The extent of mercapturic acid formation in the chlorobenzene series appears to be greatest with monochlorobenzene, and only little, if any, is formed by the polychlorobenzenes (Jondorf *et al.* 1955). In contrast, very little mercapturic acid is formed by the monochloronitrobenzenes but considerable amounts are formed by many polychloronitrobenzenes.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-chloronitrobenzene has been studied in the rabbit within the limits imposed by the toxicity of these compounds.

2. The main products excreted in urine are phenols conjugated with glucuronic and sulphuric acids. Several amino- and nitro-chlorophenols have been identified by paper chromatography. 2-Amino-5-chlorophenol has been isolated from urine of rabbits dosed with *p*-chloronitrobenzene.

3. From all three isomers about 10% of the dose was excreted as free chloroaniline.

4. Small amounts of nitrophenylmercapturic acid are formed from *o*- and *p*-chloronitrobenzene.

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Some Chemical and Physiological Properties of 3-Indolylpyruvic Acid

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Stowe & Thimann (1953, 1954) claim to have demonstrated the presence of 3-indolylpyruvic acid (IPyA) in maize endosperm and consider (1953) that it may be identical with the accelerator-α of Bennet-Clark & Kefford (1953) and with other growth promoters described by Lexander (1953) and Terpstra (1953). Our work on the preparation, chemical and biological properties and chromatographic behaviour on paper of IPyA indicates that the presence of IPyA cannot be demonstrated by the methods used by Stowe & Thimann, and that it differs from accelerator-α in its effect on root growth.

EXPERIMENTAL

Chemical

Methods for the preparation of IPyA described by Ellinger & Matsuoka (1920) and by Gränacher, Gerö & Schelling (1924) were found to be unsatisfactory.

All melting points are uncorrected and were determined on a Kofler block.

Preparation of IPyA. Indole-3-aldehyde was condensed with hydantoin to give indolylidene hydantoin (Boyd & Robson, 1935). A solution of indolylidene hydantoin (15·3 g.) in air-free 0·5N-NaOH (1 l.) was kept at 80–90° under N_2 for 20 hr. It was then cooled to 0°, treated with ice-cold 3N-H_2SO_4 (200 ml.) and extracted successively with 300 and 100 ml. of ether. The combined ether extracts were washed with water, dried over anhydrous Na_2SO_4, and the ether was boiled off under reduced pressure. The residue was treated with warm acetone (30 ml.), the solution filtered, and the filtrate treated with acetic acid (40 ml.). After leaving at 0° for 1 hr. the IPyA was filtered off, well washed with acetic acid, and dried at 100°/0·1 mm. Mg over KOH pellets. The pale-brown powder thus obtained is essentially pure IPyA, dec. 190–208°: the yield was 6·4 g. (46%). Further crystallization of this product from acetone–acetic acid (1:1), followed by a crystallization from acetone, gave pale-yellow leaflets (3·65 g.); light absorption: maxima at 2320 and 3280–3290 Å (ε 27300 and 20100 respectively) in ethanol, and at 2320 and 3280 Å (ε 27100 and 22600) in a mixture of N-HCl and ethanol (1:9). (Found: C, 64·8; H, 4·7; N, 6·8. Calc. for $C_{11}H_9O_3N$: C, 65·0; H, 4·5; N, 6·9%.) A colourless product can be obtained by two further crystallizations from acetone. Melting point cannot be used as a criterion of purity, for the compound appears to decompose with melting over a range which depends on crystal size and rate of heating; the purest specimen (finely powdered) decomposed with melting over the range 193–209° (at approx. 5°/min. rise in temp.).

Characterization of IPyA. Since the substance described by Ellinger & Matsuoka (1920) and Gränacher *et al.* (1924) as IPyA was inadequately characterized, it was necessary to show that the substance obtained by us had in fact this constitution.

Oxidative decarboxylation of IPyA. A solution of IPyA (0·03 g.) in 0·5N-NaOH (2 ml.) was treated with 15% (w/v) H_2O_2 (0·5 ml.). After 30 min. the solution was acidified with N-H_2SO_4, and extracted exhaustively with ether. The extract was dried over anhydrous $MgSO_4$, evaporated to dryness, and recrystallized from $CHCl_3$ as colourless plates, m.p. 165–167°, unchanged by admixture with an authentic sample of 3-indolylacetic acid.

Preparation of the methyl ester of IPyA. A solution of diazomethane in ether was added to IPyA (0·15 g.) until the yellow colour of diazomethane persisted. The solution was immediately evaporated under reduced pressure, and the crystalline residue crystallized from a mixture of equal quantities of ether and light petroleum, b.p. 40–60°, to give the ester (0·1 g.) as colourless needles. After recrystallization from $CHCl_3$ the *methyl 3-indolylpyruvate* had m.p. 156–158°, light-absorption max. 2300 and 3280 Å (ε 20500 and 21300 respectively) in 95% ethanol. (Found: C, 66·6; H, 5·1; N, 6·3. $C_{12}H_{11}O_3N$ requires C, 66·35; H, 5·1; N, 6·4%.) This compound reduced ammoniacal $AgNO_3$ immediately in the cold, and gave a purple colour with ethanolic $FeCl_3$.

Reduction of methyl 3-indolylpyruvate. Methyl 3-indolylpyruvate (0·1 g.) was dissolved in ethanol (10 ml.) and sodium borohydride (0·02 g.) added in portions with shaking. After allowing the mixture to stand for 1 hr., the excess sodium borohydride was decomposed with a few drops of dilute acetic acid. Ethanol was removed *in vacuo*, water (6 ml.) added, and the mixture extracted with ether. After drying over anhydrous $MgSO_4$ the ethereal extract was evaporated to give methyl 3-indolyllactate as a low-melting glass characterized by hydrolysis to 3-indolyllactic acid and by further reduction to 3-(3′-indolyl)propane-1:2-diol.

Hydrolysis of methyl 3-indolyllactate. Methyl 3-indolyllactate (0·1 g.) was refluxed for 30 min. with 3% NaOH solution (8 ml.). After cooling, acidification followed by extraction with ether yielded 3-indolyllactic acid, which crystallized from ethyl acetate as prisms, m.p. 146–147° (Bauguess & Berg, 1934, give 144–145°). (Found: C, 64·3; H, 5·4; N, 6·6. Calc. for $C_{11}H_{11}O_3N$: C, 64·4; H, 5·4; N, 6·8%.) The compound can be obtained directly but less conveniently by sodium amalgam reduction of IPyA.

Preparation of 3-(3′-indolyl)propane-1:2-diol. Methyl 3-indolyllactate (0·2 g.) was refluxed in ether with lithium aluminium hydride (0·07 g.) for 1½ hr. The reaction mixture was cooled and the excess of lithium aluminium hydride decomposed by addition of water. A portion of 10% (w/v) NaOH solution (10 ml.) was added, the ether layer separated, and the aqueous layer extracted three times with ether. The combined ether extracts were dried (anhydrous $MgSO_4$) and evaporated to dryness. The residue crystallized from benzene as colourless plates, m.p. 99°, undepressed on admixture with an authentic specimen prepared by osmium tetroxide hydroxylation of 3-allylindole, as described by Brown, Henbest & Jones (1952). Yield 0·08 g. (47%). (Found: C, 68·7; H, 6·6; N, 7·4. Calc. for $C_{11}H_{13}O_2N$: C, 69·0; H, 6·8; N, 7·4%.)

Paper chromatography

The solvent systems used were (*A*) *iso*propanol–NH_3 soln. (sp.gr. 0·880)–water (8:1:1, by vol.); (*B*) acetic acid–water (1:3, v/v). Whatman's no. 1 paper was used throughout. All runs were carried out at room temperature (approx. 15°), except where otherwise stated.

Reagents for spraying. (1) Ammoniacal $AgNO_3$ was made by adding 50 ml. of NH_3 soln. (sp.gr. 0·880) to 100 ml. of N-$AgNO_3$. (2) Ehrlich's reagent. A solution of 2 g. of *p*-dimethylaminobenzaldehyde in a mixture of 20 ml. of HCl (sp.gr. 1·18) and 80 ml. of ethanol. (3) Nitrous–nitric acid. 1 g. of KNO_2 dissolved in 20 ml. of HNO_3 (sp.gr. 1·42) was diluted to 200 ml. (4) Dilute Salkowski reagent. A mixture of 2 ml. of $FeCl_3,6H_2O$ (0·05M) and 100 ml. of 5% (w/v) $HClO_4$. (5) Concentrated Salkowski reagent. A mixture of 1 ml. of $FeCl_3,6H_2O$ (0·5M) and 50 ml. of 35% (w/v) $HClO_4$. This reagent was diluted with an equal volume of ethanol before spraying.

One-dimensional chromatography of IPyA. IPyA (2 mg.) was divided between 12 spots and run upwards in solvent *A* until the solvent front had moved 23 cm. The paper was cut into strips and each strip eluted with water (12 ml.) for the bio-assay (Fig. 1, Table 1).

Note. For the identification of spot 6 (Table 1) observed in the one-dimensional chromatogram, 3-indolylglycollic acid was prepared in alkaline solution without isolation. Ethyl 3-indolylglyoxylate (10 mg.) was dissolved in ethanol (1 ml.) and sodium borohydride (10 mg.) added. The solution was boiled for 1 min., cooled, and then made up to 2·5 ml. with N-NaOH.

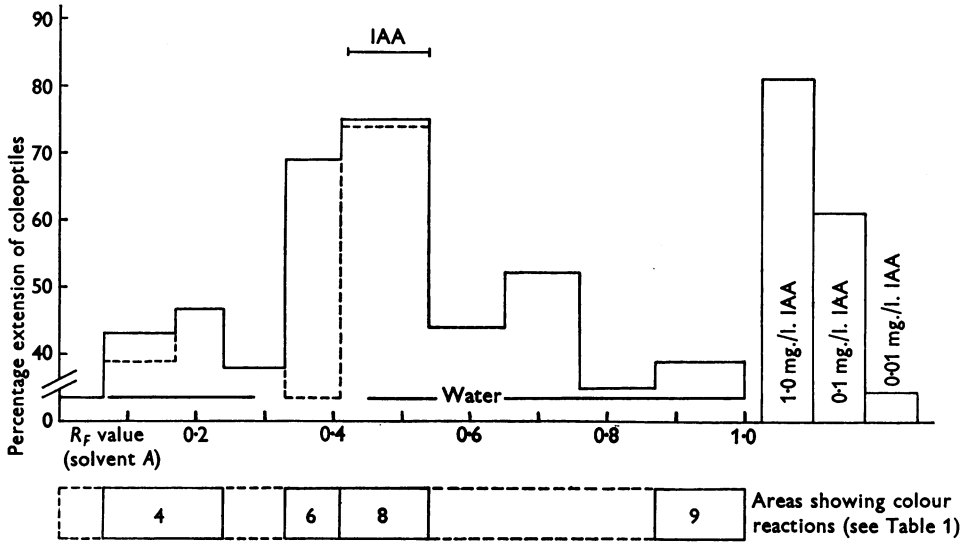

Fig. 1. Histogram of solvent *A* chromatogram of IPyA assayed on oats. Tenfold dilutions are indicated by broken lines.

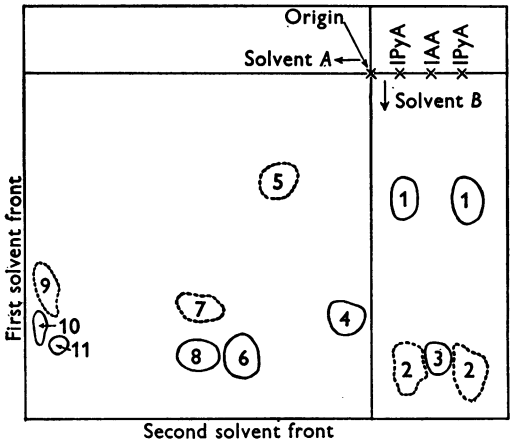

Fig. 2. Paper chromatogram obtained by developing IPyA in solvent *A* (after Stowe & Thimann, 1953), and then developing the decomposition products with fresh IPyA and 3-indolylacetic acid (IAA) as markers in solvent *B*. For key see Table 1. Broken lines indicate spots detectable only by fluorescence in ultraviolet light.

Two-dimensional chromatography of IPyA. IPyA (50–100 μg.) was chromatographed in solvent *A* until the solvent front had risen 20·3 cm. The paper was dried in a current of air for 30 min. at room temperature to remove NH_3. With solvent *B* at 27°, the decomposition products were now chromatographed in a direction at right angles to the original direction until the solvent front had risen 20·5 cm. 3-Indolylacetic acid (10–20 μg.) and a fresh sample of IPyA (10–20 μg.) were used as markers (Fig. 2, Table 1.)

Bio-assay techniques

Solutions to be tested for physiological activity were assayed by the *Avena* straight-growth method (Bentley, 1950; Bentley & Housley, 1954), except where otherwise stated. Briefly, the method consists of measuring the growth of isolated segments of oat coleoptiles in the solution to be tested. The assay is carried out at a constant temperature of 27° for approximately 18 hr., with 10 segments in 10 ml. of solution. At the stage of development at which the coleoptiles are used, growth occurs only by cell elongation.

The effect of IPyA on root growth was examined by a modified form of the technique of Moewus (1949). Cress seedlings, initial root length 5 mm., were grown on 1·5% agar into which the test solution had been incorporated. Growth was measured after approximately 18 hr. at 27°.

RESULTS AND DISCUSSION

Chromatography

Stowe & Thimann (1953, 1954) found that a growth-promoting constituent of maize (var. Country Gentleman) had R_F 0·12 in solvent *A* and gave a crimson colour with the concentrated Salkowski reagent. These authors chromatographed synthetic IPyA in solvent *A*, and on spraying with the concentrated Salkowski reagent found a crimson spot with R_F 0·12 compared with a similar spot of R_F 0·25 given by 3-indolylacetic acid under these conditions. On the basis of these experiments they identified the growth-promoting substance they had found in maize as IPyA.

Although Stowe & Thimann (1953, 1954) noticed some formation of 3-indolylacetic acid from IPyA

on chromatograms, and also found that on keeping their corn extracts for some time other breakdown products could be observed, they considered that IPyA could be chromatographed in solvent *A* without complete destruction. Our results on the chromatography of IPyA in solvent *A* are not in agreement with those of Stowe & Thimann, and show that IPyA is rapidly and completely broken down by this procedure.

Fig. 1 and Table 1 show the results of colour-spray tests and bio-assays on a one-dimensional chromatogram of IPyA in solvent *A*. Several physiologically active zones are formed, but no part of the chromatogram gives the immediate reduction of ammoniacal $AgNO_3$ in the cold, which is a characteristic reaction of IPyA. There is a region of rather low biological activity between R_F 0·065 and 0·24. Since this lies approximately half-way between the starting line and the 3-indolylacetic acid region (R_F 0·475 in this experiment), its position relative to 3-indolylacetic acid corresponds to that found for IPyA by Stowe & Thimann (1953).

With the spray reagents, part of the region between R_F 0·065 and 0·24 shows the colour reactions indicated in Table 1 (spot 4).

Spot 4 gives a yellow colour with the dilute Salkowski reagent, and a brownish yellow colour with the more concentrated Salkowski reagent used by Stowe & Thimann (1953). This indicates that spot 4 is not IPyA, which gives a purplish red colour with the concentrated Salkowski reagent.

When the decomposition products of IPyA from chromatography in solvent *A* are chromatographed in a second direction in solvent *B*, spot 4 has R_F 0·71, and no reducing zone is found with the R_F value of freshly applied IPyA (0·37). Solvent *B* causes very little breakdown of freshly applied IPyA. In this solvent, apart from the main IPyA spot (R_F 0·37, spot 1, Table 1), only one other spot can be distinguished (R_F 0·87, spot 2, Table 1). Spot 2 gives no reactions with any of the spray reagents and can only be detected because it shows a yellow fluorescence in ultraviolet light. The spray reagents give only one well-defined spot (spot 1) on solvent *B* chromatograms of IPyA, but sometimes a faint trailing pink colour can be seen between the IPyA region and the solvent front. This may be due to the formation of traces of 3-indolylacetic acid.

Since IPyA is stable in solvent *B*, any IPyA remaining after chromatography in solvent *A* would be expected to show up after further chromatography in solvent *B* as a reducing spot (R_F 0·37). Spot 5, which can be distinguished on the two-dimensional chromatogram, has R_F 0·31 in solvent *B* but cannot be IPyA since it gives no reaction with the spray reagents. It can only be detected because of its light-blue fluorescence in ultraviolet light.

Table 1. *R_F values and colour reactions observed in the chromatography of 3-indolylpyruvic acid (IPyA) (Figs. 1, 2)*

No. of spot	Ultraviolet fluorescence	Reaction with ammoniacal $AgNO_3$	Reaction with dilute Salkowski reagent	Reaction with nitrous–nitric acid mixture	Reaction with Ehrlich's reagent	R_F values (mean) Solvent *A*	Solvent *B*
1. IPyA	Bluish purple	Immediate reduction	Yellow with mauve border	Orange	Yellow changing to green	—	0·37
2.	Yellow	—	—	—	—	—	0·87
3. IAA*	Purple	Reduction overnight	Pink	Red	Mauve	0·50	0·83
4.	Orange–pink	Reduction during 10–20 min. after spraying	Very faint greyish yellow	Yellow	Faint green	0·07	0·71
5.	Light blue	—	—	—	—	0·27	0·31
6.†	Reddish mauve	Reduction after a variable period (15 min. to overnight)	Pink	Red	Pink	0·37	0·82
7.	Purple	—	—	—	—	—	0·68
8.	Purple	Reduction overnight	Pink	Red	Mauve	0·48	0·82
9.	Dark purple	—	Faint pink	Faint pink	—	0·93	0·63
10.	—	Very slight on long standing	—	—	Yellow changing to green	0·95	0·74
11.	—	Very slight on long standing	—	—	Mauve	0·89	0·79

* IAA: 3-indolylacetic acid.

† The intensity of the colour reactions given by spot 6 was reduced after running in solvent *B*.

Bio-assay of a two-dimensional chromatogram showed that spot 5 had no biological activity.

The region of the solvent *A* chromatogram showing the highest physiological activity is that associated with spots 6 and 8 (R_F 0·33–0·54). The zone corresponding to spot 8 (R_F 0·41–0·54) gives a solution with an absorption maximum at 280 mμ. and an intensity in agreement with an approximately 10% conversion of IPyA into 3-indolylacetic acid; the bio-assay results obtained with this zone are consistent with a 3-indolylacetic acid concentration of this order. The reactions of spot 8 with the spray reagents are completely consistent with the identification of this spot as 3-indolylacetic acid.

Bio-assays on solutions diluted tenfold show that the physiological activity of the spot 6 zone is less than one-tenth of that of the immediately adjacent spot 8 region. In an attempt to separate further the zones of activity associated with spots 6 and 8, IPyA was run downwards for 40 hr. in solvent *A*, but the results were essentially the same as those of Fig. 1. There was no clear-cut zone of lower activity between spots 6 and 8, and the possibility that the physiological activity associated with spot 6 is due to tailing of spot 8 is not ruled out.

Since spot 6 gives a red colour with the Salkowski reagent, it is possible that this is the spot observed by Stowe & Thimann (1953) between the starting line and the 3-indolylacetic acid region. However, the ratio of the R_F value of spot 6 to that of spot 8 is always close to 0·8 instead of approximately 0·5 as observed by Stowe & Thimann. Moreover, on chromatography in solvent *B*, spot 6 has R_F 0·82, whereas IPyA has R_F 0·37.

Spot 6 was compared with a number of indole compounds of similar R_F value. It differed from 3-indolylcarboxylic acid, 3-indolylacetic acid and 3-indolylglyoxylic acid in its colour reactions, but gave colour reactions identical in every respect with those of 3-indolylglycollic acid.

As described in the experimental part, 3-indolylglycollic acid was prepared and used in alkaline solution. This procedure is used because 3-indolylglycollic acid rapidly becomes red on acidification of its alkaline solutions. This decomposition in acid solution is shown by spot 6, which gives less intense colour reactions after chromatography in acetic acid solution, and a red tailing streak down the paper. The two growth-promoting zones between the 3-indolylacetic acid region and the solvent front (Fig. 1) show some faint colour reactions with the spray reagents, but have none of the reactions characteristic of IPyA.

It is evident that chromatography in solvent *A* decomposes IPyA completely, and it follows that the substance (R_F 0·12 in solvent *A*) shown by Stowe & Thimann to occur in maize cannot be IPyA; nor can the substance with similar properties observed by these authors in their solvent *A* chromatograms of IPyA be IPyA itself. It is possible that an impurity was present in Stowe & Thimann's preparation, which cannot have been as pure as our material, since they say that 'it darkens rapidly on exposure to air', whereas our crystalline IPyA appears unchanged after keeping in air in a stoppered tube at room temperature for some months.

We are of the opinion that the presence of IPyA in maize has not been convincingly demonstrated, and do not consider that the presence of IPyA in any biological extract could be shown by the chromatographic procedure of Stowe & Thimann (1953).

Bio-assays

To examine the effect of IPyA on cell elongation, bio-assays of IPyA without chromatography were carried out (Table 2). The IPyA was dissolved in a drop of ethanol to facilitate solution; low concentrations of ethanol have no effect on the bio-assay. It is seen from these results that solutions of pure IPyA have low activity, and if this is expressed in terms of equivalent 3-indolylacetic acid concentrations, then at optimum activity it is less than 1% of the 3-indolylacetic acid.

Since it had already been shown that IPyA broke down on chromatography in ammoniacal solution, it seemed possible that some breakdown might also be occurring in the test solutions of the assay. To investigate this possibility a chromatogram of IPyA was run in water over a period of 2 hr., and bio-assayed. Results (Fig. 3) clearly indicate that there is breakdown of IPyA even in water over a short period of time. There appears to be continuous production of a growth-promoting substance, most concentrated near the solvent front in a region having the same spray reactions and

Table 2. *Activity of 3-indolylacetic acid (IAA) and IPyA in the* Avena *straight-growth test*

Figures represent final length (mm.) of sections initially 10 mm. long.

Expt.	Compound	Concn. (mg./l.) 0	0·01	0·1	1·0	10	25	50	100
a	IAA	14·0	14·2	15·9	16·9	17·8	—	—	14·2
	IPyA		13·3	13·2	13·8	14·9	—	—	—
b	IAA	13·1	14·5	16·6	17·4	15·6	—	—	—
	IPyA		13·3	13·3	14·3	15·3	—	—	—
c	IAA	12·4	13·3	15·8	16·2	16·8	—	—	13·4
	IPyA		12·8	12·6	13·2	13·6	14·5	14·3	13·7

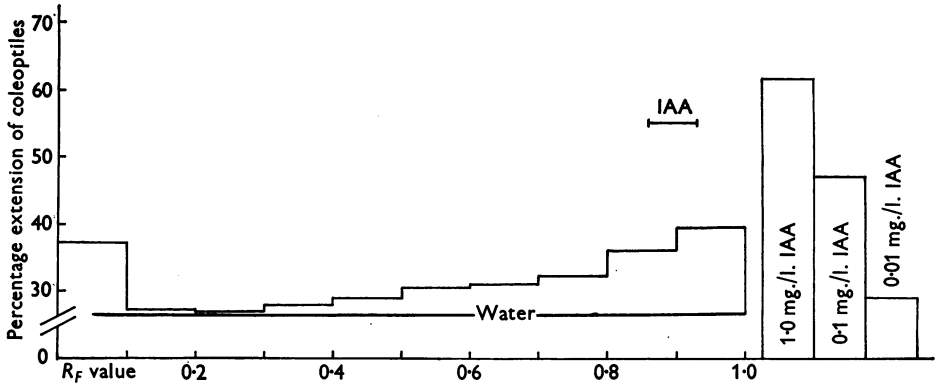

Fig. 3. Histogram of IPyA chromatographed in water for 2 hr.

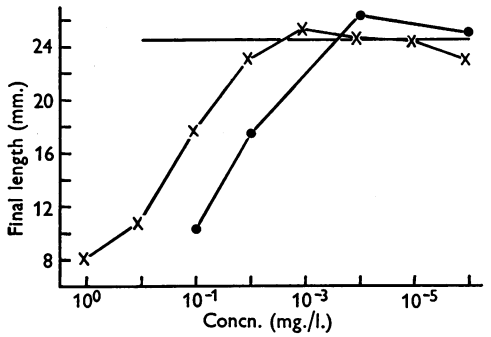

Fig. 4. Effect of 3-indolylacetic acid (IAA) ● and IPyA × on root growth. The horizontal line is at the final level given by a water control.

R_F value as 3-indolylacetic acid. On the starting line spray reactions characteristic of IPyA are shown: activity in this zone may be ascribed to continuous breakdown of unchanged IPyA during the bio-assay.

When IPyA was bio-assayed on roots, only inhibition of growth was obtained, the activity corresponding approximately to a 10% conversion into 3-indolylacetic acid (Fig. 4). This result shows clearly that the accelerator-α which promotes root growth (Bennet-Clark & Kefford, 1953) is not IPyA, as postulated by Stowe & Thimann (1953).

SUMMARY

1. 3-Indolylpyruvic acid (IPyA) has been synthesized by an improved method and fully characterized.

2. It has been shown to break down completely on chromatography under the conditions used to demonstrate its alleged presence in maize.

3. The physiological activity of synthetic IPyA in the *Avena* straight-growth test has been investigated and shown to be mainly, if not entirely, due to its breakdown products.

4. From an examination of its activity on root growth it has been demonstrated that it is not identical with accelerator-α.

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