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20. Yo Isogai, Toshihiko Okamoto, and Tôru Koizumi : Studies on Plant Growth Regulators. I.*1, Isolation of Indole-3-acetamide, 2-Phenylacetamide, and Indole-3-carboxaldehyde from Etiolated Seedlings of Phaseolus.*2

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Plant growth promoting substances, indole-3-acetamide and phenylacetamide and related compound, indole-3-carboxaldehyde were isolated from the aqueous extract of etiolated seedlings of *Phaseolus mungo*.

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Although many papers have appeared reporting the presence of plant growth regulators,*5 only a few have reported the isolation of new plant growth regulators.

One of the present authors (Yo Isogai) had found that neutral (F-1) and acidic (F-2) fractions obtained from aqueous extract of Moyashi*6 by the separation method[1] outlined in Fig. 1 had growth promoting action on Avena coleoptile sections. When

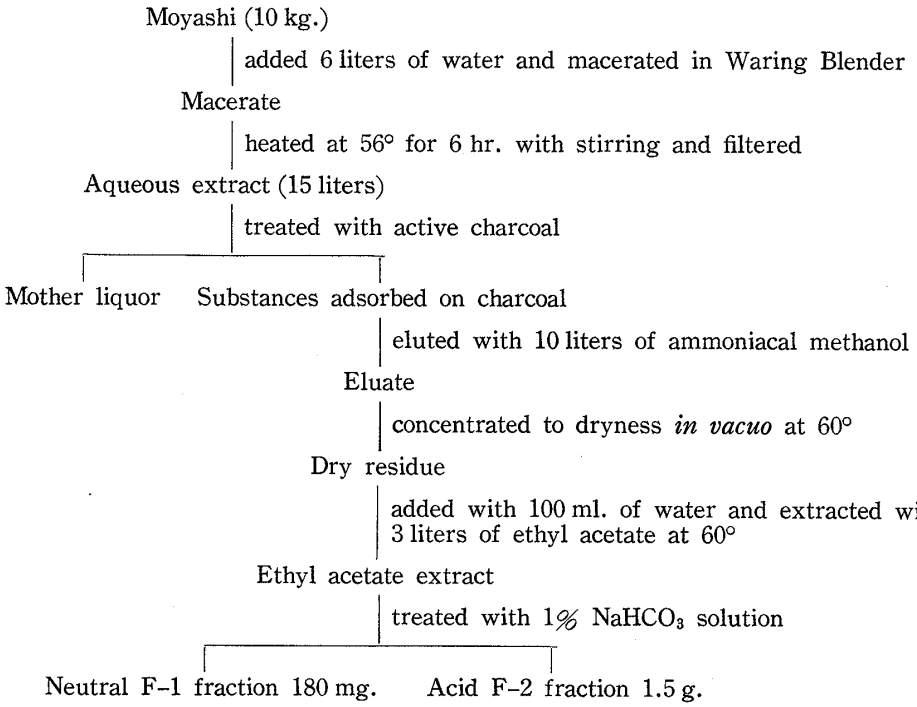

Fig. 1. Extraction and Separation Method by Isogai

*1 This paper constitutes a part of a series entitled "Studies on Plant Growth Regulators" by T. Okamoto and Y. Isogai.

*2 A Part of this work was presented at the International Symposium on Plant Growth Regulators held at Gif-sur-Yvette, July 1963. The preliminary communication of this paper was briefly reported in This Bulletin, **11**, 1217 (1963).

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*5 In this series all the substances having the activity which controls plant growth are designated as plant growth regulators.

*6 Japanese name "Moyashi" is given for etiolated seedlings of *Phaseolus mungo* Linne, sprouted at factory and commercially sold in green grocers as a sort of vegetable in Japan.

1) Y. Isogai : Sci. Pap. Coll. Gen. Educ., Univ. Tokyo., **10**, 73 (1960).

these two fractions were developed by paper chromatography*7, they gave many distinct spots on the chromatogram as shown in Fig. 2. Among them, the zone from Rf 0.54~0.79 in the case of the neutral fraction (F-1) and the zone corresponding to the purple spot at Rf 0.35 in the case of the acidic fraction (F-2) gave promotion, while that of Rf 0.14 and the pink spot from Rf 0.46~0.82 in the chromatogram of the acidic fraction (F-2) had growth inhibitory activity. The purple spot at Rf 0.35 in the case of the (F-2) fraction was thought to correspond to that of indoleacetic acid, but the compounds corresponding to the other active spots could not be identified.

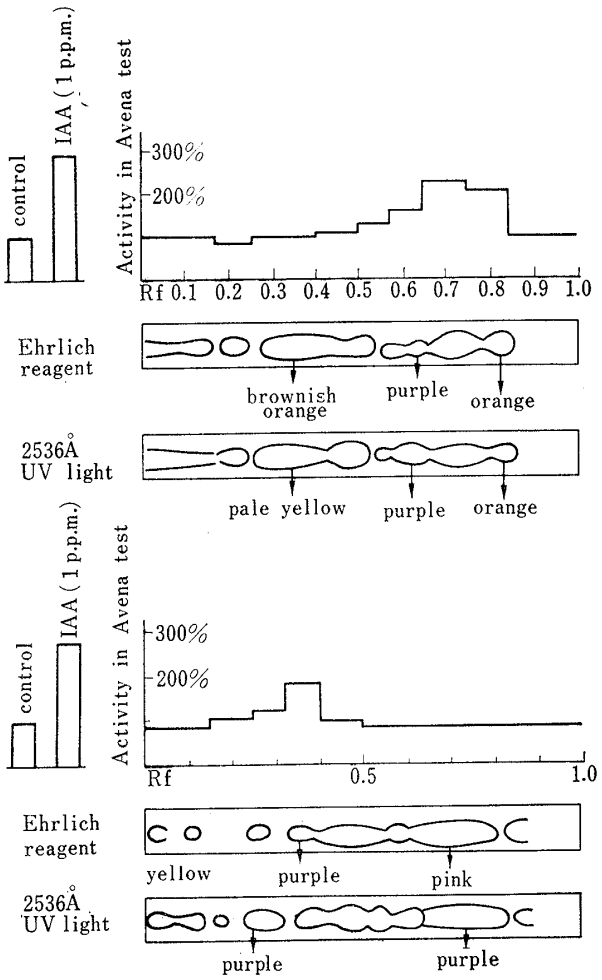

Fig. 2. Paper Chromatography of the Neutral F-1 Fraction (above) and the Acidic F-2 Fraction (below)

Activity is represented as % elongation of the control growth.

This report describes the isolation of several growth promotors in crystalline form from the neutral fraction (F-1) of aqueous extract of Moyashi. In order to isolate several plant growth regulators from this fraction, large scale extraction techniques were developed. Starting from 145 kg. of Moyashi each time, the whole extraction process was repeated eight times as shown in Table I (September, 1961 ~ February, 1962) and a total of 33 g. of the neutral fraction (F-1) and 68.5 g. of acidic fraction (F-2) was obtained.

During these procedures, the two F-1 fraction from the seventh and eighth extraction gave partially different result from that of the others in the paper chromotography.*7 In the two cases, the purple spot of Rf 0.61 became quite strong in the F-1 fraction, and in the F-2 fraction the purple spot at Rf 0.35 became intense. Further in the latter fraction on additional purple spots appeared at 0.68 in the middle of the pink spot at Rf 0.6~0.82. Moreover crystals were formed from the F-1 fraction from the 8 th extraction when the ethyl acetate solution was concentrated and kept in the refrigerator. This was recrystallized from ethyl acetate and yielded 30 mg. of crystal (m.p. 148~151°) which gave purple spot at Rf 0.67 on its chromatogram*7 and showed growth promoting activity on Avena coleoptile sections. (This substance was tentatively designated as crystal X.) Owing to this characteristics, the samples from the 7 th and 8 th extractions were excluded for further study from the other six extractions.

The neutral F-1 fraction obtained from the first to the 6 th large scale extraction (24 g./ 900 kg. of Moyashi) was subjected to adsorption column chromatography with Woelm neutral alumina. From a total of 3.1 g. of F-1 extract obtained in the second large scale extraction a small amounts of sample were first tested for recovery of

*7 Solvent system, iso-PrOH : NH_3 : H_2O (8:1:1).

Table I. Large Scale Extraction

		NF-1 Fraction (g.)	AF-2 Fraction (g.)
I	1961 Sept. 28～29	2.5	—
II	1961 Oct. 12～13	3.1	10.5
III	1961 〃 21～28	6.8	11.0
IV	1961 Nov. 8～ 9	3.1	11.5
V	1961 〃 24～25	4.4	7.9
VI	1961 Dec. 8～ 9	3.8	8.1
VII	1962 Jan. 24～25	3.5	13.5
VIII	1962 Feb. 14～15	5.5	6.0

activity in the eluate. This is shown in Fig. 3. The recovery of activity in the eluate was nearly complete, and the eluate from fraction number 2～12 (Fr. 2～12) gave growth promotion in the Avena straight growth test. Fr. 2～6, Fr. 10～12 yielded colorless oily material and Fr. 7～9 gave a crystal which has characteristic ultraviolet spectra. (It was tentatively designated as crystal A.) The eluate from Fr. 25～45 also gave growth promotion and yielded an amorphous solid which has characteristic peak at 220, and 280 mμ. (It was tentatively called as crystal C though it was not pure at this stage.) This chromatographic treatment was repeated 3 times using the same sample and gave similar results.

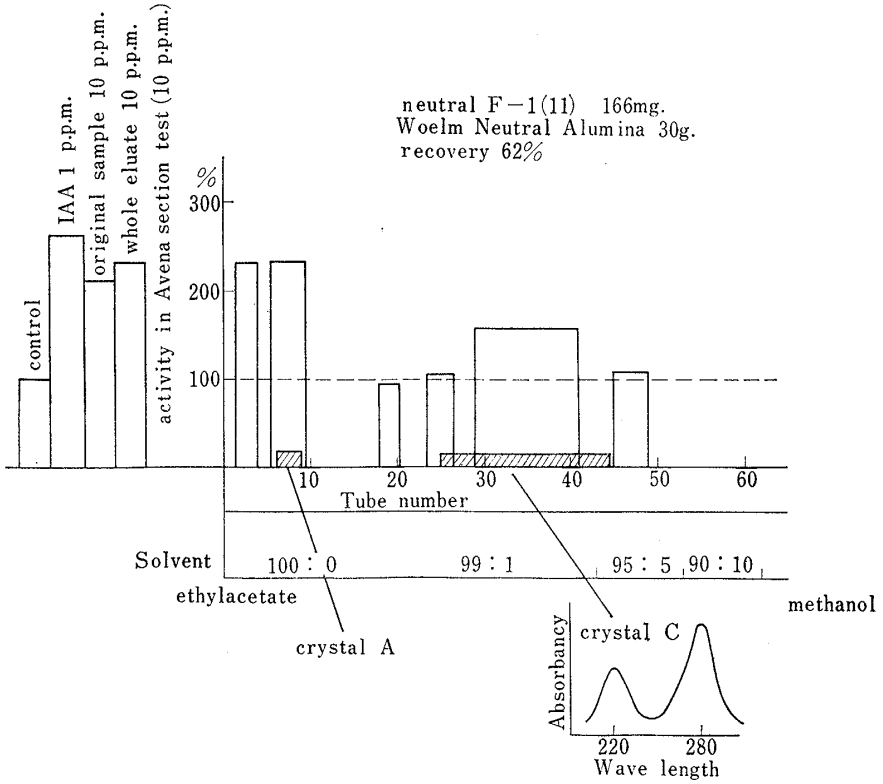

Fig. 3. Chromatography of Neutral F-1 Fraction (166 mg.)

Since no other active zones were found in the chromatogram the active substances were studied using the combined F-1 fraction from 1 st to 6 th extraction (except 2 nd extraction) for column chromatography using ethyl acetate as eluting solvent.

In this case crystal A was obtained but crystal C could not be isolated. Instead a new crystalline substance which was tentatively designated as crystal D was obtained. The result of the column chromatography is shown in Fig. 4. This treatment was repeated 10 times and gave similar results.

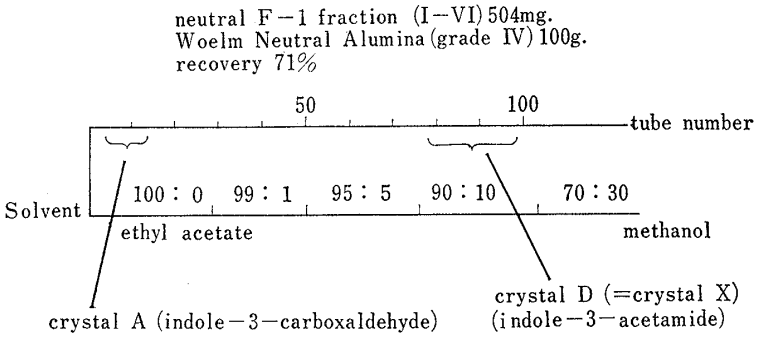

Fig. 4. Chromatography of Neutral F-1 Fraction (I-VI)

All the crystal D which was obtained in these procedure was recrystallized from a mixture of ethyl acetate and hexane yielding 8.4 mg. of crystal D (m.p. 150.5~151.5°). Ultraviolet and infrared spectra suggest crystal D as indole-3-acetamide (IAM) and this identification was confirmed by the mixed melting point and the comparison of the infrared spectra with that of authentic specimen. Furthermore crystal X was also confirmed as indole-3-acetamide by admixture and by comparing their infrared spectra.

The remaining F-1 fraction (17 g.) was chromatographed employing a relatively small amount of Woelm neutral alumina (Fig. 5). The separation was not perfect but the eluate obtained from 5% methanolic ethyl acetate contained crystal A and C as determined by the thin-layer chromatography. At the same time 509 mg. of indole-3-acetamide (Crystal D) was obtained. Crystal A and C seemed to have plant growth promoting activity from the preceding experiment, so this fraction was rechromatographed as shown in Fig. 5. The residue obtained from Fr. 21~28 contained crystal A as determined by the ultraviolet spectrum and this was purified by the chromatography and recrystallization, yielding 7 mg. of indole-3-carboxaldehyde (IAlD, m.p. 189.5~190.5°). This was confirmed by admixture with authentic indole-3-carboxaldehyde and by comparing their infrared spectrum. Pure indole-3-carboxaldehyde gives no growth promoting activity and the apparent action of crude crystal A might be attributed to some impurities.

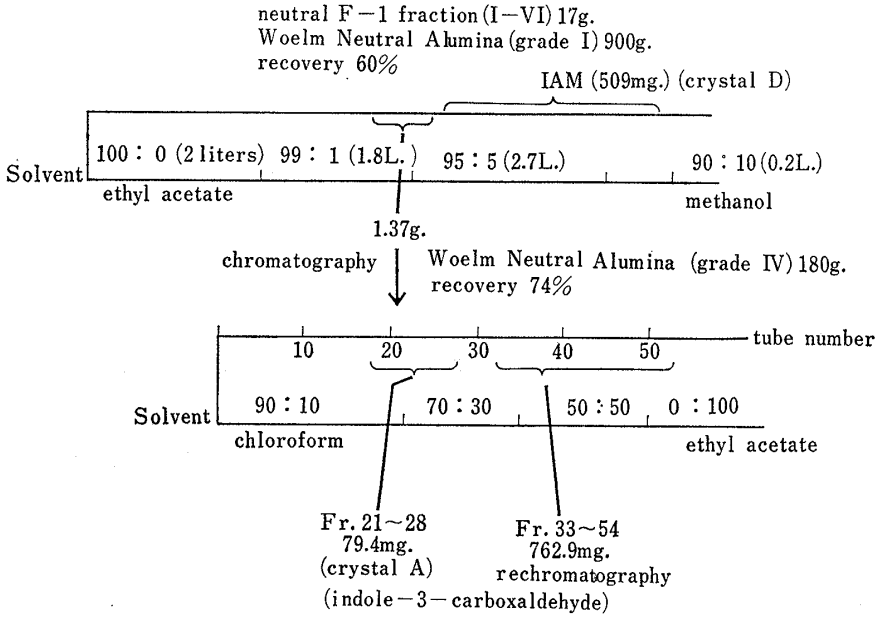

Fig. 5. Chromatography of Neutral F-1 Fraction

The deposit obtained from Fr. 33~54 had an ultraviolet spectrum similar to that of crystal C, but it was a mixture of several substances. It was rechromatographed as shown in Fig. 6 yielding two substances, crystal C (m.p. 112~115°). and a new crystalline substance, which was tentatively disignated as crystal B (m.p. 156.8~157.3°). Crystal B was identified as phenylacetamide (PAM) by its infrared spectrum and this was confirmed by admixture with authentic specimen and by comparing their infrared spectra. The chemical composition of crystal C is now under study, though pure crystal C has no growth promoting activity. The example of the bioassay of crystal A, B, C, D are shown in Table II.

Fr. 33~54 762 mg. Woelm Neutral Alumina (grade IV) 80 g. recovery 73%

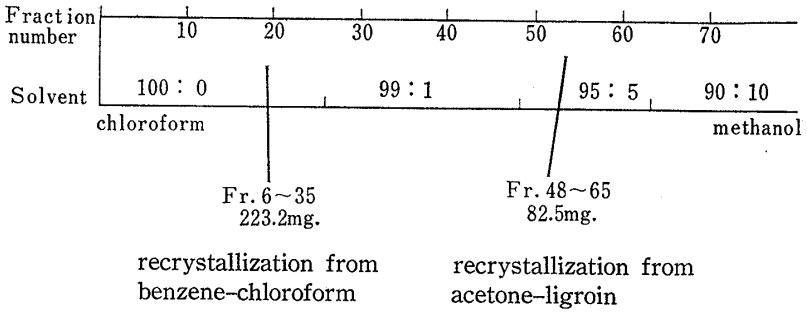

Fig. 6. Chromatography of Fraction 33~54

Table II. Biological Activity of Crystal A, B, C and D in Avena Straight Growth Test

Concn.	Crystal A (indolecarboxaldehyde)	Crystal B (phenylacetamide)	Crystal C	Crystal D (indole-3-acetamide)
100 p.p.m.	6.9±0.22	8.6±0.26	7.0±0.19	10.1±0.36
10 p.p.m.	7.4±0.20	9.4±0.27	7.0±0.14	8.7±0.30
1 p.p.m.	7.5±0.25	8.0±0.20	6.9±0.12	8.1±0.29
Control	7.2±0.21	7.8±0.20	7.0±0.15	8.1±0.29
IAA (1 p.p.m.)	10.2±0.41	10.3±0.29	9.5±0.21	10.4±0.26

Each value is the average length of 15 coleoptile section in mm.

Since the neutral F-1 fraction was eluted with ammoniacal methanol, there is a possibility that both IAM and PAM are artifacts, that is, they may be produced from some precursors by the action of ammonia. In order to elucidate this question, F-1 fraction obtained by elution with methanol was chromatographed. Though pure indole-3-acetamide and phenylacetamide have not been obtained in this case, the presence of IAM and PAM was confirmed by thin-layer chromatography.

Experimental

Bioassay——The following method was always employed for bioassay. Dehasked oat seeds (*Avena sativa* var. Victory) were soaked in water for two hours, and grown for 72 hr. at 25° in darkness, except for occassional exposure to red light. Section of 5 mm. length were cut from coleoptiles at 3 mm. below the tip, and 15 sections per dishes were floated on 3 ml. of aqueous solution containing the test substance. After 24 hr. growth in the dark the length was measured and compared with that of control.

Material——The whole Moyashi plant was employed for extractions. Moyashi is the Japanese name of etiolated seedlings of *Phaseolus mungo* which are sold at vegetable stores all the year round. These seedlings are grown in a building at nearly 25° in darkness for 6 days and have radicles and young stems with shrunken cotyledons and small leaves. Although the seedlings are grown in the dark, they are exposed to light while they are being carried from the grower to the laboratory. Thus, the light conditions were not completely controlled in this material.

Extraction Method——After washing, Moyashi was treated as shown in Fig. 1. 10 kg. of material was mixed with 8 L. of water, macerated in a Waring Blender (10,000 r.p.m.), and the macerate was heated at 56° for 6 hr. with constant stirring. It was filtered first through thick cotton cloth with a hand press, and the turbid solution was then filtered through filter paper precoated with talcum powder.

The clear filtrate was treated with active charcoal for 5 hr., which was separated from the mother liquor by filtration. Substances adsorbed on charcoal were eluted for 12 hr. with 2 L. of ammoniacal methanol (0.5% NH_3 in MeOH) at room temperature, and after repeating the same procedure 5 times, 10 L. of combined eluates were evaporated to dryness *in vacuo* at 60°. The dry residue was suspended in 100 ml. of H_2O and 1L. of AcOEt and heated to 60°. The whole solution was shaken vigorously, and poured into a separatory funnel. When the upper layer became clear, it was separated. After repeating the same procedure 3 times, 3L. of combined AcOEt extract were treated with 1% $NaHCO_3$ solution.

The AcOEt portion which contained substances not transferred to the $NaHCO_3$ solution was evaporated to dryness *in vacuo* at 60°, and gave brown syrup which was tentatively designated as the F-1 fraction. $NaHCO_3$ solution was then adjusted to pH 3.5 with 2*N* H_2SO_4, and was extracted first with 500 ml. and then with 250 ml. of AcOEt. After evaporating the solvent, brown syrup which was tentatively designated as F-2 fraction was obtained. Both F-1 and F-2 fraction were soluble in ether, Me_2CO, AcOEt, $CHCl_3$ and MeOH and sparingly soluble in benzene and H_2O. Both fractions have growth promoting activity. When they were chromatographed at 25° on Whatman No. 1 paper with iso-PrOH-NH_3-H_2O (8:1:1) (v/v) by the ascending method, many distinct spots appeared after the paper was sprayed with Ehrlich reagent or illuminated with 2563 Å ultraviolet light.[1)]

Eluates from section of the chromatograms corresponding to these spots were tested by bioassay. In this case of the F-1 fraction, the eluate from Rf 0.54～0.79 had growth promoting activity.

In the F-2 fraction, growth inhibition was found at Rf 0.14 and between 0.46 and 0.82 while the Rf 0.35 region gave growth promotion. The properties of these fractions remained almost the same in samples obtained at different time during the year and also remained practically unchanged when the fractions were kept in a refrigerator for a year.

Large Scale Extration——Starting from 145 kg. of Moyashi each time, the whole extraction process was repeated 8 times, and a total of 33 g. of F-1 fraction and 68.5 g. of F-2 fraction was obtained as shown in Table I.

Alumina Column Chromatography——Woelm neutral alumina (Wn alumina) was used in the present study and activity grade Ⅳ was used with one exception. The recovery of the sample was always checked.

Chromatographic Separation. 1) Chromatography of the F-1 Fraction from the Second Large Scale Extraction——166 mg. of F-1 fraction from second large scale extraction was chromatographed on a column containing 30 g. of Wn alumina (grade Ⅳ) (see Fig. 3). Eluting solvent system and recovery were as follows (1 Fr. 15 ml.).

Fraction No.	Solvent system	
1～20	AcOEt	
21～43	AcOEt-MeOH (99:1)	
44～53	AcOEt-MeOH (95:5)	
54～62	AcOEt-MeOH (90:10)	
63～	MeOH	(Recovery 62%)

The eluate from Fr. 2～12 gave growth promotion and Fr. 2～6 yielded 53 mg. of oily substance and Fr. 7～9 gave 3 mg. of crystal A which has characteristic ultraviolet (UV) spectra (peak 205, 242, 260, 295 mμ). Fr. 25～45 also gave growth promotion and yielded 14 mg. of a white amorphous solid which has characteristic peaks at 220, 280 mμ ($\varepsilon_{220\,m\mu} < \varepsilon_{280\,m\mu}$).

2) Chromatography of the F-1 fraction obtained in I～VI large scale extractions——504 mg. of F-1 obtained from I～Ⅵ extractions (except the Ⅱ sample which was used up by this time) was chromatographed using 100 g. of Wn alumina (grade Ⅳ) (Fig. 4.). Eluting solvent system and recovery were as follows (1 Fr. 15 ml.).

Fraction No.	Solvent System	
1～29	AcOEt	
30～51	AcOEt-MeOH (99:1)	
52～76	AcOEt-MeOH (95:5)	
77～103	AcOEt-MeOH (90:10)	
104～121	AcOEt-MeOH (70:30)	
122～	MeOH	(Recovery 70.5%)

The eluate from Fr. 4～7 yielded 267.7 mg. of oily material and Fr. 8～10 gave 2.1 mg. of crude crystal A. The material corresponding to Fr. 80～95 was 26.2 mg. of crystal D and this was recrystallized from a mixture of hexane and ethyl acetate.

Structure and Properties of Crystal D——71 mg. of crude crystal D which was obtained in several chromatographic separation was recrystallized from the mixture of AcOEt and hexane, yielding 8.4 mg. of

pure crystal D (m.p. 150.5~151.5°). *Anal.* Calcd. for $C_{10}H_{10}ON_2$: C, 68.95; H, 5.79; N, 16.08. Found : C, 68.95; H, 5.89; N, 15.79. IR ν_{max}^{KBr} cm^{-1} : 3418, 3240, 1640, 1617, 740. UV λ_{max}^{EtOH} mμ (ε) : 220 (35,400), 280 (6,280), 289 (5,400). These results suggested that crystal D was indole-3-acetamide, and this was confirmed by the mixed melting point determination and the comparison of the infrared (IR) spectrum with that of authentic specimen. Furthermore crystal X which separated out as crystal from the F-1 fraction of the Ⅷ sample, was also confirmed as indole-3-acetamide (IAM) by admixture and by comparing their IR spectra. IAM gives a violet spot of Rf 0.67 when it was chromatographed with iso-PrOH-NH_3-H_2O (8:1:1) (v/v) and sprayed with Ehrlich reagent. Also it gives the following results in the thin-layer chromatography.

	Solvent	Rf
Tosinlayer G (silica gel)	AcOEt	0.54
〃	Me_2Co	0.45
〃	$CHCl_3$-MeOH (9:1)	0.63

3) Large Scale Column Chromatography of the F-1 fraction (I~VI extraction except II——17 g. of F-1 was chromatographed with 900 g. of Wn alumina (grade I) (Fig. 5). Eluting solvent system and recovery were as follows (1 Fr. 200 ml.).

Fraction No.	Solvent system
1~25	AcOEt
26~36	AcOEt-MeOH (99:1)
37~44	AcOEt-MeOH (95:5)
45~50	AcOEt-MeOH (90:10)
51~	AcOEt-MeOH (70:30) (Recovery 60%)

The eluate from Fr. 35~41 yielded 1.37 g. of oily material which was confirmed to contain crystal A and C by thin-layer chromatography (TLC). Fr. 42~45 gave 509 mg. of IAM and the material corresponding to Fr. 5~34 was 8.4 g. of oily material.

4) Chromatography of the deposit obtained in 5% methanolic AcOEt eluates——The eluate containing crystal A and C in the large scale chromatography (Fig. 5) yielded 1.37 g. of residue which was rechromatographed with 180 g. of Wn alumina (grade Ⅳ). Eluting solvent system and recovery were as follows (1 Fr. 15 ml.).

Fraction No.	Solvent system
1~21	$CHCl_3$-AcOEt (9:1)
22~35	$CHCl_3$-AcOEt (7:3)
36~49	$CHCl_3$-AcOEt (1:1)
50~63	AcOEt
64~76	AcOEt-MeOH (99:1)
77~86	AcOEt-MeOH (95:5) (Recovery 74%)

The residue obtained from Fr. 21~28 yielded 79.4 mg. of amorphous solid which has the same UV spectrum as that of crystal A and rechromatographed as follows.

Rechromatography of Crude Crystal A and Structure of Crystal A——79.4 mg. of the crude crystal A was passed through a column containing 14 g. of Wn alumina (grade Ⅳ), and eluted with benzene and $CHCl_3$ as eluting solvent. Crystal A was obtained from the eluate with 1% $CHCl_3$ in benzene and was recrystallized from Me_2CO-ligroin, yielding 7.3 mg. of pure sample (m.p. 189.5~190.5°). *Anal.* Calcd. for C_9H_7ON : C, 74.47; H, 4.86; N, 9.65. Found : C, 74.59; H, 4.86; N, 9.63. mol. wt. (Rast method) : 166 (calcd. : 145). IR ν_{max}^{KBr} cm^{-1} : 3220, 1628, 784, 755. $\nu_{max}^{CHCl_3}$ cm^{-1} : 1670.

These results suggest that crystal A was indole-3-carboxaldehyde and this was confirmed by admixture with authentic IAID and by comparing their IR spectra.

Rechromatography of Fr. 33~54 and Structure of Crystal B——The deposit obtained from Fr. 33~54 (Fig. 5) was a yellowish amorphous material and had an UV spectrum similar to that of crystal C, but since this material was identified as a mixture of several substances by TLC, it was rechromatographed. The dry material 763 mg. obtained from Fr. 33~54 was rechromatographed with 80 g. of Wn alumina (Fig. 6).

Eluting solvent system and recovery were as follows.

Fraction No.	Solvent system
1~24	$CHCl_3$
25~50	$CHCl_3$-MeOH (99:1)
51~63	$CHCl_3$-MeOH (95:5)
64~	$CHCl_3$-MeOH (90:10) (Recovery 73%)

82.5 mg. of crude crystal C was obtained from the eluates in Fr. 48~65 and was recrystallized two times from Me_2CO-ligroin, yielding 9.3 mg. of fairly pure sample (m.p. 112~115°). This sample has no growth

promoting activity. In addition, 223 mg. of another amorphous solid was obtained from the eluates in Fr. 6～35 and was recrystallized from benzene–$CHCl_3$, yielding 85.4 mg. of pure crystal B (m.p. 156.8～157.3°) *Anal.* Calcd. for C_8H_9ON: C, 71.09; H, 6.71; O, 11.84; N, 10.36. Found: C, 71.16; H, 6.71; O, 11.58; N, 9.81. IR ν_{max}^{KBr} cm^{-1}: 3396, 3216, 1634, 1417, 746, 700. UV λ_{max}^{EtOH} mμ (log ε): 248 (2.06), 252.5 (2.18), 258 (2.28), 264.5 (2.16), 267.5 (1.90).

These various results suggested that crystal B was phenylacetamide (PAM), and this was confirmed by admixture with an authentic sample of PAM and by comparing their IR spectra. PAM gives a spot of Rf 0.72 when it was chromatographed on paper with iso-PrOH–NH_3–H_2O (8:1:1). Although no color reaction was used for PAM, it was detected by the following procedure. 1～2 mg. of PAM was spotted and paper-chromatographed. The zone corresponding to Rf 0.72 gave a faint purple fluorescence under 2563 Å of UV light and pure PAM was recovered from the same Rf region by elution with AcOEt. Also it gives following result in TLC (30% H_2SO_4 as spray reagent).

Toshinlayer G (silica gel) $CHCl_3$–MeOH (97:3) Rf 0.25

Detection of IAM and PAM in the F-1 fraction which was eluted with MeOH only——1 kg. of Moyashi was macerated in a Waring Blender and was treated according to the extraction method by Isogai. Charcoal residue which was obtained was eluted with 1 L. of MeOH at room temperature. After repeating the same procedure 5 times, the 5 L. of combined eluate were evaporated to dryness *in vacuo* at 60°. The dry residue was extracted with AcOEt and separated to F-1 and F-2 fraction. 32.5 mg. of F-1 fraction was obtained. IAM and PAM were detected by TLC in the following solvent system and adsorbent.

IAM (spray reagent: Ehrlich reagent)

Toshinlayer G	AcOEt
〃	3% MeOH–$CHCl_3$
〃	5% MeOH–$CHCl_3$
〃	10% MeOH–$CHCl_3$
〃	Me_2CO

PAM (spray reagent: 30% H_2SO_4)

Toshinlayer G	3% MeOH–$CHCl_3$
Fluka Aluminiumoxyd	25% AcOEt–$CHCl_3$

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