and the above-named callus tissues, the ascorbic oxidase is largely recovered in the wall fraction. While many studies of this enzyme in the past have been carried out on centrifugated saps, the presence of ascorbic oxidase in such preparations may not be inconsistent with the

7. Crook, E. M., *Biochem. J.*, 1941, v35, 226.

8. Crook, E. M., and Morgan, E. J., *Biochem. J.*, 1944, v38, 10.

9. Wagenknecht, A. C., abstr. program 22nd ann. mtg., Am. Soc. Plant Physiol., 1947.

10. Rothchild, M. L., and MacVicar, R., *Fed. Proc.*, 1949, v8, 245.

11. Burris, R. H., and Little, H. N., p. 166 in Respiratory Enzymes, Burgess Publ. Co., Minneapolis, 1949.

12. Newcomb, E. H., in Plant Growth Substances, University of Wisconsin Press, Madison, 1951.

present results since more drastic preparative technics than those employed here may displace considerable activity into the supernatant, even though in the intact tissues the enzyme occurs in the peripheral cytoplasm.

Ascorbic oxidase may be, of course, only one of a number of enzymes which change in amount or activity in response to auxin. The following points, however, are pertinent in this regard: (1) the magnitude of the response of ascorbic oxidase to auxin; (2) the increase in activity of this enzyme prior to a detectable increase in tissue size or weight; (3) the rapid increase in enzyme activity which in the early stages parallels the rapid cell enlargement. These facts together with demonstration of an association of the enzyme with cell wall material in several tissues and of its high activity in growing tissues of a variety of plants suggest that ascorbic oxidase is causally concerned in cell enlargement and that its synthesis or activation may be controlled by auxin generally.

The function of ascorbic oxidase in the metabolism of the plant cell is unknown, although it has frequently been suggested that this enzyme may serve as a respiratory terminal oxidase(11,13). If ascorbic oxidase functions in the pith tissue as a respiratory terminal oxidase, then its increase may represent part of a change in respiration essential to the enlargement process. It is difficult to see how a modest increase in respiration occurring in the cytoplasmic interior, *e.g.*, in mitochondria, could lead to the burst in wall growth which occurs in these parenchyma cells on exposure to auxin. Such an effect would be more understandable if the increase in respiration were localized in the cytoplasm adjacent to or interpenetrating the primary wall, since this respiration might be the immediate source of energy for wall growth, active absorption of water, or other mechanism underlying cell enlargement. For instance, the ascorbic oxidase system might serve to generate energy-rich phosphate: linkage of inorganic phosphate to ascorbate followed by oxidation of the phosphoascorbate could generate an energy-rich phosphate bond, as suggested by Lipmann(14) for compounds of the ascorbic acid type.

Another possibility is that the increase in ascorbic oxidase activity alters the properties of the plasma membrane by increasing the potential difference across the membrane. This could be accomplished by maintenance of ascorbate in the oxidized form at the cell surface if it is largely in the reduced form in the cell interior.

It is also conceivable that this enzyme plays some specific, though as yet unknown role in the organized, active growth of the primary wall which may control cell enlargement. Whatever the mechanism may be, insofar as auxin and ascorbic oxidase are causally related in growth, study of plant growth substances in terms of the action of this enzyme system constitutes a new experimental approach which may aid in elucidating the growth process.

Summary. Auxin (3-indoleacetic acid) at a concentration of 3.5 mg/l in the medium causes a striking increase in the ascorbic oxidase activity of cultured tobacco pith sections. This is subsequently accompanied by a lesser respiratory increase, by cell enlargement, and by gains in fresh and dry weight. No cell divisions occur. Since the ascorbic oxidase activity of homogenates is localized in the cell wall fraction obtained by differential centrifugation, it is suggested that the enzyme occurs in cytoplasm intimately associated with the wall. Vigorous ascorbic oxidase activity is characteristic of a variety of actively growing plant tissues examined. It is suggested that ascorbic oxidase may be causally related to cell growth.

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Received February 5, 1951. P.S.E.B.M., 1951, v76.