ON THE PLANT GROWTH HORMONE PRODUCED BY RHIZOPUS SUINUS

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(Received for publication, January 17, 1935)

Since it was first discovered that cell elongation in the *Avena* coleoptile is controlled by a hormone, our understanding of the nature and rôle of this substance has progressed considerably. Apart from the elucidation of its functions in promoting growth, tropisms, and other reactions of the plant, the chemical nature of the substance has been extensively studied. The active substance produced by cultures of the mold *Rhizopus suinus* was shown by Nielsen (1930) to be ether-soluble, and by Dolk and Thimann (1932) to be an unsaturated organic acid, decomposed by strong acids but not by alkalies, and readily inactivated by oxidation. Its dissociation constant, as measured by Dolk and Thimann, is $10^{-4.75}$. Previously, Went (1928) had shown the molecular weight of the active substance in *Avena* coleoptiles to be about 376. The active substance in human urine was isolated by Kögl and Haagen-Smit (1931) and by Kögl, Haagen-Smit, and Erxleben (1933), and shown to be an acid, $C_{17}H_{28}(OH)_3COOH$ (auxin A), whose lactone is also active, while from malt these workers later isolated (1933) a ketohydroxy acid, $C_{17}H_{28}O(OH)COOH$ (auxin B), which had the same activity per unit weight.

On account of the rather small amount of substance available from *Rhizopus* cultures, and also since the bulk of the partially purified product was lost through spontaneous inactivation (see section, "Concluding stages"), the chemical investigation of the active substance, begun earlier, was dropped. However, the many experiments on purification which had meanwhile been carried out showed that the active substance from *Rhizopus* did not behave in quite the same way as that from urine.

Recently, however, it was shown by Kögl, Erxleben, and Haagen-Smit (1934) that there is in urine a second active substance, identical with β-indolylacetic acid, and Kögl and Kostermans (1934) showed that the molecular weight of the substance produced by *Aspergillus* and by *Rhizopus* is that of β-indolylacetic acid rather than that of the C_{18} compounds.

Since preparations from *Rhizopus* have been extensively used for physiological work, both in this laboratory and elsewhere, the exact nature of the active substance is of considerable interest. The present paper will give evidence that the active substance produced by *Rhizopus suinus* is in fact β-indolylacetic acid. Identification by the preparation of derivatives and by mixed melting points with the pure synthetic substance was not possible on account of the small amount of material available. Nevertheless, the evidence given below is fairly conclusive. The method of purification, since it differs to some extent from that adopted by Kögl and his coworkers, will also be outlined. Finally, it will be shown that some of the peculiar conditions previously found to be necessary for the production of this growth substance (Thimann and Dolk, 1933) find a simple explanation on this basis.

Purification of Active Substance from Rhizopus suinus

The conditions necessary to obtain maximum yields of active substance from *Rhizopus suinus* were studied by Bonner (1932) and by Thimann and Dolk (1933). The organism has its temperature optimum at 35–37°, and its growth is inhibited by high acidity. It was found that high yields of growth-substance could only be obtained from media containing peptone, and further that certain peptones only were effective. On ammonium salts the yield was only about one-tenth of that on the peptone media. The yield was also proportional to the extent to which the culture was aerated.

By observing these conditions, *i.e.* by growing the mold in a medium containing 1 per cent of the effective peptone (Witte's) and 2 per cent of dextrose, with salts, at 35°, and aerating at the optimum rate of about 25 liters of air per hour, yields of from 100 to 200 units of growth substance per cc. were obtained (*cf.* Thimann and Dolk, 1933).

Since 1 mg. of pure β-indolylacetic acid is equal to about 3×10^5

of our volume units, it follows that 200 units per cc. is 6.7×10^{-4} mg. per cc., and since 1 cc. of the medium contains 31 mg. of solids, we have a concentration of the active substance of about 1 part in 50,000 of solids. Correspondingly, about 1500 liters are needed to give a gm. of the active substance, as against 9400 liters quoted by Kögl for *Rhizopus reflexus*.

It is worthy of note that the *Avena* test method as carried out in this laboratory does not give the large variations of several hundred per cent found by the Dutch workers, and ascribed by them to a periodic change of some kind in climatic conditions. At Pasadena, on the contrary, results from day to day are constant within about 10 to 20 per cent, tests being always carried out at about the same time of day; there may, however, be some variation over long periods. This constancy of the test enables quite small losses in activity to be determined.

Preliminary Extractions.—The mold was grown in a hot room in large vessels under the above conditions. About 140 liters of the medium were obtained altogether, and this was worked up in several lots. After being filtered from mycelium and spores, it was evaporated under diminished pressure to 1 to 2 per cent of its volume, filtered from precipitated peptone, etc. (Precipitate A), acidified to pH 3, and extracted about seven times with half its volume of peroxide-free ether each time. From the extracts (B and C) the ether was evaporated off and the residue taken up in hot water. On cooling, a precipitate of wax-like substances separated; this was reextracted with warm chloroform (Extract E) and the active substance so obtained brought back into water and added to the main aqueous extract.

On account of the relatively small amount of active material available an attempt was made to carry out these processes quantitatively; *i.e.*, without loss of activity. It was found that the original activity could be checked to within 1 per cent (see Table I), while 90 per cent of the original growth substance was obtained in the combined Extracts B, C, and E.

Fractional Extraction at Controlled pH—The aqueous solution from the extractions, containing in all 21.9 million units, was evaporated, filtered from a bulky inactive precipitate, and brought to pH 4.80 electrometrically. Another precipitate separating at this point carried down considerable activity, and it was, there-

fore, after washing, redissolved in hot water and worked up with $CHCl_3$, the $CHCl_3$ extract being taken up in water with considerable loss of activity and added to the main fraction. The solution, containing now 15.97 million units, was extracted five times with an equal volume of $CHCl_3$ each time, since this procedure should give 88 per cent extraction of an acid of dissociation constant $10^{-4.75}$ and partition coefficient between $CHCl_3$ and water of 1.5. The extract contained 12.58 millions, and the aqueous residue 2.90 millions; *i.e.*, 81.3 per cent was extracted. The residue, containing citric, oxalic, and other stronger acids, was discarded. The $CHCl_3$ extract was shaken five times with half of

TABLE I

Quantitative Procedure in Preliminary Extractions

Solution	Total activity
	growth substance units $\times 10^4$
Original medium	1040
A. Peptone ppt	29
B. First 3 ether extracts (filtered)	614
C. Further 4 " "	79
D. Extracted liquid	66
E. $CHCl_3$ extract of wax-like ppt. from ether extracts	241
Total	1029
" in extracts (B + C + E)	934 = 90%
Loss of active substance	1%

its volume each time of 0.5 M $NaHCO_3$ solution, which extracted 12.26 millions or 98 per cent of the activity; the alkaline solution was reacidified and extracted repeatedly with fresh $CHCl_3$. The use of $CHCl_3$ for these extractions was preferred to ether on account of the extreme sensitivity of the active substance to traces of peroxide in the ether. Although freshly distilled before use, the $CHCl_3$ no doubt developed traces of acid on keeping, and this probably accounts for the inactivation of growth substance in $CHCl_3$ solution which was later observed. Traces of alcohol were of course always added.

Treatment with Organic Solvents, Etc.—The extracts were evaporated to dryness (4.2 gm.) and extracted repeatedly with boiling

petroleum ether, b.p. 40–60°. The extracts contained 0.49 million units in 565 mg. and were discarded. The insoluble residue was extracted with warm ligroin, which removed a further small amount of inactive substance. Many other solvents were tried but gave little increase in purity.

Precipitation of the active substance as lead salt gave unsatisfactory results. Only a small part of the substance was precipitated by addition of basic lead acetate in alcoholic solution, and in addition considerable inactivation occurred, from 30 to 50 per cent of the activity being lost in the trial experiments. This was traced to the use of warm acid solution for removal of lead with H_2S, the active substance being readily decomposed by warm dilute acid (*cf.* below). Even when this procedure was avoided by extracting precipitate and filtrate directly with ether, the lead precipitation gave no increase in purity and was therefore abandoned.

At this stage the procedure of Kögl, Haagen-Smit, and Erxleben (1933), *i.e.* conversion of the acid to a lactone by boiling with 1 per cent HCl in methanol, was tested, but was also found to cause considerable inactivation. Since, however, the product showed no tendency to crystallize, it was decided to attempt distillation.

Fractional Distillation in Vacuo—Contrary to the findings for auxentriolic acid, it was soon found that this active substance could be distilled as the free acid almost without loss, in a high vacuum. After numerous trials a still of the Hickman type was adopted (*cf.* Hickman, 1932). It is designed to realize pressures below 10^{-3} mm. in the still itself, and is a micromodification of those of Hickman, with the difference that condensation takes place on a removable inner tube (see Fig. 1). This tube fits into the main vessel—a sphere of 5 cm. radius—with a ground joint protected by a mercury seal, and its base is 2.5 cm. from the bottom of the bulb. The still was heated by immersing to about half its depth in a paraffin bath, the inner condenser tube being cooled with crushed ice. The side tube was connected through a liquid air trap to a two-stage mercury pump backed by an oil pump. The pressure was from 3×10^{-4} to 1×10^{-4} mm. of Hg.

With this "molecular still" the oil was distilled in quantities of about 300 mg. at a time. The procedure was to raise the bath temperature until a faint cloudiness appeared on the condenser; then to hold the bath at the same temperature or a few degrees

higher until no more substance appeared to distil. The vacuum was then released and the condenser tube removed and rinsed down with $CHCl_3$ or acetone into a small dish. The condenser was replaced, a vacuum again established, and another fraction taken. Since the still is small, high vacuum is quickly reestablished. In all the distillations the bulk of the activity appeared in one fraction, that distilling at a bath temperature between 95–105°,

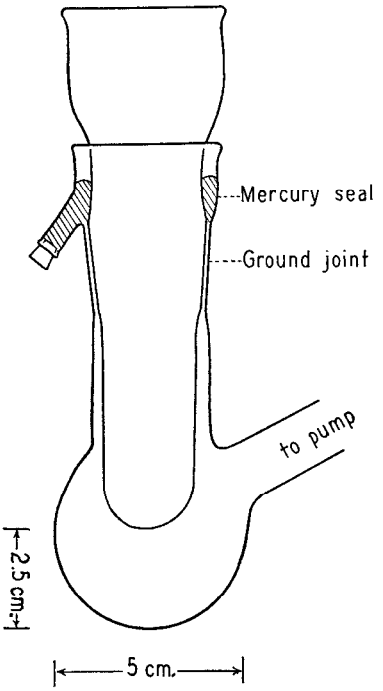

FIG. 1. Still for high vacuum fractional distillation of small quantities

and the inactivation was negligible. In the sample distillation shown in Table II, the loss of activity was 6 per cent only, while the purity was increased between 3 and 4 times in the main fraction.

The residues left above 115° from all twelve distillations were finally combined, reextracted with petroleum ether, and again distilled; the active distillate was, however, not combined with the other material, since its purity was lower.

Concluding Stages—The combined active distillates, totaling 5.0 million units, were evaporated once with CCl_4 to remove the last traces of chloroform, and then extracted cautiously with CCl_4 at 0°. The extracts on standing at 0° for a few days precipitated an insoluble yellow oil which was added to the other insoluble fraction. The insoluble material contained 2.5 million units in 135 mg. This was dissolved in methanol, a small amount of insoluble material being removed, and 186 mg. of brucine dissolved in methanol were added. On cooling to −30° the salt was precipitated; it was filtered off and washed quickly with MeOH cooled in the same freezing mixture. The precipitate was dissolved in water, made to pH 10, and extracted with CCl_4 till the extract no

TABLE II

Sample Distillation

Fraction No.	Weight	Activity	
	mg.	*total units*	*units per mg.*
1. 60–72° for 40 min.	36.7	58,000	1,600
2. 80–97° " 10 " 95–97° for 18 min.	48 9	900,000	18,000
3. 95–104° " 8 " 104–115° " 57 "	55.9	372,000	5,000
4. Residue above 115°	135.5	159,000	1,200
Total	277.0	1,489,000	
Initial	294	1,580,000	5,400
Recovery	94%	94%	

longer gave the HNO_3 test for brucine. The solution was then brought to pH 3 and extracted with $CHCl_3$. The extract, 12.8 mg., contained 1.5 million units or 117,000 units per mg. This figure, compared with 310,000 units per mg. for synthetic β-indolylacetic acid (Thimann and Koepfli, 1935) shows that the preparation was about 40 per cent as active as the synthetic product. However, the substance lost activity rapidly on being kept in $CHCl_3$ in the dark and before it could be further studied the bulk of the activity had disappeared.

The filtrate,[1] which carried 900,000 units in 34.5 mg., *i.e.* was one-fourth as pure, was therefore worked up by redistillation *in*

[1] This solution, indicated as Br. F. (brucine filtrate), was used in the experiments on root formation of Thimann and Went (1934).

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Medium, about 0.14 mg. per unit; evaporated *in vacuo* and filtered

Ppt. discarded

Filtrate extracted seven times with ether at pH 3

Aqueous layer discarded

Extract taken back into water and chilled

First ppts. worked over with ether, extracts being combined with rest of active material; later ppts. discarded → Solution brought to pH 4.80 and filtered

Ppt. discarded

Filtrate extracted five times with $CHCl_3$

Aqueous layer discarded

Chloroform layer shaken with $NaHCO_3$

Chloroform layer discarded

Alkaline solution acidified and extracted with chloroform; extract has 5000 units per mg.

Aqueous layer discarded

Extract evaporated and extracted with petroleum ether

Petroleum extract discarded

Residue extracted with ligroin

Ligroin extract discarded

Residue transferred to molecular still

Fractions below 95° discarded

Fraction 95–110° contains most of the activity; purity 20,000 units per mg.

Combined active fractions extracted with CCl_4, at 0°, successively

Extract chilled and active ppt. recovered; remainder discarded → Residue pptd. with brucine in MeOH at −30° and filtered

Precipitate freed from brucine with CCl_4; purity 110,000 units per mg., but rapidly inactivated; lost

Filtrate therefore freed from brucine by CCl_4 extraction; purity 26,000 units per mg.; reextracted with petroleum ether

Extract discarded

Residue redistilled

Fraction 70–85°

Fractions 85–95° and 95–102°

Above 102°

Needles, m.p. 109° after recrystallization twice from acetone; inactive

Platelets, m.p. 160° (*ca.*) but only about 1 mg.; purity 130,000 units per mg., or 4×10^{-8} mg. per plant unit, before recrystallization

Nothing

vacuo, after it had been first freed from brucine as above and again extracted with petroleum ether. Three fractions were obtained. That distilling at a bath temperature below 85° crystallized in long needles, m.p. 109°, but was almost inactive. The fractions distilling at 85–95° and 95–102° were semisolid, weighed 3.5 and 1.2 mg. respectively, and had an activity of 130,000 units per mg. They were apparently the same. On cooling, they crystallized in platelets, which on account of their small amount, could not be properly separated from the syrup. However, recrystallization from acetone was attempted. Under the microscope the crystals melted, not sharply, at 160°. Their nature will be considered below.

A summary of the purification procedure is shown in the accompanying diagram.

Chemical Nature of the Active Substance

There are thus three points of difference between the active substance from *Rhizopus* and the C_{18} auxins from urine. The *Rhizopus* substance (1) is destroyed by warm dilute acids (this was earlier shown by Dolk and Thimann (1932)); (2) is not precipitated by basic lead acetate in neutral alcoholic solution; (3) can be distilled without destruction at about 100° *in vacuo*.

In connection with the lead precipitation, a remarkable phenomenon occurred when the same procedure was tried for comparison on a urine concentrate. This had been extracted with ether at pH 3, and the ether extracts evaporated down and freed from a quantity of crystals (hippuric acid). The extract was purified by the first three stages described above and then treated with neutral lead acetate in weakly alkaline alcoholic solution just as described by Kögl *et al.* (1933). The precipitate in this case contained one-third of the activity but at a great increase in purity. Repeated addition of lead acetate gave no further precipitate; hence the filtrate carried the bulk of the activity, but a small amount of highly active substance had been removed (Table III). The only reasonable explanation for this behavior is the presence of two different active substances. In view of the subsequent findings of Kögl *et al.* these must be auxentriolic and β-indolylacetic acids respectively, since only the former forms an insolu-

ble lead salt under these conditions, while the latter is always present to some extent in urine.

Examination of the active preparations showed them to contain nitrogen. In many cases the amount was so small as barely to reach the limits of detection, but it was definitely present.

Color Reactions—All the active preparations gave the Salkowski reaction with nitrous acid, the color having a yellowish cast compared with that given by indole. In the impure preparations the intensity of the color reactions was proportional to their biological activity. A solution containing 1 part in 10,000 of the active substance, in about 10 per cent purity, gave a color equal to that of a solution of β-indolylacetic acid of the same concentration. Positive tests were given with the Ehrlich and $FeCl_3$-HCl reactions, the latter

TABLE III

Lead Acetate Precipitation of Partially Purified Urine Concentrate

Solution	Total activity	Weight	Purity
	growth substance units × 10^4	*mg.*	*units per mg.*
Ppt. 1	13.5	9.6	14,000
" 2	5.5	4.0	13,500
" 3	3.4	9.0	3,800
" 4	1.5		
Filtrate	28.0	92.1	3,040

of which is the more specific (*cf.* Frieber, 1922). The intensity of these tests was also strictly proportional to the activity.

The crystalline distillate, Fraction II of the redistilled brucine filtrate, which was evidently the active substance in only slightly impure state, gave positive tests in all three reactions and must therefore certainly be an indole acid derivative. Since indole, indolecarboxylic acid, indolepropionic acid, and tryptophane are all inactive, the substance must be either indoleacetic acid, or, what is very improbable, another unknown indole acid of the same activity.

Vacuum Distillation—Since the active substance distilled in a high vacuum at about 100° without much loss of activity, the same treatment was given to some synthetic β-indolylacetic acid, prepared by Majima and Hoshino's method (1925). In the mo-

lecular still described above no distillation occurred below 96°, but at a bath temperature of 99–103°, the substance distilled rapidly and quantitatively; 1.2 mg., activity 310,000 units per mg., yielded 1.2 mg. of distillate, activity 224,000 units per mg. The behavior is thus identical with that of the *Rhizopus* substance and different from that of the urine auxins.

Sensitivity to Acid—A dilute solution of pure β-indolylacetic acid was treated with 0.5 N HCl for 30 minutes at 100°. The activity before treatment was 99.2 and 24.8 units per cc.; after treatment, 3.5 and 0 units per cc., respectively. The destruction is thus practically complete.

Melting Point—Little emphasis can be placed on this property, since the crystals could not be satisfactorily separated from the syrup, but the melting point of 160° observed is close to that of 164.5° of synthetic β-indolylacetic acid.

Loss of Activity on Keeping—The loss of activity of the purified *Rhizopus* growth substance in solution is paralleled by the loss of activity of synthetic β-indolylacetic acid, which proceeds steadily, though apparently not so rapidly as that of auxin A. The following results were obtained with an aqueous solution of β-indolylacetic acid in the dark at 0°.

Day	Activity
	units × 10^3 *per cc.*
0	53.2
3	40.8
7	35.2
17	16.2
29	9.4

Conditions for Production of Active Substance by Rhizopus

The principal findings previously reported (Thimann and Dolk, 1933) were as follows: (1) On peptone media, the yield of growth substance depends on specific substances in the peptone; these were almost completely absent from the Merck product but present in the Witte product. This mysterious substance is clearly *tryptophane,* whose presence in peptones is known to vary with their history and origin. Qualitative tests on samples of the peptones used in that study show that the Merck product used gives

almost a negative Adamkiewicz reaction in 1 per cent solution; *i.e.*, its tryptophane content is extremely low. The Witte peptone, on the other hand, gives a strong test in 1 per cent solution, even stronger than that given by so called tryptophane broth preparations. Further, the "precursor" substance was shown to be reduced in amount by repeated autoclaving. (2) The yield of growth substance is proportional to the extent to which the culture is aerated. This follows from consideration of the reaction tryptophane $\rightarrow$ β-indolylacetic acid, as carried out by microorganisms:

$$RCH_2CHNH_2COOH \xrightarrow{\frac{1}{2}\,O_2} RCH_2COCOOH + NH_3 \xrightarrow{\frac{1}{2}\,O_2} RCH_2COOH + CO_2$$

The reaction is thus a typical oxidative deamination. The mold is also capable of oxidizing the substance further, as is shown by the fall in activity of the medium after 10 days at 35°.

Both these findings are thus satisfactorily explained.

Further, Boysen-Jensen (1932) found that, with *Aspergillus*, tryptophane and the 6-carbon-containing amino acids lysine, leucine, tyrosine, and phenylalanine could be converted to growth hormone. The relationship between these compounds and β-indolylacetic acid is, as pointed out by Kögl and Kostermans (1934), obvious.

It may be added that, as previously reported (Thimann and Went, 1934) the activity in growth promotion was paralleled in all preparations by activity in promoting root formation, root formation being determined by the method of Went (1934). Since the activity for both functions remained approximately proportional up to the final stages of purification, the conclusion was drawn that the two hormones are either identical or very closely related. However, the fact that the crystalline C_{18} auxins and the synthetic β-indolylacetic acid are all about equally active in promoting root formation proves that the two hormones are identical. The synthetic substance has an activity varying between about 40,000 and 280,000 root units per mg.

SUMMARY

1. The purification of the plant growth-promoting substance produced by cultures of *Rhizopus suinus* is described.

2. Although the preparation of derivatives could not be carried out on the small amount of substance available, it is shown that there is no doubt that the active substance is identical with β-indolylacetic acid. This identity is supported by specific color reactions, the melting point (approximate), distillation temperature, and various chemical properties, as compared with those of the synthetic substance.

3. The mechanism of the production of the active substance from peptones by the mold is reviewed, and showed to be largely explained by this finding.

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