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Interactions of Phenolic Acids, Metallic Ions and Chelating Agents on Auxin-Induced Growth

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Summary. By growth experiments in indoleacetic acid-1-^{14}C (IAA), and determination of the $^{14}CO_2$ evolved, it has been shown directly that polyphenols synergize IAA-induced growth by counteracting IAA decarboxylation. Sinapic and ferulic acids act like polyphenols. Endogenous polyphenols doubtless exert the same influence in intact plants. Monophenols stimulate the decarboxylation of IAA under conditions where they depress growth. When Mn^{++} is present as well, this effect is enhanced. All these growth effects are paralleled by effects on the isolated IAA oxidizing enzyme of *Avena*.

EDTA acts like the polyphenols in depressing the decarboxylation of IAA, and not synergizing with the growth induced by naphthalene-acetic acid (NAA) and 2,4-D. However, since EDTA synergizes with IAA for growth even at optimal IAA concentrations, its growth promotion probably involves an additional effect.

DIECA inhibits powerfully the destruction of IAA, but without causing much growth promotion, apparently because its decomposition products inhibit respiration.

Mn^{++} alone stimulates the decarboxylation of IAA, i.e. this ion promotes the IAA oxidase in vivo as it does in vitro. Nevertheless, it does not inhibit elongation, but at relatively high concentrations even stimulates it, both at low and high IAA levels. Since Mn^{++} also promotes the growth induced by NAA and 2,4-D, its growth action cannot rest primarily on modifying the metabolism of the auxins.

Cobalt somewhat decreases the decarboxylation of IAA, but this cannot explain its growth promotion, since Co^{++}, like Mn^{++}, stimulates elongation even at optimal IAA concentrations, and acts with NAA just as well as with IAA. Ferrous ion, on the other hand, acts like the polyphenols.

Floating pea stem sections exude enough organic matter to support bacteria which after 7 hours cause considerable decarboxylation of IAA. *Avena* coleoptile sections have a comparable though smaller effect after 12 hours.

The present experiments, with those of others, point to a major role for polyphenolase in controlling hormone balance, since the introduction into a phenolic molecule of a second, adjacent hydroxyl group changes the action from auxin-destroying to auxin-preserving. Thus the phenol oxidizing enzymes must act as general growth controllers.

It is now becoming clear that naturally occurring phenolic compounds may markedly modify growth in several bioassays. Caffeic acid, ferulic acid, chlorogenic acid, quercetin, rutin and some other polyphenolic substances have been found to synergize the action of IAA in the *Avena* curvature test (10, 36, 37), while monophenolic compounds such as *p*-hydroxybenzoic acid and others antagonize IAA in the same bioassay (23, 37). Essentially similar results have been obtained in straight growth tests, using sections of oat coleoptiles (17, 37), oat first internodes (19), rice coleoptiles (40), sunflower hypocotyls and pea internodes (37). Polyphenolic substances also promote the growth of explants in tissue cultures (2, 5, 18, 25) and it has been shown (18) that this promotion rests on a synergism between the polyphenol and IAA. Vendrig and Buffel (4, 38) reported that the elongation of coleoptile sections may be promoted by caffeic

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acid without added IAA, but it was pointed out that in such cases the phenols are probably interacting with the endogenous IAA (33). Both phenols and IAA oxidases are known to be widespread throughout the whole plant kingdom, and therefore interactions of this sort probably take place under natural conditions. Such responses may be expected to differ in different plants according to the particular phenolic substances synthesized by them. The IAA oxidase, which in most cases is a peroxidase acting as oxidase (22), is a multiple enzyme whose fractions vary not only in their ability to oxidize the auxin (15, 16) but also in their peroxidative activity (24). The general significance of a second IAA-destroying system, that of tyrosine coupled with its phenolic substrates (3), is not yet known. Tyrosinase does not occur in pea or oat seedlings (6).

Some indication of the probable general importance of phenolic substances in controlling growth activity is given by their action in promoting or inhibiting bud formation in tissue cultures. Thus tyrosine (512 μM) and 4-hydroxybenzoic acid (8 μM) particularly strongly promoted bud formation in presence of kinetin and IAA, while ferulic and protocatechuic acids, among others, strongly inhibited it (14). These effects were interpreted as due primarily to their promoting or inhibiting, respectively, the oxidation of IAA. Paulet and Nitsch (20) had earlier described comparable effects due to chlorogenic acid and rutin on tobacco callus.

Some metallic ions have also been shown to modify IAA-induced growth; Co^{++} and Fe^{++} are the most effective, acting in concentrations as low as 10^{-5}M (26, 31, 32). The fact that externally applied chelating agents like EDTA may also promote IAA-induced growth supports the idea that metallic ions represent another natural factor involved in growth regulation (32). The mechanism by which these agents act is not yet clear, and Fe^{++} at least appears to promote auxin transport (26), but there are indications that EDTA (32) and Fe^{++} (26) may counteract the destruction of IAA. In the case of EDTA this interpretation is strongly supported by the fact that it synergizes with IAA but not with NAA or 2,4-D (32).

The aim of the present work was to reinvestigate in a comparative way the mode of action of the 3 types of synergists, namely phenols, metallic ions and chelating agents. To make the results as generally valid as possible, 3 types of seedling sections have been used, namely oat coleoptiles and green and etiolated pea stems. It will be shown that polyphenols and Fe^{++} manifest their activity by counteracting the IAA oxidase, but the action of other metals such as Co^{++} and Mn^{++} rests on a quite different mechanism. The action of EDTA apparently involves the IAA oxidase plus a second system. The importance of polyphenol oxidase as a growth-modifying enzyme is stressed. Comparable conclusions about the role of the polyphenols in vivo have been expressed by Zenk and Müller (45, 46) but with a less extensive experimental basis.

Materials and Methods

Avena coleoptile sections 10 mm long were cut about 3 mm below the tip from seedlings of Victory oats 72 hours old, grown in water at 25° in total darkness, after being exposed to 4 hours of weak red light beginning from the third hour after soaking. The sections were floated for 3 to 6 hours on distilled water and then on the test solutions, which contained 1 % sucrose.

Sections of the third internode of 7-day-old etiolated pea seedlings, var. Alaska, were cut 20 mm long from very close below the apical hook.

Sections of the fifth internode of green Alaska seedlings were cut similarly, 20 mm long, from very close below the terminal bud (within 1 mm of the base of the hook). In both cases the peas were grown on perforated plastic sheets with their roots dipping in water at 25°. The light-grown plants were maintained on 16-hour photo-periods in about 600 ft-c of fluorescent light, and uniform seedlings were selected 12 to 14 days after sowing. Both types of sections were floated 3 hours on distilled water prior to use. Growth solutions for the pea sections contained no sucrose or buffer (except for table IV).

^{14}C Carboxyl-labeled IAA of specific activity 0.95 curies per mole was synthesized and purified as described by Stowe (29). The sections were put into 50 ml Erlenmeyer flasks along with 5 ml of the radioactive IAA solution. The flasks were equipped with small test tubes containing 1 ml of 10 % NaOH as a CO_2 trap; they were tightly closed with stoppers and gently shaken in the dark room at 25°. After a suitable time interval, the stoppers were removed and the distribution of radioactivity was assayed. Aliquots of the NaOH solution containing the trapped $^{14}CO_2$ were introduced into 15 ml of the DAM 611 scintillator solution and suspended in Cab-O-Sil for assay in a Packard Tri-Carb liquid scintillation counter. Similarly, aliquots of the test solutions were used to determine the radioactivity remaining in the solution. The sections were prepared for assay by first rinsing with distilled water on a Buchner funnel and then homogenizing with ether in a small mortar at 25°. The homogenate was subsequently extracted by vigorous shaking 4 times with ether (for about 1 min each time), the combined ether extracts evaporated to dryness, dissolved in 0.5 ml of ethanol and mixed with the DAM solution as above. Since the pH of the homogenate was between 4.5 and 5.0, some IAA would be freed from its conjugates in this extraction (1); however, the amount of radioactivity in the ether extract of 10 pea stem sections corresponds to 0.01 μg of IAA, far below the sen-

Table I. *The Fate of* IAA-*1*-^{14}C *in Internodes of Etiolated Alaska Peas*

Initial concentration of IAA-1-^{14}C applied in 5 ml of ambient solution was 3.4×10^{-6} M, corresponding to 8525 cpm. Each group comprised 10 sections each 20 mm long. In distilled water alone the etiolated sections elongated 7.1 %.

Substance added	Solution	Distribution of radioactivity as cpm after 6 hrs incubation: $^{14}CO_2$	Extracts from tissues: Ether	Ethanol	Solid	Total in tissue	Recovery %	Elongation, % of initial length
			Etiolated epicotyls					
Control:								
IAA alone	4134	1973	31	882	260	1173	85.4	45.0
Chelating agents:								
DIECA*, 2×10^{-4} M	6338	282	40	910	255	1206	91.8	45.6
EDTA*, 5×10^{-5} M	4137	1165	46	1531	333	1909	84.6	46.9
Polyphenols:								
Chlorogenic acid, 5×10^{-5} M	5134	286	42	1114	299	1455	80.6	47.1
Caffeic acid, 5×10^{-5} M	4843	417	46	1285	293	1624	80.7	47.0
Sinapic acid, 5×10^{-5} M	4226	296	74	1839	412	2325	80.3	48.9
Monophenols:								
p-Coumaric acid, 5×10^{-6} M	4703	1857	39	601	228	868	87.1	45.3
p-OH-Benzoic acid, 1×10^{-4} M	1286	4621	27	816	173	1016	81.2	42.0

* Average of 2 experiments.

Table II. *The Fate of IAA-1-*^{14}C *in Green Epicotyls of Light-Grown Alaska Peas*

IAA as in table I. Ten 20mm sections. In distilled water alone, the green sections elongated 13.0 %.

Substance added	Solution	Distribution of radioactivity as cpm after 6 hrs incubation: $^{14}CO_2$	Extracts from tissues: Ether	Ethanol	Solid	Total in tissue	Recovery %	Elongation, % of initial length
Control:								
IAA alone	7370	282	48	208	34.8	291	93.2	27.0
Chelating agents:								
EDTA, 5×10^{-5} M	7737	240	61	272	31.8	364	97.8	34.4
Polyphenols:								
Chlorogenic acid, 5×10^{-5} M	7275	145	78	286	26.8	391	91.6	35.0
Caffeic acid, 5×10^{-5} M	7460	255	54	252	18.2	324	94.3	29.4
Sinapic acid, 5×10^{-5} M	7915	153	65	266	25.5	356	98.8	33.2
Monophenols:								
p-Coumaric acid, 5×10^{-6} M	6970	404	48	229	21.9	299	90.0	26.3
Vanillic acid, 1×10^{-5} M	7340	449	43	240	24.4	307	94.9	28.1
p-OH-Benzoic acid, 1×10^{-4} M	5757	916	32	154	33.9	220	80.9	26.2
2,4-Dichlorophenol, 1×10^{-4} M	6625	590	22	177	34.7	233	87.4	27.2
Mannitol, 0.3 M	7790	357	51	241	25.0	317	99.3	5.0

sitivity of the Salkowski reaction used in reference 1. The homogenized tissues were then extracted 3 times with 96 % ethanol. The combined ethanol extracts were reduced to 0.5 ml, mixed with the DAM scintillator and counted. The insoluble residue was spread uniformly over aluminum planchettes and assayed for radioactivity in a windowless gas-flow counter Model C 110B (Nuclear, Chicago).

A preparation of IAA oxidase was made from *Avena* coleoptiles (37); the enzyme solution contained 0.71 μg protein/ml as estimated spectrophotometrically (13). The destruction of IAA was followed, using the Salkowski reagent, colorimetrically with the Klett No. 54 green filter.

Results and Discussion

The Influence of Phenols and Chelating Agents on IAA Metabolism in Pea Epicotyls. Table I summarizes the fate of IAA-1-^{14}C administered with various test substances to etiolated sections of pea epicotyls. Table II gives corresponding data for the green sections. A general comparison of the 2 tables shows that under the same experimental conditions (both were in darkness) the etiolated sections absorbed much more IAA than did the green sections. The radioactivity in the etiolated tissue averages 1500 cpm; that in the green tissue 310 cpm. This fact complicates the direct comparison of the data on the distribution of radioactivity within the tissues. Nevertheless it is evident not only that the etiolated sections yielded a greater absolute amount of $^{14}CO_2$, but also that the CO_2 they gave off represented a greater fraction of the IAA which disappeared from the solution than in the case of the green sections.

Polyphenols such as chlorogenic acid and caffeic acid caused a drastic reduction in the $^{14}CO_2$ output in both kinds of tissue, and sinapic acid was about as effective as the nonmethoxylated polyphenols. The monophenol *p*-hydroxybenzoic acid caused a marked increase in the $^{14}CO_2$ output, and other monophenols in table II had similar though smaller effects. Vanillic acid acted like a typical monophenol, and indeed it has been found previously that at 10^{-5}M it stimulates IAA oxidation by the extracted enzyme (37). Table II shows that 2,4-dichlorophenol, a monophenol (but one which does not occur in nature) also stimulates the decarboxylation of IAA-1-^{14}C. Goldacre et al. (9) early showed that this monophenol promotes the IAA oxidase from peas and correspondingly lowers the elongation of pea epicotyl sections. The present data show that the inhibition of IAA destruction by polyphenols in vitro is paralleled by inhibition of the oxidative decarboxylation of IAA in the intact tissue. The opposite parallel holds for the promotion caused by monophenols. The fact that there was more intensive decarboxylation in etiolated than in green tissues also supports this assumption, since etiolated pea apices synthesize largely monophenolic substances, whereas the green apices produce both mono- and polyphenols (7, 37). Recently Pilet (21) has found chlorogenic acid to increase the indole auxin content of lentil roots.

Tables I, II and III bring out 1 more important fact, namely that the action of the phenolic compounds on decarboxylation does not depend on their promoting or inhibiting the uptake of IAA. For example, in table I, the diphenols and EDTA strongly inhibit the decarboxylation, yet they somewhat increase the radioactivity in the tissue. Correspondingly the first 2 monophenols in table II promote the decarboxylation while not significantly affecting the radioactivity in the tissue.

One may also deduce that the sections incubated with monophenols probably absorbed more IAA from the solution, for they destroyed far more auxin than those treated with polyphenols, and the amounts remaining in the solution were less than with the latter.

The parallelism between decarboxylation and elongation is not quite perfect, for in table II vanillic acid, which clearly promotes decarboxylation, does slightly promote elongation, while in table III ferulic acid shows just the opposite peculiarity. Also in table II, EDTA produces almost maximal synergism of the elongation, yet inhibits decarboxylation only slightly. Nevertheless the general trend is clear enough.

It is well known that etiolated sections elongate better than do green ones, whether in plain water or in auxin solution (8). The absorption of IAA by green tissues is strongly promoted by light (34) but light effects were not included in the present experiments. The fact that the green sections here absorbed less IAA than did the etiolated ones is probably not directly related to their growth, since the radioactivity of the ether extracts from green epicotyls was even somewhat higher than that of the etiolated tissues. Since these ether extracts represent the free IAA, the lesser elongation of the green segments could hardly be due to a poorer auxin supply. On the other hand the radioactivity of the alcohol extracts from etiolated tissues is clearly greater than that of the green sections, yet the alcohol fractions seem to contain mainly indoleacetylaspartic acid (1) and perhaps a little indoleacetylglucose (44). These conjugates exert little or no physiological activity. The radioactivity insoluble in 95 % ethanol might be due to IAA-1-^{14}C bound to protein (43, 44). The radioactivity of this fraction was so much higher in the etiolated tissue than in the green that it was at first thought that perhaps the IAA-protein might be an active form of auxin, but this assumption was ruled out by applying a higher concentration of IAA to green sections, which, as a result, accumulated considerable radioactivity without any evident increase in elongation (table III). The protein-bound IAA of *Avena* coleoptiles is also without growth activity (43).

Table III. *The Fate of Higher IAA-1-^{14}C Levels in Green Internodes of* Pisum

Initial concentration of IAA-1-^{14}C applied in 5 ml of water was 7.7×10^{-6} M, corresponding to 18,595 cpm. Ten 20 mm sections used. In distilled water the sections elongated 2.64 mm or 13.2 %.

Substance added	Radioactivity as cpm after 8 hrs incubation: In $^{14}CO_2$	In tissue	Elongation, % of initial length
Control:			
IAA alone	2126	1204	28.2
Polyphenols:			
Chlorogenic acid, 5×10^{-5} M	1227	1083	33.9
Caffeic acid, 5×10^{-5} M	950	1001	33.8
Ferulic acid, 5×10^{-5} M	1103	1599	27.7
Gentisic acid, 5×10^{-5} M	1098	1502	34.3
Ellagic acid, 5×10^{-5} M	1157	1030	28.9
Monophenols:			
p-Coumaric acid, 5×10^{-6} M	3032	1035	28.6
Vanillic acid, 1×10^{-5} M	2646	732	26.4
p-OH Benzoic acid, 1×10^{-4} M	4685	733	28.2

If both polyphenols and EDTA generally synergize the IAA-induced growth (with the reservation above) by the similar influence which they exert on IAA decarboxylation, then the action of DIECA must have another explanation, for DIECA reduced the $^{14}CO_2$ output even more than did EDTA, yet failed to synergize the IAA-induced growth. This paradox is discussed below.

In one experiment the sections were floated on 0.3 M mannitol and did not elongate, yet table II shows that evolution of $^{14}CO_2$ was no greater and the ^{14}C content in the tissue not markedly changed as compared with the normally growing control. This shows that neither decarboxylation nor IAA uptake is directly linked to growth.

Tables I and II show that the radioactivity remaining in the ambient solution varied markedly with the substances added. Since the total radioactivity in the sections shows less drastic differences than the $^{14}CO_2$ output it is important to know whether the decarboxylation took place inside the tissue, at the cut surface or in the test solution. This led to a series of experiments, which revealed that after the sections had been in the solution for 8 hours, and were then removed, the decarboxylation continued; after 24 hours the amount of $^{14}CO_2$ evolved was about as great as during the 6 to 8 hours with the sections present, and reached 30 to 60 % of the radioactivity supplied. Only in the presence of DIECA was there no further decarboxylation, whereas polyphenols, which powerfully inhibited the $^{14}CO_2$ output in presence of the sections, failed completely to do so after removal of the sections, and sometimes even increased it, i.e., they acted as substrate for the microorganisms.

To facilitate this action of the exudate on added IAA, 300 etiolated pea sections (not pre-washed) were floated on 150 ml of pH 5.9 buffer for 3 hours; the sections were then removed and cold IAA added to the solution. The disappearance of IAA was followed by adding Salkowski reagent directly to aliquots of the reaction mixture. The results (table IV) show that a sudden destruction of IAA began after about 7 hours of incubation. Chelating agents effectively inhibited this destruction, as did also previous boiling of the exudate; phenols, with or without Mn^{++}, were without effect, while Penicillin GK greatly delayed it. Evidently, then, a bacterial process was involved, the exudate simply supplying organic substrate for the microorganisms. A bacterial loss of IAA from nutrient solution after prolonged incubation (1), and the excretion of both bacteria and IAA-destroying enzyme (42) have been reported before. It is important to note that in this experiment conditions were optimal for bacterial development, no prewashing, and a pH of 5.9. In the growth experiments throughout this work, the sections were washed for 3 hours and since buffer was not added the pH approached 5.0 during the growth (cf. 31). Under these conditions the lag period was 8 to 10

Table IV. *Disappearance of IAA Added to the Exudate Solution from Pea Epicotyl Sections*

Data show the percent of the initial amount of IAA which has disappeared.

The standard reaction mixture contained 6 ml of the exudate solution (sections present for 3 hours), 1 ml of IAA solution containing 200 μg IAA, 1 ml of solution of the substance to be tested, and 2 ml of phosphate buffer pH 5.9, with or without 5×10^{-4} M $MnCl_2$.

Substances added to the exudate	Time of incubation, hrs					
	4	6	7	8	9	10
Water	0	...	14	54	86	95
Water, exudate boiled	0	...	2	3	3	5
DIECA, 2×10^{-4} M	0	...	2	2	2	2
EDTA, 1×10^{-4} M	0	...	2	2	2	3
Chlorogenic acid, 4×10^{-5} M	0	...	25	58	84	93
Sinapic acid, 4×10^{-5} M	0	...	22	50	79	93
Ferulic acid, 4×10^{-5} M	0	...	12	42	66	90
p-Coumaric acid, 4×10^{-5} M	5	5	31	68	80	...
L-Tyrosine, 1×10^{-4} M	2	3	21	58	75	...
Penicillin GK, 4×10^{-5} M	0	...	...	...	11	24
Penicillin + chlorogenic acid	0	...	...	...	14	16
Penicillin + ferulic acid	0	...	...	...	7	11

Table V. *The fate of IAA-1-^{14}C in* Avena *Coleoptile Sections*

Initial concentration of IAA-1-^{14}C applied in 5 ml of solution was 1.4×10^{-6} M, corresponding to 3290 cpm. The elongation was measured on ten 10 mm long sections. In sucrose alone they elongated 26.0 %.

Substance added	Distribution of radioactivity as cpm after 12 hrs incubation							Elongation, % of initial length
			Extracts from tissues					
	Solution	$^{14}CO_2$	Ether	Ethanol	Solid	Total tissue	Recovery %	
Control:								
(IAA + 1 % sucrose alone)	442	1456	2.2	775	52	829	82.8	56.8
Chelating agents:								
DIECA 2×10^{-4} M	867	693	15.4	815	41	872	73.8	61.3
EDTA 5×10^{-5} M	695	762	17.8	1112	39	1169	79.7	68.0
Polyphenols:								
Chlorogenic acid, 5×10^{-5} M	457	804	10.5	1028	58	1096	71.6	63.0
Caffeic acid, 5×10^{-5} M	470	854	9.0	1076	50	1135	74.6	60.0
Sinapic acid, 5×10^{-5} M	325	1097	13.8	1042	51	1107	76.8	63.3
Ferulic acid, 5×10^{-5} M	370	1090	10.0	965	39	1015	75.1	59.5
Monophenols:								
p-Coumaric acid, 5×10^{-6} M	455	1350	12.1	948	47	1007	85.4	53.5
p-OH-Benzoic acid 1×10^{-4} M	337	1315	11.9	933	39	988	80.0	55.5
2,4-Dichlorophenol, 1×10^{-4} M	330	1456	11.5	719	54	784	78.0	54.8

hours.[4] For safety, however, it should be considered that only the decarboxylation which occurs within the first 6 to 8 hours of incubation is due to the tissue itself. [For this reason the data of table III (incubated for 8 hrs) are given only in summary form; since phenols do not appear to modify the action of the bacterial enzyme the values for $^{14}CO_2$ show their effects on decarboxylation by the *Pisum* system clearly enough. Bacterial growth is also less rapid with green sections than with etiolated ones.]

Apart from the exudate, additional destruction of auxin might occur at the cut surfaces, (cf. e.g. 28) although the bulk of the IAA-destroying enzyme should have been washed out when the sections were floated on water before IAA was added. Judging from the experiments of Zenk and Müller (46) the destruction caused by the cut surfaces of sunflower hypocotyl segments is relatively low. The presence of cut surfaces did increase the output of $^{14}CO_2$ (45), but this seemed to be the result of an increased uptake of IAA.

The Influence of Phenols and Chelating Agents on IAA Metabolism in Avena *Coleoptiles.* The results with etiolated *Avena* coleoptile sections were

[4] The bacterial destruction of IAA (40 mg/l) in an *Avena* exudate with phosphate added after 17 hrs. Varied with pH as follows:– pH 3.8, 0 %; pH 4.5, 12 %; pH 5.0, 80 %; pH 5.9, 100 %. At 8 hours no destruction was detectable at any pH from 3.4 to 6.8.

essentially similar to those with *Pisum* epicotyls (table V). While the total recovery leaves a good deal to be desired, the strictly parallel treatment in each experiment gives the results validity on a relative basis. The liberation of $^{14}CO_2$ was clearly less in the presence of chelating agents and polyphenols than with monophenols, showing directly that the former substances inhibit the decarboxylation. Furthermore, the table shows a somewhat higher content of ^{14}C in the tissues treated with polyphenols, which coincides with an increase in elongation. It was also found, as with the pea sections, that the $^{14}CO_2$ output did not stop after removal of the sections from the solution, although with coleoptile sections the action of bacteria did not begin to be detectable until after 12 hours or more. The bacterial process is pH-dependent also; at the pH of 4.5 which the (unbuffered) solutions reached, the decarboxylation was only just beginning at 17 hours. Boiling the exudate, even though it was then left in non-sterile conditions, delayed the onset of decarboxylation for over 24 hours. In general, phenols somewhat promoted this later development of $^{14}CO_2$, but chelating agents depressed it (table VI). Using coleoptile sections incubated with cold IAA, it was often observed in experiments of very long duration that the presence of phenols actually accelerated the appearance of turbidity in the test solution, whereas chelating agents, especially DIECA, clearly suppressed the bacterial contamination.

Since DIECA is a powerful inhibitor of the IAA-destroying enzyme in *Pisum* stems (41) and rice coleoptiles (39), its effect on the *Avena* enzyme preparation was compared with that of the phenols. As shown in figures 1 to 3, monophenols such as *p*-coumaric acid, *p*-hydroxybenzoic acid and 2, 4-dichlorophenol cause a rapid disappearance of the Salkowski reaction, whereas polyphenols inhibit this disappearance in extremely low concentrations. Both DIECA and EDTA between 10^{-4} and 10^{-5} M caused a lag period but thereafter the destruction was rapid, and after 90 minutes the

FIG. 1-3. Effect of phenols and chelating agents on the destruction of IAA by oxidase preparation from *Avena* coleoptiles. The standard reaction mixture contained 2 ml enzyme solution, 1 ml of 10^{-3} M $MnCl_2$, 1 ml of monophenol, 1 ml of the substance to be tested (indicated for each fig) and 5 ml phosphate buffer pH 5.9 containing 250 μg IAA. There was no disappearance of IAA in the absence of Mn^{++} or monophenol. The final concentrations of the test substances are as indicated:

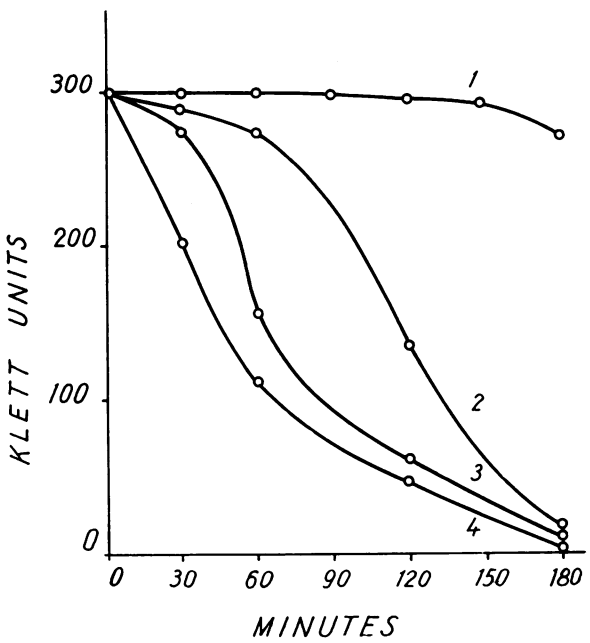

Fig. 1.
1. *p*-Coumaric acid 4×10^{-5} M, + Chlorogenic acid 1×10^{-6} M.
2. *p*-Coumaric acid 4×10^{-5} M, + Chlorogenic acid 4×10^{-7} M.
3. *p*-Coumaric acid 4×10^{-5} M, + Chlorogenic acid 2×10^{-7} M.
4. *p*-Coumaric acid 4×10^{-5} M, alone.

Table VI. *$^{14}CO_2$ Output as Percent of Initial Radioactivity of IAA-1-^{14}C Applied to Etiolated* Avena *Coleoptile Sections*

For growth conditions see Table V.

Solution	0-12 Hrs with sections	12-24 Hrs sections removed	0-24 Hrs total
Control:			
IAA alone	44.2	14.9	59.1
Chelating agents:			
DIECA, 5×10^{-4} M	21.0	5.4	26.4
EDTA, 5×10^{-5} M	22.0	8.6	30.6
Phenolic substances:			
Chlorogenic acid, 5×10^{-5} M	24.4	20.4	44.8
p-OH-Benzoic acid, 1×10^{-4} M	39.9	22.0	61.9

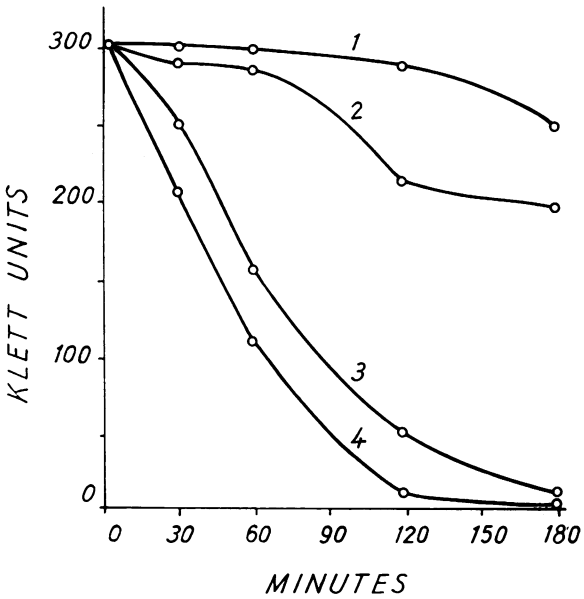

Fig. 2.
1. *p*-Hydroxybenzoic acid 4 × 10^{-4} M, + Chlorogenic acid 10^{-6} M.
2. *p*-Hydroxybenzoic acid 4 × 10^{-4} M, + Sinapic acid 2 × 10^{-6} M.
3. *p*-Hydroxybenzoic acid 4 × 10^{-4} M, + Ferulic acid 2 × 10^{-6} M.
4. *p*-Hydroxybenzoic acid 4 × 10^{-4} M, alone.

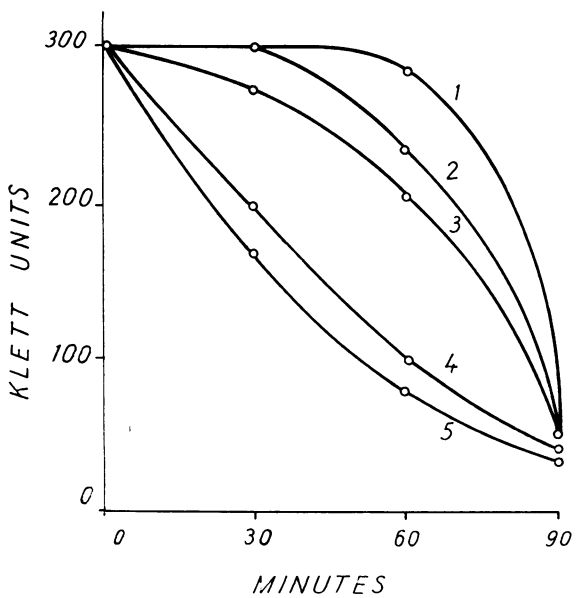

Fig. 3.
1. 2,4-Dichlorophenol 10^{-4} M, + DIECA 10^{-4} M.
2. 2,4-Dichlorophenol 10^{-4} M, + DIECA 10^{-5} M.
3. 2,4-Dichlorophenol 10^{-4} M, + EDTA 10^{-4} M.
4. 2,4-Dichlorophenol 10^{-4} M, + EDTA 10^{-5} M.
5. 2,4-Dichlorophenol 10^{-4} M alone.

level of the destroyed IAA was as high as in the absence of the chelator. Chlorogenic acid, however, shows a comparable lag period at concentrations 100 times lower, i.e. between 2 and 4 × 10^{-7} M. Actually, in 10^{-5} M chlorogenic acid there was no destruction of IAA within 24 hours of incubation. The methoxylated polyphenols were somewhat less effective than chlorogenic acid.

The behavior of DIECA is again exceptional. While EDTA markedly promotes the IAA-induced elongation of coleoptiles, DIECA shows only small effects thereon, although it is more effective as an IAA oxidase inhibitor than is EDTA (tables I and V). The explanation of this behavior is to be sought in the following way: *Avena* sections in sucrose make the solution acid, reaching a pH of about 4 (31); DIECA is unstable in a mildly acid solution and decomposes as follows: $(C_2H_5)_2NCSSH \rightarrow (C_2H_5)_2NH + COS + H_2S$. These decomposition products may act as cytochrome oxidase inhibitors (11), and it has been shown that the oxygen uptake of *Avena* coleoptiles decreases somewhat when incubated in DIECA (36). Thus, the relatively poor growth promotion may be ascribed to nonspecific inhibitory effects on the respiratory processes.

To decrease the decomposition of DIECA, experiments were performed in which 10 coleoptile sections were incubated in a large volume (20 ml) of the test solution buffered with 10^{-2} M pH 7.0 phosphate buffer. Another set was tested similarly but without sucrose and buffer, the pH being 5.6 to 6.6 depending on the concentration of the sodium salt of DIECA used (1 × 10^{-3}, 3 × 10^{-4}, 1 × 10^{-4}, 3 × 10^{-5}, and 1 × 10^{-5} M). It was found that at 3 × 10^{-4} M and above, DIECA markedly inhibited the IAA-induced growth, while at 1 × 10^{-4} M and below it had little effect. Only in 2 out of 20 experiments was the elongation due to DIECA itself significant.

Although EDTA inhibits the IAA destroying enzyme of etiolated pea stems far less effectively than do the polyphenols (table I), it is relatively more effective on *Avena* coleoptiles. Tables V and VI show that EDTA inhibits $^{14}CO_2$ production by coleoptile sections somewhat better than the most effective polyphenol (chlorogenic acid). Correspondingly, it more strongly promotes the IAA-induced growth of *Avena* coleoptiles. Since EDTA promotes the elongation even in optimal IAA concentrations (32) where polyphenols are ineffective or inhibitory (fig 4) it seems that the mechanism of its promotion may have a dual basis, a second property becoming evident where factors other than auxin become limiting.

This conclusion is strongly supported by the following observation, made in a number of growth experiments. If both EDTA and polyphenols are added, the resulting effects on growth are more than additive. For example, in one series, *Avena* sections in 2 × 10^{-7} M IAA alone elongated 73 %,

in IAA + 5×10^{-4} M sinapic acid 93 % and in IAA + 5×10^{-5} M EDTA, 88 %. These 2 increases of 20 and 15 % respectively, would be expected (when both substances were present together) to give a total increase of 35 %, or an elongation of 108 %; instead the elongation with both added was 130 %. Similar results were obtained with protocatechuic acid; alone it increased the IAA-induced growth by 17.6 %, EDTA alone by 9.9 %, and the 2 together by 37.9 %.

The Fate of IAA-1-^{14}C in the Tissues as Modified by Metallic Ions. Table VII shows the effects of metallic ions on the distribution of the radioactivity from IAA-1-^{14}C after 6 hours of incubation with coleoptile sections. Chelating agents were also included for comparison. As in table V, the depression of the $^{14}CO_2$ output caused by DIECA and EDTA resembles the results obtained with etiolated pea epicotyls. Again the decreased $^{14}CO_2$ output caused by EDTA was accompanied by an increase in radioactivity in the sections, whereas the similar decrease caused by DIECA was accompanied by a higher radioactivity in the solution. Thus, DIECA appears to prevent the uptake of the auxin. As a result, there was an appreciably increased growth in EDTA and none in DIECA.

Fe^{++} caused the greatest decrease in the decarboxylation of IAA, and at 3×10^{-4} M it apparently increased the auxin uptake. These results support the conclusions of Shibaoka and Yamaki (26) that Fe^{++} inhibits IAA oxidation in *Avena* and accelerates the uptake of auxin therein. Even the lack of any increase in elongation at 3×10^{-4} M Fe^{++} is in accordance with their findings (see their fig 1) since this ion promoted growth best when both IAA and Fe^{++} were applied in low concentrations, e.g. 10^{-5} M Fe^{++}. In our own work Fe^{++} never promoted the growth induced by optimal IAA concentrations, and at 3×10^{-4} M Fe^{++} caused loss of turgor after 24 hours of incubation.

Co^{++} caused some decrease in decarboxylation, which was accompanied by an increase in radioactivity in the ether-extractable free IAA, though the total radioactivity in the tissue was unchanged. In table VII there was no increase in elongation after 6 hours of incubation, but in other experiments with cold IAA the promotion due to Co^{++} became evident after 12 hours and was strong after 36 hours of incubation.

Mn^{++} showed little net effect on the IAA uptake but clearly enhanced the rate of decarboxylation. Consequently, it produced no increase in total radioactivity of the tissue and only a weak growth promotion. This agrees with the earlier finding (31) that Mn^{++} produces appreciable growth promotion only at relatively high concentrations, e.g. 3×10^{-3} M.

Other experiments have shown that pea epicotyl sections acted essentially like those of *Avena;* Fe^{++} at 3×10^{-5} M inhibited growth, and at 3×10^{-4} M it inhibited decarboxylation as well. Co^{++} inhibited decarboxylation, and Mn^{++} promoted it.

Since Mn enhances the rate of IAA decarboxylation both in *Pisum* and in *Avena,* its action is not simple. For the decarboxylation is in accordance with its in vitro activation of IAA oxidase, yet it does not fit with the growth promoting effects of this ion. To help elucidate this discrepancy a series of growth experiments were performed with

Table VII. *The Effects of Chelating Agents and Metallic Ions on the Fate of IAA-1-^{14}C in Oat Coleoptile Sections*

Initial concentration of IAA-1-^{14}C applied in 5 ml of solution was 3.9×10^{-6} M corresponding to 9412 cpm. The elongation was measured on thirty 10 mm sections; on the basal medium alone (1 % sucrose) they elongated 16.8 %.

	Distribution of radioactivity as cpm after 6 hrs incubation							
			Extracts from tissues			Total in tissue		Elongation, % of initial length
Substance added	Solution	$^{14}CO_2$	Ether	Ethanol	Solid		%	
Control:								
(IAA + 1 % sucrose alone)	6037	2373	107	435	127	669	96.5	45.3
Chelating agents:								
DIECA, 3×10^{-5} M	7142	1237	148	329	105	582	95.2	48.0
DIECA, 3×10^{-4} M	8332	970	99	241	26	366	102.7	41.6
EDTA, 3×10^{-5} M	6202	1803	355	602	187	1144	97.2	53.2
EDTA, 3×10^{-4} M	5482	1572	296	649	122	1067	86.3	54.6
Metallic ions:								
$FeSO_4$, 3×10^{-5} M	6360	1284	224	400	90	714	88.8	45.4
$FeSO_4$, 3×10^{-4} M	5167	1116	344	719	186	1249	80.0	39.8
$CoCl_2$, 3×10^{-5} M	5980	1774	182	270	108	560	88.3	45.0
$CoCl_2$, 3×10^{-4} M	5555	1593	239	360	114	713	83.5	41.0
$MnCl_2$, 3×10^{-5} M	4862	3607	132	376	100	608	96.4	48.6
$MnCl_2$, 3×10^{-4} M	5122	2683	205	426	95	727	90.6	50.1

FIG. 4-6. Effects of phenols, Mn^{++} and EDTA on auxin-induced growth of *Avena* coleoptile segments. Ten 10 mm long sections in 2 % sucrose and 5×10^{-3} M KH_2PO_4. Elongation as per cent increase of initial length after 24 hours. The final concentrations of the added agents are indicated below for each figure:

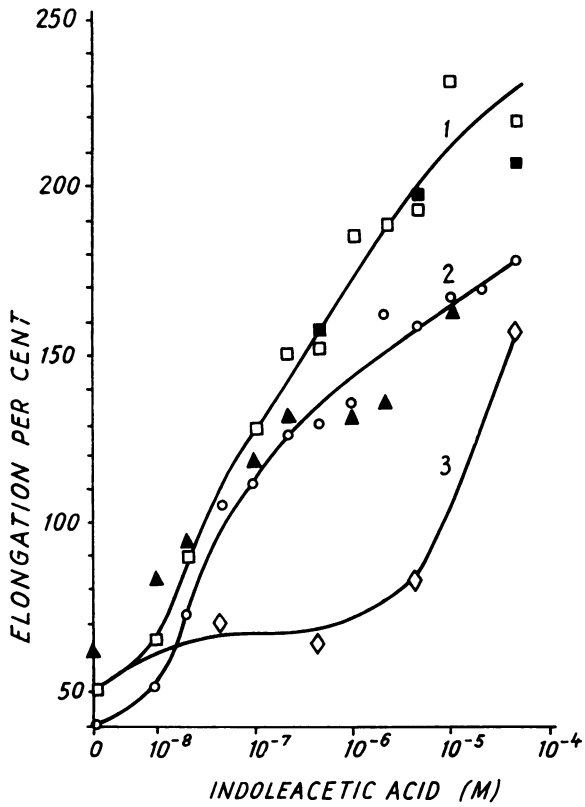

Fig. 4; auxin, IAA.
1. (Open squares) $MnCl_2$ 10^{-3} M;
   (Black Squares) EDTA 5×10^{-5} M
2. (Circle) None,
   (triangles) Sinapic acid 2×10^{-5} M
3. $MnCl_2$ 10^{-3} M plus *p*-Coumaric acid 2×10^{-5} M

coleoptile sections (figs 4, 5, 6). The following results were obtained:

Firstly, it was confirmed that the IAA-induced growth was enhanced best by relatively high Mn^{++} concentrations, the optimum being about 3×10^{-3} M. Manganese also increased the elongation of coleoptile sections in absence of IAA, the optimal increase of 40 % being achieved at 3×10^{-4} M $MnCl_2$. Since at this concentration and above, Mn^{++} causes a lag period in the kinetics of the IAA oxidase (12), this effect may be exerted via the endogenous auxin.

Secondly, Mn^{++} enhanced the IAA-induced growth even at a supraoptimal concentration of auxin, and indeed better there than at suboptimal auxin levels (fig 4). In this respect the action of Mn^{++} resembles that of EDTA rather than that of polyphenols. EDTA does not further enhance the growth in presence of optimal Mn^{++} (fig 4).

Thirdly, *p*-coumaric acid dramatically decreases the growth response brought about by IAA plus Mn^{++}. This fits with its powerful IAA-destroying action in figure 1. Sinapic acid, on the other hand, promotes elongation at the lowest IAA levels, though its effect is less than in table V (fig 4). It should be noted, though, that sinapic acid evidently exerts a secondary unfavorable effect on growth, since it produces a small but real inhibition of the growth caused by NAA and 2,4-D (figs 5, 6). Its marked promotion of growth of pea sections in tables I and II suggests that this secondary effect is slight or absent in *Pisum*.

Fourthly, Mn^{++} somewhat enhances the growth induced by NAA (fig 5) and definitely that due to 2,4-D (fig 6). In this respect its action differs from that of EDTA and polyphenols which do not synergize with these auxins, as was shown for EDTA some years ago (32). In many respects, therefore, except for the effective concentrations,

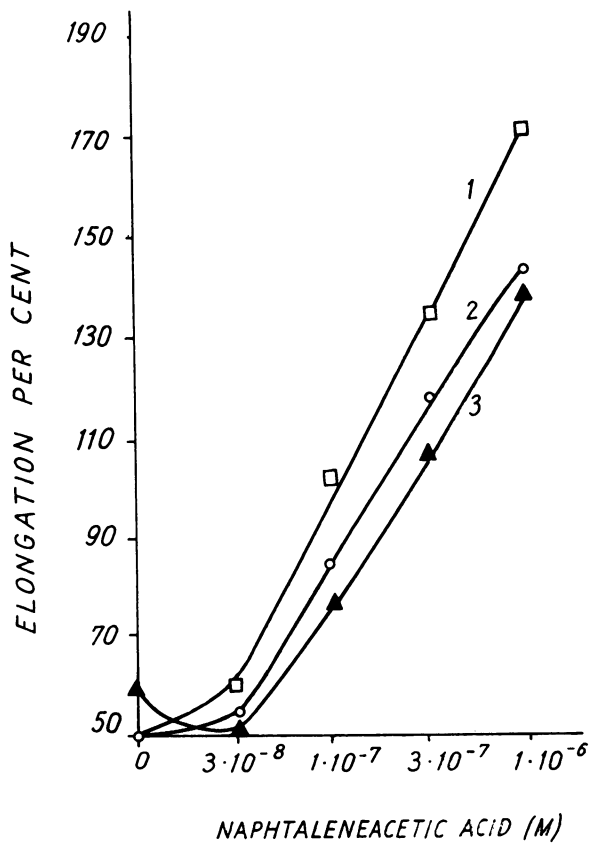

Fig. 5; auxin, NAA.
1. $MnCl_2$ 10^{-3} M
2. None
3. Sinapic acid 2×10^{-5} M

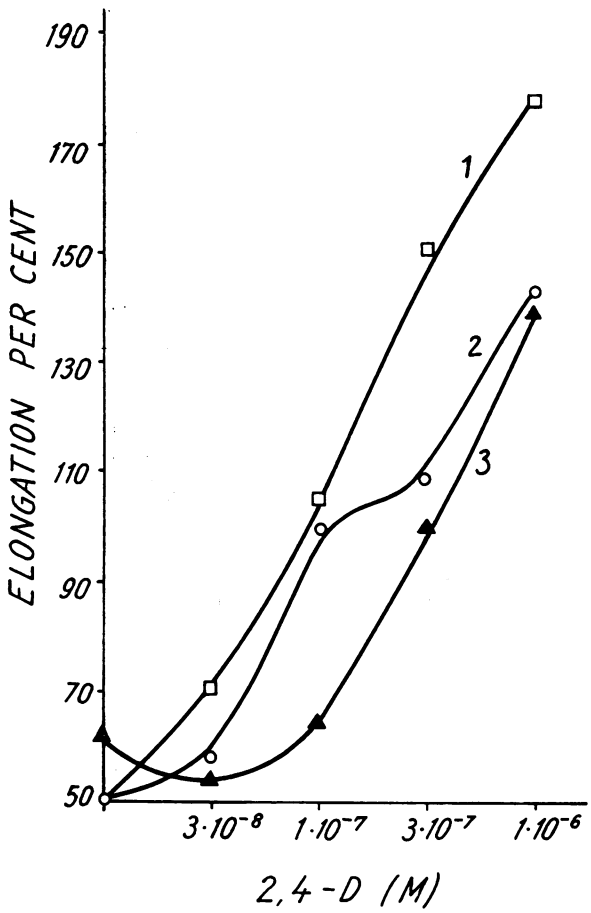

Fig. 6; auxin, 2,4-D.
1. $MnCl_2$ 10^{-3} M
2. None
3. Sinapic acid 2×10^{-5} M

which differ by 2 powers of 10, the action of manganese is comparable with that of cobalt, and evidently comprises 2 factors of opposite sign, namely an enhancement of the decarboxylation of IAA, and a promotion of some growth process which does not depend on the specific auxin used.

Literature Cited

1. ANDREAE, W. A. AND M. W. VAN YSSELSTEIN. 1960. Studies on 3-indoleacetic acid metabolism. VI. 3-Indoleacetic acid uptake and metabolism by pea roots and epicotyls. Plant Physiol. 35: 225–32.
2. BARDINSKAYA, M. S., L. D. PRUSAKOVA, AND T. A. SHUBERT. 1962. Concerning interaction between ferulic acid, gibberellin and indoleacetic acid in plant growth process. Dokl. Akad. Nauk Uz. SSR 146: 145–48.
3. BRIGGS, W. R. AND P. M. RAY. 1956. An auxin inactivating system involving tyrosinase. Plant Physiol. 31: 165–67.
4. BUFFEL, K. AND J. C. VENDRIG. 1963. The hydroxycinnamic acids and other phenols as plant growth regulators. Mededel. Koninkl. Vlaam. Acad. Wetenschap. Belg. 25: 1–34.
5. CONSTABEL, F. 1965. Physiologische Reaktionen von Gewebekulturen auf Catechin. Planta 64: 62–68.
6. EICHENBERGER, E. AND K. V. THIMANN. 1957. Terminal Oxidases and Growth in Plant Tissues. IV. On the terminal oxidases of etiolated pea internodes. Arch. Biochem. Biophys. 67: 466–78.
7. FURUYA, M., A. W. GALSTON, AND B. B. STOWE. 1962. Isolation from peas of co-factors and inhibitors of indolyl-3-acetic acid oxidase. Nature 193: 456–57.
8. GALSTON, A. W. AND R. KAUR. 1961. Comparative studies on the growth and light sensitivity of green and etiolated pea stem sections. In: Light and Life, John Hopkins Press, p 687–705.
9. GOLDACRE, P. L., A. W., GALSTON, AND R. L. WEINTRAUB. 1953. The effect of substituted phenols on the activity of IAA oxidase of peas. Arch. Biochem. Biophys. 43: 358–73.
10. HEMBERG, T. 1951. Establishment of acid growth-inhibiting substances in plant extracts containing auxins by means of *Avena* test. Physiol. Plantarum 4: 437–45.
11. JAMES, W. O. AND N. GASTON. 1952. The use of sodium diethyldithiocarbamate as respiratory inhibitor. J. Exptl. Botany 3: 310–18.
12. KENTEN, R. H. 1955. The oxidation of indolyl-3-acetic acid by waxpod bean root sap and peroxidase system. Biochem. J. 59: 110–21.
13. LAYNE, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In: Methods of Enzymology 3. Academic Press, New York. p 447–54.
14. LEE, T. T. AND F. SKOOG. 1965. Effects of substituted phenols on bud formation and growth of tobacco tissue cultures. Physiol. Plantarum 18: 386–402.
15. MANICOL, P. K. AND J. REINERT. 1963. Untersuchungen über Auxinoxydasen. II. Auftrennung und IES-Oxydaseaktivität der multiplen Peroxydase des etiolierten Erbsensprosses. Z. Naturforsch. 18b: 572–79.
16. MCCUNE, D. C. 1961. Multiple peroxidases in corn. Ann. N. Y. Acad. Sci. 94: 723–30.
17. MUMFORD, F. E. AND H. M. STARK. 1963. 4-Hydroxybenzyl alcohol, a naturally occurring cofactor of indoleacetic acid oxidase. Phytochemistry 2: 215–20.
18. NITSCH, J. P. AND C. NITSCH. 1960. Action de quelques flavonoides sur la croissance de tissue de topinambour cultivés in vitro. Bull. Soc. Botan. France 107: 326–30.
19. NITSCH, J. P. AND C. NITSCH. 1962. Composés phenoliques et croissance végétale. Ann. Physiol. Végétale 4: 211–25.
20. PAULET, P. AND J. P. NITSCH. 1963. Etude preliminaire du bourgeonnement in vitro du *Nicotiana glauca, N. suaveolens* et leur hybride. Bull. Soc. Botan. France 110: 361–66.
21. PILET, P. E. 1964. Effect of chlorogenic acid on the auxin catabolism and the auxin content of root tissues. Phytochemistry 3: 617–21.
22. RAY, P. M. 1958. Destruction of Auxin. Ann. Rev. Plant Physiol. 9: 81–118.

23. ROUBAIX, J. DE AND O. LAZAR. 1960. The inhibitory substances contained in sugar beet glomerules. In: Phenolics in Plants in Health and Disease. Pergamon Press, Oxford, p 35–41.
24. SEQUEIRA, L. AND L. MINEO. 1965. Partial purification of indoleacetic acid oxidase from tobacco roots. Plant Physiol. 40: lxxi.
25. SHANTZ, E. M. AND F. C. STEWARD. 1964. The growth promoting substances from the environment of the embryo. In: Régulateurs Naturels de la Croissance Végétale. C.N.R.S. Paris, p 59–75.
26. SHIBAOKA, H. AND T. YAMAKI. 1959. Mechanism of growth promotion by ferrous sulfate. Botan. Mag. (Tokyo) 72: 203–14.
27. STAFFORD, H. A. 1951. Intracellular localization of enzymes in pea seedlings. Physiol. Plantarum 4: 696–741.
28. STEEVES, T. A., G. MOREL, AND R. H. WETMORE. 1955. A technique for preventing inactivation at the cut surface in auxin diffusion studies. Am. J. Botan. 40: 536–38.
29. STOWE, B. B. 1963. Synthesis of high activity ^{14}C-carboxyl indoleacetic acid and ^{14}C-nitrile indoleacetonitrile. Anal. Biochem. 5: 107–15.
30. THIMANN, K. V. 1964. Studies on the movement of auxin in tissues and its modifications by gravity and light. In: Régulateurs Naturels de la Croissance Végétale C.N.R.S., Paris, p 577–85.
31. THIMANN, K. V. 1956. Studies on the growth and inhibition of isolated plant parts. V. The effect of cobalt and other metals. Am. J. Botan. 43: 241–50.
32. THIMANN, K. V. AND N. TAKAHASHI. 1961. Interrelationships between metallic ions and auxin action, and the growth promoting action of chelating agents. In: Plant Growth Regulation. Iowa State University Press, p 367–77.
33. THIMANN, K. V., M. TOMASZEWSKI, AND W. L. PORTER. 1962. Growth promoting activity of caffeic acid. Nature 193: 1203.
34. THIMANN, K. V. AND I. F. WARDLAW. 1963. Effect of light on the uptake and transport of indoleacetic acid in the green stem of the pea. Physiol. Plantarum 16: 368–77.
35. THIMANN, K. V. AND M. WICKSON. 1960. The antagonism of auxin and kinetin in apical dominance. II. The transport of IAA in pea stems in relation to apical dominance. Physiol. Plantarum 13: 539–54.
36. TOMASZEWSKI, M. 1961. A relationship between the phenol-phenolase system and the processes of respiration, formation of lignin and auxin inactivation in apricot and peach shoots. Arboretum Kórnickie 6: 169–225.
37. TOMASZEWSKI, M. 1964. The mechanism of synergistic effects between auxin and some natural phenolic substances. In: Régulateurs Naturels de la Croissance Végétale. CNRS, Paris, p 335–51.
38. VENDRIG, J. C. AND K. BUFFEL. 1961. Growth activity of *trans*-caffeic acid isolated from *Coleus rhenaltianus*. Nature 192: 276–77.
39. WADA, S. 1961a. Some characteristics of indoleacetic acid oxidase in rice coleoptiles. Sci. Rep. Tôhoku University, Biol. 27: 223–35.
40. WADA, S. 1961b. IAA-oxidase inhibitors contained in rice coleoptiles. Sci. Rep. Tôhoku University, Biol. 27: 237–49.
41. WAGENKNECHT, A. C. AND R. H. BURRIS. 1950. Indoleacetic acid inactivating enzymes from bean roots and pea seedling. Arch. Biochem 25: 30–53.
42. WICHNER, S. AND E. LIBBERT. 1964. Hinweise zur Exoenzym-Natur einer IES-Oxydase. Naturwiss. 51: 268.
43. WINTER, A. AND K. V. THIMANN. 1966. Bound indoleacetic acid in *Avena* coleoptiles. Plant Physiol. 41: 335–42.
44. ZENK, M. H. 1964. Isolation, biosynthesis and function of indoleacetic acid conjugates. In: Régulateurs Naturels de la Croissance Végétale. CNRS, Paris, p 241–50.
45. ZENK, M. H. AND G. MÜLLER. 1963. In vivo destruction of exogenously applied indolyl-3-acetic acid as influenced by naturally occurring phenolic acids. Nature 200: 761–63.
46. ZENK, M. H. AND G. MÜLLER. 1964. Über den Einfluss der Wündflachen auf die enzymatische Oxydation der Indol-3-Essigsäure in vivo. Planta 61: 346–51.