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INHIBITORY PRODUCTS OF THE ACTION OF PEROXYACETYL NITRATE UPON INDOLE-3-ACETIC ACID

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Abstract—Peroxyacetyl nitrate reacted *in vitro* with indole-3-acetic acid giving five products, three of which inhibited 2,4 dichlorophenoxyacetic acid-induced growth of oat coleoptiles. One product was identified as 3-hydroxymethyloxindole

INTRODUCTION

INVESTIGATIONS on peroxyacetyl nitrate (PAN) a component of polluted urban air, have shown a variety of effects upon biologically important compounds. It may inhibit enzymes both *in vivo* and *in vitro*.[1–5] and it has been shown to oxidise various amino acids and peptides.[6] Ordin and Propst[7] showed that PAN could irreversibly abolish the biological activity of the plant growth hormone, indole-3-acetic acid (IAA); further, Fukuyama and Moyed[8] demonstrated that light-catalysed oxidation of IAA led to the formation of oxindolic products which had similar spectral characteristics to those given by the products of PAN oxidation of IAA. The work described here had the purpose of determining the nature of the reaction of PAN with IAA, isolating and identifying the products of the reaction as far as possible and testing their effect in a biological system.

RESULTS AND DISCUSSION

Figure 1 shows the time course of oxidation of IAA by PAN as seen by u.v. spectra. The extinction of the IAA peak at 280 nm is parallelled by the appearance of a peak at 250 nm.

It was found that gassing a range of concentrations of IAA produced substances which inhibited 2,4-D induced extension growth of *Avena* coleoptiles.

Table 1 shows the results of such experiments. The range of concentrations found to be effective was from $1{\cdot}2 \times 10^{-5}$ M converted IAA and upwards although some slight inhibition was observed at $2{\cdot}3 \times 10^{-6}$ M converted IAA, but only after 20 hr incubation. In general, at the highest concentration of 2,4-D (3×10^{-4} M) values for elongation in all

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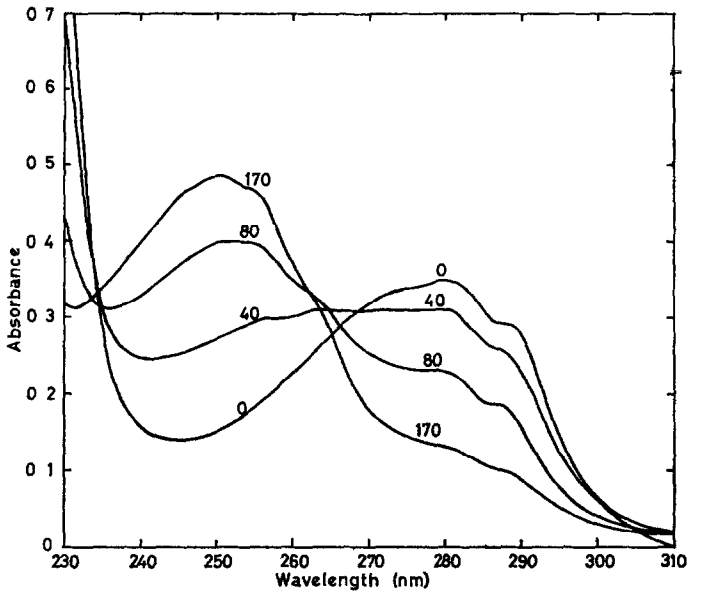

FIG. 1. U.V. SPECTRA OF SAMPLES DERIVED FROM IAA GASSED WITH PAN FOR VARIOUS TIMES. IAA, 1·2 × 10^{-5} M in 100 ml of solution was gassed with 32 ppm PAN flowing at 400 ml/min and samples removed at intervals between 0 and 170 min. Gassed buffer was used in the reference cuvette.

treatments tend to converge—probably as a consequence of a general inhibition by the growth regulator at this level. At the lowest concentration of 2,4-D used (6 × 10^{-7} M), although substantial inhibition occurs in inhibitor treated sections, a large proportion of this seems to be due to dissolved PAN or its breakdown products in that there is a considerable inhibition in the gassed buffer controls. At concentrations between these extremes however, the inhibition seems to be due to the IAA breakdown products.

Chromatography of oxidation products in isopropanol–water (5:95, v/v) yielded five discrete bands visible in u.v. light, designated compounds 1–5 respectively (see Table 3). In both solvents used, Compound 4 had the same R_f as the compound reported by Fukuyama and Moyed[8] to be 3-hydroxymethyloxindole. Its u.v. spectrum showed peaks at 210 and 248 nm and a shoulder at 280 nm. This is the same as that reported by Fukuyama and Moyed. Its i.r. spectrum was characteristic of an oxindole; furthermore, incubation of the compound in 0·1 M phosphate buffer pH 8·8 caused a change in the spectral pattern from the maximum at 248 nm to the double maxima at 248 and 254 nm characteristic of 3-methyleneoxindole.[9] It seems likely that compound 4 is 3-hydroxymethyloxindole.

The remaining compounds were not obtained in sufficiently large amounts to permit extensive chemical analysis, but i.r. and u v. spectra (Table 2) indicate they are similar to 3-hydroxymethyloxindole.

The effect of the five compounds upon *Avena* coleoptile extension growth is shown in Table 3. It may be seen that compounds 1, 3 and 4 inhibit growth to some extent, although none of the compounds gives as substantial an inhibition as the same concentration of

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TABLE 1. EFFECT OF IAA GASSED WITH PAN UPON 2,4-D INDUCED GROWTH OF *Avena* COLEOPTILES

Conc. of 2,4-D M	Change in length of coleoptiles (mm)														
	$2{\cdot}2 \times 10^{-6}$ M converted IAA					$1\,2 \times 10^{-5}$ M converted IAA					$5\,7 \times 10^{-5}$ M converted IAA				
	C	GB	GB as a % of C	I	I as a % of C	C	GB	GB as a % of C	I	I as a % of C	C	GB	GB as a % of C	I	I as a % of C
3×10^{-4}	1·33	1·24	93	1 40	95	0 90	0 33	37*	0 80	89	0 73	0 66	91	0·70	96
6×10^{-5}	1·73	1 50	87	1 71	99	1 55	1 50	97	1 15	75*	1 47	1·42	97	1 05	71*
3×10^{-5}	1 48	1·87	127*	1 54	104	1 45	1 50	103	1 05	72*	1 56	1 65	106	1·11	71*
$1{\cdot}2 \times 10^{-5}$	1 84	1 87	102	1·44	77*	1·75	1 60	92	0 90	51*	1 53	1 51	99	0 68	44*
6×10^{-6}	1·78	1·88	106	1 29	73*	1 40	1 40	100	0 60	43*	1 46	1 41	97	0 64	44*
6×10^{-7}	1 45	1 16	80†	0 87	60*	1 20	0 80	67*	0 30	25*	1 09	0 75	69*	0 26	24*

IAA at the concentrations shown was gassed with PAN until its absorption peak at 280 nm was extinguished A range of concentrations of 2,4-D were then added to portions of the gassed IAA, 20 5-mm coleoptile sections were put in and their growth recorded after 20 hr.

C—plain buffer.

GB—gassed buffer.

I—gassed IAA.

* Significantly different from control at 0·01 level of probability.

† Significantly different from control at 0 05 level of probability.

TABLE 2 SPECTRAL CHARACTERISTICS OF PRODUCTS FORMED BY THE ACTION OF PAN UPON IAA

Compound	Ultraviolet absorption (95% ethanol) max (nm)			Infra-red absorption, cm^{-1} (KBr)					
I	205	245	287sh	3450 s 1480 w 830 w	2930 w 1460 w 780 w	2860 w 1380 s 750 m	1720 w 1345 w	1660 w 1115 m	1610 m 1035 w
II	214	244	290sh	3410 s 1680 w 1380 s 1130 m 945 w 770 w	2930 w 1615 w 1345 s 1110 m 870 w 750 w		1720 w 1510 w 1285 w 1030 w 830 w 660 w		1710 w 1430 w 1200 m 1000 w 785 w
III	210	243 248	298	1620 s 1290 w 940 w	3460 s 1460 m 1190 w 760 w		2920 w 1380 s 1110 w 750 w		1730 s 1330 m 1090 m 650 w
IV	210	248	280sh	3420 s 1620 m 1115 m	2930 w 1480 m 1040 w		2850 vw 1385 s 750 m		1715 m 1190 w
V	210	246	294	3360 s 1620 m 1350 w 750 m	2930 w 1505 w 1310 w		1710 m 1470 m 1190 w		1660 w 1380 m 1065 m

TABLE 3 EFFECT OF OXIDATION PRODUCTS OF IAA UPON GROWTH OF *Avena* COLEOPTILES

Compound	R_f in *iso* PrOH–H_2O (5 95)	Growth 0–20 hr (% of control)
1	0 28	72*
2	0 37	95
3	0 60	66*
4	0 72	76*
5	0 85	96
1,2,3,4 and 5 combined	—	46*
Unseparated mixture	—	54*
IAA	0 86	104
None	—	100

Coleoptiles were incubated in pretreatment solution containing 20 ppm of the compound tested in 25 mM KCl and 2 5 mM KH_2PO_4, pH 4 8. For the combination of compounds 1, 2, 3, 4 and 5 each was at a concentration of 4 ppm After 2 hr the solutions were made 10^{-5}M with respect to 2,4-D. Measurements were made of total increase in length after a further 18 hr.

* Significantly different from control at 0 01 level of probability.

unseparated mixture (20 ppm). Recombination of all five compounds each at a concentration of 4 ppm also gave a much greater inhibition than the compounds singly.

Gassing of indole-3-acetic acid 2-^{14}C with PAN showed that the primary stable product was 3-hydroxymethyloxindole followed by compound 3 and then by compound 5. Radioactivity at the origin increased as gassing proceeded, possibly as a consequence of polymerisation of oxindoles, an effect which has been noted by Hinman and Bauman[10] with 3-methylene oxindole. Compounds 1 and 2 did not appear as radioactive peaks and it therefore seemed likely that these compounds lacked a methylene carbon at the 3 position.

In order to determine whether the effect of PAN upon IAA could be attributed to action by the NO_2 anion alone, different concentrations of IAA were incubated with 10^{-3} or 10^{-4} M KNO_2 at pH 4·8. Oxidation occurred but the nature and relative amounts of the compounds formed were largely different from those formed by the action of PAN. Changes in IAA and NO_2 concentrations caused substantial variation in the ratio of products. formed. Hinman and Lang[11] report that 3-methyleneoxindole is formed from IAA under these conditions, but that the reaction does not occur at high concentrations of nitrite and IAA. The peroxy moiety of PAN therefore seems to account for a large proportion of the oxidation.

The results we have obtained on the oxidation of IAA by PAN are similar to results reported by Fukuyama and Moyed[8] on photo-oxidation of IAA and the results of Hinman and Lang[11] on the action of horseradish peroxidase upon IAA. In all three cases it seems that 3-hydroxymethyloxindole is an important primary product. Further, it was shown by Still, Fukuyama and Moyed[9] that the action of 3-hydroxymethyloxindole upon plants and bacteria may be attributed to its conversion to 3-methyleneoxindole, a potent inhibitor of sulphydryl groups. In a recent paper Moyed and Tuli[12] summarized the effects of 3-methyleneoxindole showing that it could react with certain enzymes causing desensitization to end product control, suggesting thereby stimulation of processes held back by excessive feedback inhibition. They have further shown that 3-hydroxymethyloxindole and 3-methyleneoxindole occur naturally in some higher plants.

If the products described above are formed *in vivo* from endogenous IAA the results could offer a partial explanation of the inhibitory effects of PAN upon plants.

EXPERIMENTAL

PAN was produced by the method of Stephens, Darley, Taylor and Scott[13] and dispensed from nitrogen pressurised stainless steel cylinders. IAA-2^{14}C with a specific activity of 37 8 mcm/M was obtained from Nuclear Chicago Corporation. This preparation ran as a single band in both chromatographic systems used.

Oat seedlings (*Avena sativa* L. var Segerhavre) were grown and prepared for bioassay as described by Ordin and Propst.[7]

Radioactive IAA (2μc) was gassed with PAN delivered directly from a cylinder IAA-2^{14}C was combined with carrier and converted to the potassium salt The radioactive solution (8 ml) was contained in a glass vessel into which were inserted electrodes connected to a radiometer titrator. The reaction mixture was agitated by means of a magnetic stirrer PAN was used at a concentration of 1580 ppm and a flow rate of 70 ml/min During gassing, samples were removed at intervals, immediately frozen and lyophilized The lyophilizates were extracted thrice with EtOH–Et_2O (3 1) Chromatography was carried out by the descending technique (at 2°) on Whatman 3MM strips using *iso*-PrOH–H_2O (5·95); at other times *n*-BuOH–benzene–MeOH–H_2O (2 3·4:1) was used. Chromatograms were scanned with a Vanguard autoscanner.

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For large scale preparations, 100 mg of the K salt of IAA was dissolved in 2 l. of water contained in special gas washing cylinders through which PAN diluted with air was passed. The pH was maintained at 4 8 by the addition of 1N KOH. Washing cylinders were fitted with an electrode (Radiometer GK 2021 C) and a capillary side arm, the latter permitting entrance of KOH into the cylinder from a burette via a magnetic valve. Samples were removed periodically and the progress of the reaction followed spectrophotometrically. Gassing was continued for various periods of time always at least until the absorption due to IAA at 280 nm ceased to decrease

When compounds were to be extracted and analysed the final reaction mixture was lyophilized and then extracted thrice with 500 ml portions of EtOH–Et_2O (3 1) The solvent was reduced in volume in a flash evaporator at 30°, applied to sheets of Whatman No 3MM and chromatographed as described above. The compounds which separated were located under u.v light, cut out and eluted with 80% ethanol. Rechromatography and re-elution were carried out and the compounds taken to dryness over P_2O_5 U v. spectra were obtained in 95% aq. EtOH. I.r. spectra were obtained by making KBr discs.

In growth experiments, different concentrations of IAA in 100 ml of 25 mM KCl + 2 5 mM KH_2PO_4 solution were gassed with 30–45 ppm PAN at 400 ml per minute approximately 4–5 hr as above until the absorption peak at 280 nm was extinguished. 2,4-D was then added to these solutions. 2,4-D was used instead of IAA because slow oxidation of the latter occurs in the presence of nitrite which is present in PAN treated samples Solutions containing no inhibitor were used as controls. Buffer which has been gassed with PAN at the same rate and over the same period as the IAA was used as a further control. Coleoptile sections were added to each treatment and growth was measured over a 20 hr period

In some cases compounds isolated by chromatography were tested in the *Avena* straight growth test. The compounds were taken up in 20 ml 25mM KCl and 2 5 mM KH_2PO_4 pH 4 8 at a final concentration of 20 ppm. Sections were then put in and after 2 hr the solutions were made 10^{-5}M with respect to 2,4-D. After 20 hr the change in length of the coleoptiles was measured.

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