In the early life of the organism when x is small the value of this fraction may be large. If α is small in comparison with x the value of the ratio will rapidly approach unity and the decrease of K will be observed only for the early values of x. However, if α is large with respect to x the ratio will approach unity more slowly and the curve will have an increased asymmetry.

The use of equation (3) where a constant quantity, designated α, is added to each value of x seems justified, therefore, by quantitative as well as physiological considerations.

* Paper No. 264, University of California Graduate School of Tropical Agriculture and Citrus Experiment Station, Riverside, California.

[1] Reed, H. S., and R. H. Holland. "The Growth Rate of an Annual Plant, Helianthus," *Proc. Nat. Acad. Sci.*, **5**, 135–144 (1919).

[2] Reed, H. S., "The Nature of the Growth Rate," *J. Gen. Physiol.*, **2**, 545–561 (1920).

[3] Bray, W. C., and P. R. Davis. "The Autocatalytic Reduction of Bromate by Hydrogen Peroxide in Acid Solution," *J. Am. Chem. Soc.*, **52**, 1427–1435 (1930).

[4] I am grateful to Professor Bray for his interest and assistance contributed to this study.

[5] Reed, H. S., "Intra-seasonal Cycles of Growth," *Proc. Nat. Acad. Sci.*, **14**, 221–229 (1928).

STUDIES ON THE GROWTH HORMONE OF PLANTS. I

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In recent years the process of cell elongation in plants has been studied by various investigators, Paal,[10] Went,[12] Cholodny,[3] and others. In these investigations it was proved that this process takes place under influence of a special substance, the so-called growth-substance ("Wuchsstoff"). Its physiological action, since it is assumed to be present in small quantities, is more or less comparable to that of the animal hormones. The presence of this substance in the coleoptiles of grasses was first established by Paal, and it was Went who extracted it from the tips of coleoptiles and who worked out a quantitative method for testing its activity. The hormone is not only found in higher plants, but similar substances are produced in relatively large quantities by various fungi, yeasts and bacteria (Nielsen[8,9], Boysen-Jensen[1,2]). The recent investigations of Heyn[7] have shown that the growth substance influences the cell elongation by changing the plasticity of the cell wall. In order to get a better idea

of the mechanism of its action, further knowledge about its chemical structure is necessary, and the following study is therefore a first attempt to establish the chemical nature of the substance.

§ 1. *Methods.*—The substance can be obtained from various sources. Its extraction out of the tips of coleoptiles did not appear very promising to us, as very large numbers of tips would have to be worked up in order to get a reasonable yield. At first an attempt was made to isolate it from malt syrup, as investigations of Seubert[11] have shown that this contains the substance in rather large amounts. However, it was soon found that the various commercial samples of malt give very different yields, some of them being quite inactive, probably through small differences in the method of preparation. It was therefore decided to use, as source of the substance, cultures of fungi. A pure culture of *Rhizopus suinus*, for which we have to thank Dr. Nielsen of Copenhagen, was used. In suitable media this mold produces large amounts of growth substance, as was shown by the careful and thorough studies of Nielsen. To what extent this substance is identical with that produced in the coleoptiles of grasses is uncertain, but this is not important for our purposes, as its physiological action is the same. According to Nielsen, the mold produces the hormone only when grown on solid media.* Under such conditions, however, the concentration of growth substance in the medium soon reaches a maximum and the production stops. The following arrangement was therefore made to avoid this.

The fungus was grown in large Petri dishes, 18 cm. in diameter, on filter paper soaked in nutrient medium. To one side a hole was drilled in the cover of the dish, through which by means of a syphon fresh sterile medium continuously dripped into the dish. On the other side, between cover and dish, was inserted a sterilized strip of filter paper, which acted as a syphon. By this arrangement a continuous stream of nutrient medium passed through the filter paper and in 24 hours about 150 cc. of active medium was collected from each dish. Under these conditions the fungus continues to produce the growth substance over a period of several days. In order to get still larger quantities of material this arrangement was later on altered in the following way. A large bell-jar with an opening in the top was filled with wood shavings soaked with nutrient medium. The opening was fitted with a stopper with two exit tubes, and the wide end of the bell-jar was covered with a glass plate in which four holes were drilled. The whole was inverted and sterilized in the autoclave, the shavings then inoculated with a suspension of the mold spores, and the apparatus placed in a room at 37° for two days to allow the mold to develop. After this time sterile nutrient medium was allowed to drip through the holes in the glass plate by means of syphons, and in order to keep the culture oxygenated, a current of sterile air was blown into the bell-jar

through one of the lower exit tubes. About 1 liter of medium dripped through in 24 hours. In all experiments, the medium described by Nielsen was used. Its composition is as follows: 10 g. dextrose, 10 g. ammonium tartrate, 0.5 g. $MgSO_4$, 0.5 g. KH_2PO_4, and a trace of $FeCl_3$, in 1 liter. In spite of all precautions, after some days the collected liquid often became infected with bacteria. In order to remove these the liquid was filtered through filter paper and a Berkefeld candle. The liquid was then concentrated under reduced pressure to about one-tenth of its volume, and stored at a temperature of 0° until required.

Its activity was tested on coleoptiles of *Avena sativa* by means of the method described by Went. In this method the active liquid is mixed with agar, small blocks of the agar are put on one side of the decapitated plants, and the difference in growth between the two halves of the coleoptiles measured by means of the curvature produced. A pure line of "Sieges hafer," obtained through the kindness of Dr. Åkermann of Svalöf was used.

The seeds, deprived of their husks, were soaked in water for four hours, placed on filter paper for 24 hours to germinate, and planted in glass holders as described by Went. The seedlings were grown for 48 hours in a dark room at a constant temperature of 25° and a relative humidity of 85%. The coleoptiles were then decapitated at 5 mm. from the tip, the primary leaf pulled loose, and after 40 minutes the agar blocks were put on one side of the stump by means of a drop of 10% gelatin. 110 minutes after the agar was put on, the curvatures were photographed by making a shadow print on bromide paper. To wait any longer than 150 minutes between decapitating and photographing is not advisable, since the formation of the new physiological tip would interfere with the test (Dolk[4]).

The agar blocks were prepared by mixing 1 cc. of active liquid with an equal volume of 3% agar, and pouring $^1/_2$ cc. of the mixture into a brass ring 20.6 mm. in diameter. After setting, a small rectangular plate 8.0 mm. × 10.7 mm. was cut out and this divided into 12 equal smaller blocks, special tools similar to those already described by Dolk[5] being used. Each resulting block had a volume of 10.7 $mm.^3$ As was found by Went, and confirmed by Nielsen, up to a certain concentration the curvature is directly proportional to the amount of active material present in the agar. Above this concentration it becomes less and less, and reaches finally a constant value, ("maximum angle"). In our case it was found that up to an angle of 14° there was direct proportionality, therefore in testing the active material it was always diluted beforehand to such a concentration that the angle obtained was between 5° and 14°. Each test was made on 8–12 plants to get a satisfactory mean value.

The curvatures obtained were determined by measuring the angle between the uppermost part of the stump and the uncurved base, with a

tangimeter. This gives a direct measure of the difference in growth between the two sides of the coleoptile, providing the thickness does not vary considerably. As was shown by Went, the thickness of this type of oat varies only between very narrow limits. This method has the advantage that only one measurement has to be made, while that used by Nielsen and by Boysen-Jensen involves at least two, i.e., the length of the curved part and the radius of curvature. Small growth differences, especially, cannot be determined accurately by this method, since radii longer than 8 cm. have to be neglected. This is presumably the reason why the curves relating reaction of the plant to concentration of growth substance, given by Nielsen, do not pass through the origin, but meet the abscissa at a point some distance away, which should mean that no reaction is produced by small concentration of growth substance. This is, of course, not the case, since Went and also van der Wey[13] found for the same relation a straight line passing through the zero point.

In order to be able to bring all the data on to a quantative basis, some kind of a unit system had to be introduced. As *unit* was chosen that quantity of growth substance which has to be present in 1 cc. of solution to give, after mixing with 1 cc. of agar, an angle of 1°. The total number of units per cc. of solution is then found by multiplying the angle measured by the dilution in which the test was carried out. In the experiments below, the first figure gives always the dilution, the second the angle actually measured. The actual amount of material in the block applied to the plant is only $^1/_{200}$ of that which is present in 1 cc., and this quantity is termed a "plant unit." The units defined above have only an arbitrary value, since their application is limited to measurements in which the procedure described above is rigidly adhered to. The same dependence upon the conditions of the test applies to the biological assay of all animal hormones. The variety of oat used, the culture conditions, the size of the agar blocks and the time relations between the various manipulations, will all exert a considerable influence upon the final result. Van der Wey, for instance, showed that variations in the time relations alone can alter the curvatures by as much as 10 times. This is the reason that the unit as defined above cannot be compared with that proposed by Boysen-Jensen. Duplicate determinations on the same solution, however, showed that if the standardized conditions are followed, the closest possible agreement can be obtained.

In order to obtain active solutions of growth substance, the concentrated culture media were always extracted with ether to get rid of most of the salts and impurities, since Nielsen found the growth substance to be soluble in ether. The most efficient extraction can be obtained by equilibrating the solution three times with half its volume of ether. Most of the ether was distilled off, water was added, and the solution boiled to remove the

last traces of ether. As already pointed out by Nielsen, great care has to be taken to use pure ether, since traces of peroxides readily destroy the growth substance. At the beginning, ether freshly distilled over sodium was used, but even with this, considerable destruction of growth substance took place, probably due to traces of peroxides still remaining. Various methods of purifying the ether were therefore tried out:

(1) One hundred and fifty cc. ether (Merck blue label) was kept overnight over fresh sodium wire, and then distilled. Two cc. of solution, with an activity of 16 $\times$ 12.0° $\times$ 2 cc. = 384 units, + 3 cc. water, were shaken well with 5 cc. of this ether, the ether layers mixed with 10 cc. water and the ether boiled off. Activity of the ether extract, 3 $\times$ 9.0° $\times$ 10 cc. = 270 units; activity of the water layer; 5.5° $\times$ 5 cc. = 28 units. Total activity, 298 units, hence loss = 20.4%. In most of the extractions made with ether so purified, a similar loss of about 20% was found.

(2) One hundred and fifty cc. ether was shaken well with an equal volume of 30% NaOH solution, and washed five times with distilled water to remove the NaOH. Two cc. of the same solution as above + 3 cc. water were shaken in the same way with 5 cc. ether. Activity of ether layer, 3 $\times$ 9.1° $\times$ 10 cc. = 273 units; activity of the water layer, 6.5° $\times$ 5 cc. = 33 units. Total activity; 306 units, hence loss = 20.0%.

(3) One hundred and fifty cc. ether were allowed to stand overnight with 7 g. $FeSO_4 \cdot 7H_2O$ + 1 g. CaO and then distilled. Two cc. of the same solution + 3 cc. water were shaken with 5 cc. ether as above. Activity of ether layer, 3 $\times$ 10.2° $\times$ 10 cc. = 306 units. Activity of water layer, 6.5° $\times$ 5 cc. = 33 units. Total activity, 339 units, hence loss = 11.5%.

From these experiments it is clear that if ether is purified with ferrous sulphate and lime (Garbarini's method[6]), the best results are obtained. A small loss was still found with this ether, but this is at any rate partly due to mechanical loss on the walls of the vessel.

For the determination of the total dry matter in the solution, $^1/_2$ cc. was pipetted by means of an Ostwald pipette into a small platinum dish. This was placed on top of a small Erlenmeyer flask containing boiling redistilled water, and evaporated to dryness. It was then transferred to the oven at 110° for five minutes, cooled in a micro-desiccator and weighed on the micro-balance. Since the amounts available for weighing were very small, the error was somewhat high, but duplicate determinations usually agreed within 2–3%, when the total amount weighed was 0.2 mg. or more. After weighing, the dish and contents were heated to redness for a moment, cooled in the desiccator and again weighed to obtain the ash. For the loan of a Christian Becker micro-balance the authors are greatly indebted to Dr. Chester Bliss of the U. S. Bureau of Entomology at Whittier, Calif.

§ 2. *Determination of the Partition Coefficient and Dissociation Constant.*—In the preliminary experiments on the effect of pH, it was found that the active solutions of the growth substance were somewhat acid, having a pH of about 4. On the addition of alkali and extraction of the alkaline solution with ether, the ether extract was found to be inactive. The growth substance was not destroyed by the alkali, for the remaining aqueous layer was still highly active.

To 25 cc. active solution was added 0.1 M $Ba(OH)_2$ to a pH about 10 (colorimetric), 1.0 cc. being required. The solution was extracted three times with 12–13 cc. freshly redistilled ether. Activity of the original solution $8 \times 6.8° \times 25$ cc. = 1360 units. Ether extract $8 \times 0° = 0$ units. Aqueous layer $16° \times 25$ cc. = > 400 units. (max. angle). A second experiment gave identical results.

It is clear from the above that formation of a salt has occurred with an accompanying loss of solubility in ether. The growth substance must therefore be considered as an acid. The pH limits within which salt formation occurs were determined as follows: to 5 cc. of an active solution were added 5 cc. McIlvaine phosphate-citrate buffer of pH 4, 5, 6 and 7. The mixture was extracted 3 times with 5 cc. freshly distilled ether and the extract made up to 10 cc. Activities of extracts:

pH 4	$2 \times 9.6° \times 10$ cc. = 192 units
pH 5	$2 \times 9.7° \times 10$ cc. = 194 units
pH 6	$2 \times 5.7° \times 10$ cc. = 114 units
pH 7	$1 \times 0° \times 10$ cc. = 0 units

It should be added that confirmatory tests on the remaining aqueous layers were carried out, but for reasons which are given below, did not give satisfactory curvatures, owing to the effect of the buffer on the test. The region of pH concerned is from 5 to 7. It is clear that by narrowing the range, the actual dissociation constant of the acid could be found, and this was the next step.

In order to determine accurately the dissociation constant of the growth substance, however, account must be taken of the fact that the extraction with ether displaces the equilibrium between the free acid and its salt. The amount by which the equilibrium is displaced depends on the amount of the acid which is extracted, and therefore on its partition coefficient between ether and water. This constant must therefore be determined in order to calculate the dissociation constant of the acid.

If the growth substance is an acid, then

$$\frac{[H][A]}{[HA]} = K \qquad (1)$$

where K is the dissociation constant. If HA, the free acid, is the ether-soluble form, and T the total amount of growth substance, then we have

$$\frac{[HA]}{[A]} = \frac{[H]}{K}$$

and hence

$$\frac{[HA]}{[A] + [HA]} = \frac{[H]}{[H] + K}. \tag{2}$$

Since $[A] + [HA] = [T]$, the left-hand side of (2) is $[HA]/[T]$, the fraction of growth substance extractable with ether, which can be written F, i.e.,

$$F = \frac{[H]}{[H] + K} \tag{3}$$

or

$$K = \frac{[H]\,(1 - F)}{F} \tag{3a}$$

The partition coefficient, P, is defined as the ratio of HA in ether and in water at equilibrium, i.e.,

$$P = \frac{[HA]_e}{[HA]_w}. \tag{4}$$

If the volumes of ether and water are the same, concentrations can be replaced by amounts. After one extraction with an equal volume of ether,

then

$$T = HA_w + A_w + HA_e. \tag{5}$$

From (1)

$$[A] = \frac{K[HA]}{[H]}.$$

Replacing concentrations by amounts, and considering only the water phase,

$$A_w = \frac{K\,HA_w}{H_w}.$$

Equation (5) therefore becomes

$$T = HA_w\left(1 + \frac{K}{H_w}\right) + HA_e$$

or from (3)

$$T = HA_w\left(\frac{1}{F}\right) + HA_e$$

Substituting from (4)

$$T = \frac{HA_e}{PF} + HA_e$$

whence
$$\frac{HA_e}{T} = \frac{PF}{1 + PF} \tag{6}$$

or
$$F = \frac{HA_e}{P(T - HA_e)}. \tag{6a}$$

The left-hand side of (6) is the fraction of the total growth substance to be found in the ether extract after one equilibration with an equal volume of ether. If half the volume of ether is used,

$$\frac{HA_e}{T} = \frac{PF}{2 + PF}$$

By similar treatment, with an initial concentration of $[HA]_w$, the equation for the second extraction was developed, and also for the third. These, in the case of half the volume of ether, were found to be, respectively,

$$\frac{HA_{e_2}}{T} = \frac{2PF}{(2 + PF)^2}$$

and
$$\frac{HA_{e_3}}{T} = \frac{4PF}{(2 + PF)^3}.$$

The total fraction extracted by three equilibrations with half the volume of ether is obtained by adding

$$\frac{HA_{e_1} + HA_{e_2} + HA_{e_3}}{T} = \frac{PF}{2 + PF}\left(1 + \frac{2}{2 + PF} + \frac{4}{(2 + PF)^2}\right). \tag{7}$$

However, no greater accuracy is obtained by several extractions, and a single extraction was therefore used in all those experiments from which P and pK were calculated.

Partition Coefficient.—For the determination of the partition coefficient, the following experiments were carried out. To 2 cc. active solution were added 3 cc. water, and the solution shaken thoroughly with freshly distilled ether. The experiments were carried out in the cold room at 1° to minimize evaporation of the ether. Water was added to the ether layer, and both ether and aqueous layers were then boiled until free from ether, made up to volume, and the activities of both determined. The results are shown in table 1.

Dissociation Constant.—For these experiments a measured volume of an active solution was pipetted into a slightly larger volume of Sørensen's M/15 phosphate buffer, the total volume in each case being 5 cc. The activity of the original solution having first been determined, the number

of units per cc. of the mixture of buffer and growth substance was thence obtained. (Column 5 of table 2.) The mixture was then equilibrated with 5 cc. of ether freshly distilled from $FeSO_4$ + CaO and sodium wire, the operation being carried out in the cold room at 1° as before. Finally 3 cc. water was added to the ether extract, the ether boiled off, the solution made up to 5 cc. and the activity determined. On account of the modifying effect of the buffer on the activity tests (see below), only the ether extract could be tested. In the more acid solutions, where the extraction was more or less complete, the value of $T - HA_e$ in equation (6a) becomes

TABLE 1

PARTITION COEFFICIENT OF GROWTH SUBSTANCE BETWEEN ETHER AND WATER AT 1°C.

ACTIVITY OF ETHER EXTRACT	FINAL VOL., CC.	TOTAL ACTIVITY IN ETHER, UNITS	ACTIVITY OF AQUEOUS RESIDUE	FINAL VOL., CC.	TOTAL ACTIVITY IN WATER, UNITS	P
12.0°	20	240	2.6°	10	26	9.2
{ 12.8°	10	128 + 129 }	2.7°	10	27	9.5
{ 12.9°	10	= 257 }				
3 × 10.2°	10	306	6.5°	5	33	9.3
3 × 9.0°	10	270	5.5°	5	28	9.6

Mean value of P = 9.4.

a small difference between two large numbers. Since this is then divided into a large number, HA_e, the error becomes large. On this account most of the measurements were made toward the alkaline side of the dissociation curve. One point, however, was determined at rather high acidity, and this was in general agreement with the others. The buffer for this point was an improvised KH_2PO_4-HCl mixture, whose pH was determined with the hydrogen electrode. The value obtained from this experiment was not used in determining the mean, for the reasons given

TABLE 2

DISSOCIATION CONSTANT OF GROWTH SUBSTANCE

I	II	III	IV	V	VI	VII	VIII	IX
PH OF BUFFER	VOLUME OF BUFFER SOLUTION, CC.	ACTIVITY OF ORIGINAL SOLUTION, UNITS/CC.	VOLUME TAKEN, CC.	ACTIVITY OF MIXTURE = $[T]$, UNITS/CC.	ACTIVITY OF ETHER EXTRACT = $[HA_e]$ UNITS/CC.	FRACTION OF G. S. IN HA FORM = F	DISSOC. CONSTANT OF G. S. = K (× 10^5)	−LOG. DISSOC'N. CONSTANT = pK
4.51	4	16 × 12° = 192	1	38.4	4 × 8.4° = 33.6	11.1 %	1.07	4.97
5.29	3	96 × 5.7° = 547	2	219	16 × 9.0° = 144	74.5 %	1.99	4.70
5.59	4	192	1	38.4	2 × 9.8° = 19.6	20.4 %	1.99	4.70
5.91	4	192	1	38.4	1.5 × 10.2° = 15.3	7.05%	1.82	4.74
6.25	4	192	1	38.4	1 × 8.9° = 8.9	3.22%	1.70	4.77
6.47	4.5	547	0.5	54.7	1.5 × 6.0° = 9.0	2.10%	1.58	4.80
6.64	4.5	547	0.5	54.7	1 × 6.0° = 6.0	1.32%	1.74	4.76
6.98	3	547	2	219	1 × 10.5° = 10.5	0.53%	1.95	4.71
				Mean of all except first determination			1.82	4.75

above. Since the volumes of the initial mixture and of the ether were the same (5 cc.), the ratio of column 6 over column 5 gives at once the value of HA_e/T. The values of F in column 7 were obtained from equation (6a), using 9.4 as the value of P. The calculation of K from equation (3a), and hence of pK, follow in columns 8 and 9. It should be noted that the pK is for solutions containing up to $M/15$ phosphate.

The fraction dissociated at varying pH is plotted in figure 1, in which the curve is the theoretical dissociation curve for an acid of pK 4.75.

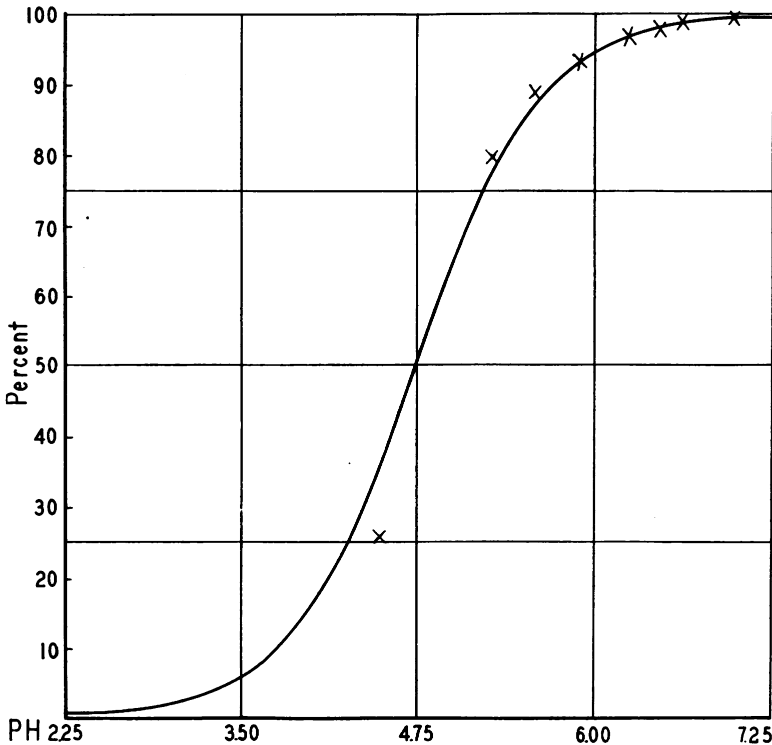

FIGURE 1

Dissociation curve of the growth substance.

The fact that the growth substance is only extractable with ether in acid solutions is very important. When media in which various organisms have grown are extracted in order to test their activity, great care has to be taken to adjust the pH beforehand, or else serious errors can be introduced. For the same reason, it was important to determine whether the growth substance in salt form had the same effect on the plant as in the free acid form. In the first experiments to determine the pK, the activity both of the water and of the ether layer were tested, as men-

tioned above. At pH 6 and 7 the activity of the water layer was in each case too low, which indicated that the state in which the growth substance was present affects the tests. In order to establish this further, the following experiments were carried out. 0.5 cc. growth substance solution of an activity 32 × 12.6° × 0.5 cc. = 202 units, were diluted 1:32 and 0.5 cc. of this solution was mixed with 0.5 cc. water and 1 cc. agar. Activity found: 32 × 12.6° × 0.5 cc. = 202 units. Another 0.5 cc. mixed with 0.5 cc. $M/15$ phosphate buffer pH 7.0 and 1 cc. agar gave activity 32 × 3.3° × 0.5 cc. = 53 units. Hence the presence of the buffer seriously affects the test. That this effect is due to the pH and not to the high salt content of the buffer can be shown by adding, instead of phosphate, $M/15$ NaCl to the solution to be tested. The activity in this and in the blank were identical within experimental error. The pH effect on the test is principally found in buffered solutions; unbuffered solutions give much better results even in the alkaline range. This is probably due to the fact that the growth substance will be rapidly converted from its salt into the free acid by the acid cell-sap.

§ 3. *Chemical Properties of the Growth Substance.*—The outstanding characteristic of the growth substance, beside its acidity, is, as mentioned above, its susceptibility to oxidation. Since the traces of peroxide present in freshly distilled ether are sufficient to destroy some 20% of the activity, strong positive reactions were to be expected with laboratory oxidizing agents, and experiments were therefore carried out to determine what type of oxidation was involved.

Permanganate.—To $^1/_2$ cc. of an active solution containing 79 units per cc. was added $^1/_2$ cc. of a 0.0024 M solution of alkaline $KMnO_4$. The solution was very gently warmed, then cooled and acidified. Reduction had taken place, a faint pink color remaining. The solution was extracted with 4 cc. freshly distilled ether, 1 cc. water added to the ether extract and the ether boiled off. The resulting solution was entirely inactive. Destruction was therefore rapid and complete.

Silver Nitrate.—A preliminary test having shown that $AgNO_3$ was reduced by the growth substance, $^1/_2$ cc. of an active solution containing 180 units per cc. was treated with $^1/_2$ cc. of 0.5 M $AgNO_3$, i.e., a considerable excess, and warmed in hot water until precipitation of Ag was complete. The solution was cooled, acidified and extracted with 5 cc. freshly distilled ether, the extract freed from ether and tested as before. A very slight angle, 10 × ?1° = ?10 units per cc., indicated that the activity was almost if not quite destroyed. The oxidation is probably somewhat slower with $AgNO_3$ than with $KMnO_4$.

Fehling's Solution.—Since the silver reaction is principally given by aldehydes, oxidation by Fehling's solution was tried. In this case a blank to compensate for the possible effect of the alkali was necessary.

To $^1/_2$ cc. of a solution containing 59 units per cc. was added $^1/_2$ cc. each of the two solutions comprising the Fehling's mixture, and as a blank, to another $^1/_2$ cc. was added $^1/_2$ cc. of the alkaline tartrate solution alone and $^1/_2$ cc. water. Both tubes were placed in boiling water for 10 minutes, cooled, acidified with HCl to pH 4–5, and extracted once with 5 cc. freshly distilled ether. The ether extracts were freed from ether and tested. Activity of solution treated with copper and alkali: $2 \times 17.4° = > 35$ units per cc. (max. angle); activity of solution treated with alkali only; $2 \times 19.6° = > 39$ units per cc. (max. angle). Hence the growth substance is not oxidized by Fehling's solution.

Oxime.—An experiment to test whether any destruction was caused by hydroxylamine gave inconclusive results. To $^1/_2$ cc. of a solution containing 120 units per cc. was added 1 cc. of a strong solution of $NH_2OH.HCl$ and after standing with gentle shaking the solution was extracted with 3 cc. freshly distilled ether and both extract and aqueous layer were tested. Activity of the ether extract: $12 \times 7.8° = 94$ units per cc. Activity of water layer: $8 \times 0.7° = 5.6$ units per cc. It is, however, possible that the equilibrium between the substance and its oxime, if formed, is displaced by the ether extraction in the same way as the equilibrium between the acid and its salt. Or, since most oximes are soluble in ether, the plant may be able to reconvert the oxime to the original growth substance. It may, however, be concluded that no definite evidence was obtained to indicate that the growth substance is an aldehyde. The extreme sensitivity to $KMnO_4$ and $AgNO_3$ indicates, however, that double linkings of some sort must be present. It is hoped to obtain further light on the nature of the double bond or bonds in subsequent work.

Effect of Acid and Alkali.—The stability with regard to acid was tested in the cold and in the hot. To $^1/_2$ cc. of an extract containing 180 units per cc. was added $^1/_2$ cc. of 2 *N* HCl. After standing about 3 hours at room temperature the solution was extracted once with an equal volume of ether and the activity of the ether extract tested. This should take out 90% of the active substance. In case the high acidity should have affected the test, another $^1/_2$ cc. similarly treated with HCl was first neutralized with NaOH and then an equal volume of pH 6 buffer added. Activity of the first extract $20 \times 5.1° = 102$ units per cc. Activity of the 2nd extract $20 \times 4.6° = 92$ units per cc. In each case the activity is reduced by about a half through the acid treatment.

Another $^1/_2$ cc. of the same extract was heated in boiling water 30 minutes with $^1/_2$ cc. 2 *N* HCl, cooled and extracted once with an equal volume of ether. From the above experiment it was clear that the 1 *N* HCl was not extracted sufficiently in the ether to affect the test, hence the acid solution was extracted directly. Activity of original solution $20 \times 8.7° = 174$ units per cc. Activity of acid extract $10 \times 1.1° = 11$ units per cc. Hence

destruction by 1 *N* acid proceeds rather slowly, requiring more than 30 minutes at 100° and more than three hours at 25°.

It was mentioned above that a blank was carried out for the alkali of the Fehling's solution, indicating that no effect occurred. However, a further experiment was carried out to confirm this. To one cc. of an active extract was added 1 cc. 2 *N* NaOH, and the liquid heated 20 minutes in boiling water, cooled, acidified with H_2SO_4 and extracted with three volumes freshly distilled ether. The ether extract was boiled and the activity tested as before. A similar tube containing 1 cc. of the same active extract and 1 cc. water, heated for the same time, acidified and extracted, served as blank. Activity of alkali heated extract; $5 \times 18.5° = 92$ units per cc. Activity of blank $5 \times 17.8° = 89$ units per cc. Hence no destruction is caused by 1 *N* NaOH in 20 minutes at 100°.

§4. *Experiments on Purification. Dialysis.*—Various attempts were made to get the substance free from impurities. This was first tried by means of dialysis. As Nielsen had shown that on dialysis through a collodion membrane the substance is destroyed, presumably by peroxides still present, a cellophane membrane was used.

TABLE 3

RESULTS OF DIALYSIS

	AFTER 24 HOURS	AFTER 3 × 24 HRS.		
SOLUTION	ACTIVITY PER CC.	ACTIVITY PER CC.	DRY WEIGHT PER CC.	DRY WEIGHT PER UNIT
Inner solution	42 units	8.8 units	0.45 mg.	0.051 mg.
Outer solution	6 units	8.0 units	0.49 mg.	0.061 mg.

A piece of glass tubing, 15 mm. bore was ground at one end and fitted into a rubber stopper so that the ground end was just flush with the surface of the stopper. Over the tube and stopper a cellophane membrane was stretched and fastened with rubber bands. Ten cc. of growth substance solution was brought into the tube and dialyzed against 25 cc. redistilled water. The inner and outer solutions were stirred by bubbling nitrogen through, and dialysis allowed to take place in the cold room at 1°. After 3 days, equilibrium was reached and the solutions were tested. A test with carmine solution, after the experiment, showed the membrane to be intact.

A second experiment gave similar results.

Hence the activity as well as the total dry matter per cc. is about equally distributed over both solutions, so that in this way no appreciable purification can be obtained.

Evaporation.—After extracting the active medium with ether, evaporating off the ether and taking up in water, Nielsen observed that the growth

substance was more soluble than the rest of the oil and obtained a partial separation by this means. In our experiments the whole of the oil was dissolved in water, but on cooling the solution a large part of it again separated. Thus, in one extraction, the aqueous solution contained 96 × 5.7° = 547 units per cc. or 13,700 units in the whole volume of 25 cc. The oil which separated on cooling was redissolved in chloroform and taken up in 10 cc. water, when part of it was dissolved, to give 8 × 7.4° = 59 units per cc. or 590 units in the whole 10 cc. The remaining oil was again dissolved in chloroform and taken up in 10 cc. water to give a solution containing 1 × 3.9° = 3.9 units per cc. or 39 units in 10 cc. Hence the whole of the oil, representing the bulk of the solid matter, contained only 631 units, while the aqueous solution contained 13,700 units. On this basis it was hoped to achieve further purification of the aqueous solution by evaporation and cooling. For this purpose 16 cc. of active solution containing 293 units per cc. was evaporated to about 1 cc., cooled to 1°C.

TABLE 4

	I SEPARATED OIL DISSOLVED IN CHLOROFORM		II AQUEOUS REMAINDER	III AQUEOUS REMAINDER REËXTRACTED WITH ETHER
Activity (units/cc.)	24 × 10.4° = 250		24 × 5.0° = 120	8 × 9.7° = 77.6
Dry weight (mg./cc.)	0.78	0.82	0.59	0.123
Ash (mg./cc.)	0	0.04	0.21	0.001
Dry weight organic matter only	0.78	0.78	0.38	0.122
Weight 1 unit/cc. (mg.)	0.00312		0.00317	0.00157
Weight 1 unit on plant (mg.)	$15.6 \cdot 10^{-6}$		$15.8 \cdot 10^{-6}$	$7.8 \cdot 10^{-6}$

and the separated oil skimmed off the surface with a fine glass rod and redissolved in chloroform. Water was added, the $CHCl_3$ evaporated off and the solution made up to 10 cc. The 1 cc. of clear yellow aqueous solution was made up to 10 cc. again. The activities and dry matter of the two solutions were then determined, I and II in table 4. Since the aqueous solution carried all the dissolved salts present, the ash was 36%. The weight of organic matter was corrected for this ash, since the fact that active solutions could be obtained without ash indicates that the growth substance is wholly organic. However, this correction is necessarily a minimum one, since the metallic impurity which is determined as ash probably has a higher weight when present in undecomposed form.

A comparison of the dry weight per unit indicates that these two solutions were of identical purity and therefore no purification resulted. The separation of oil which took place in the early stages of the preparation must therefore have removed all of the less water soluble oil. It may be deduced from this experiment that the solubilities in water of the

growth substance and its inactive contaminants at the later stages are the same.

Lastly the remainder of the aqueous solution was extracted three times with an equal volume of ether and made up back to the same volume with water. Though the activity of this solution had fallen to about $^2/_3$, the dry weight had fallen still more and a considerable purification resulted. (III in table 4.) Though it is not possible, therefore to purify the extract further by simple evaporation and chilling, yet reëxtraction with ether is effective, probably on account of the high partition coefficient.

Fractional Extraction.—It follows from the value of the dissociation constant that at pH 7 only a very small fraction of the growth substance will be extracted with ether. It can be calculated from the formula, equation (7), that the total fraction extracted by three times equilibrating with $^1/_2$ the volume of ether will be 6.7%. Any other substance present, unless also an acid of similar strength, will however be removed in the ether, and the ratio of activity to dry matter in such an extract should be lower if impurities are present. The usefulness of this method for purification was therefore examined as follows: two cc. of an active

TABLE 5

SOLUTION	ACTIVITY UNITS/CC.	TOTAL UNITS	DRY WEIGHT MG./CC.	MG./UNIT
pH 6.98 ext	2 × 5.3° = 10.6	65.7 = 11%	0.159	0.0150
pH 5.29 ext.	16 × 6.4° = 102.4	512 = 88%	0.426	0.00416

solution containing 24 × 12.2° = 293 units per cc. were mixed with 4 cc. Sørensen's phosphate buffer of pH 6.98. The McIlvaine buffer could not be used owing to the solubility of citric acid in ether. The mixture was thoroughly shaken three times with 3 cc. of freshly distilled ether in the cold at 1°C., the extract freed from ether, made up to 6.2 cc. and the activity and dry weight determined. An exactly similar experiment was then carried out using buffer of pH 5.29, at which pH about 86% should be extracted. The results, given in table 5, show that there is a considerable difference in the activity : dry weight ratio.

By subtracting the activities and dry weights of the pH 7 extract from those of the pH 5.3 extract it is clear that the 91.8 units per cc. difference corresponds to a weight of 0.267 mg. per cc., or 1 unit is 0.00291 mg. Hence if everything is assumed to be extracted at pH 5.3, then by first extracting at pH 7 and then acidifying and reëxtracting, an increase in the purity given by (1/0.00291 − 1/0.00416)0.00416 = 42.7% would be obtained.

This method was therefore applied to a considerable amount of highly active solution. This solution was prepared in the ordinary way by ether extraction of active culture medium, dissolving in water, thoroughly

chilling, and then decanting from separated oil. It contained 64 × 4.5° = 288 units per cc. and was already of high purity, the dry weight being 0.52 mg. per cc., so that 1 unit per cc. = 0.0018 mg. per cc., or 1 plant unit = 9.10^{-6} mg. 21.5 cc. of this solution were mixed with pH 6.98 phosphate buffer and extracted three times with 25 cc. freshly distilled ether at 1° C. The extract was freed from ether and made up to 10 cc. The aqueous solution was acidified with HCl to a pH about four (colorimetric) and again extracted three times with an equal volume of ether. The activity and dry weight of both extracts were determined. A portion of the pH 4 extract was then reëxtracted three times with $^1/_2$ the volume of ether and the activity and dry weight again determined. This agreed satisfactorily, showing approximately a constant weight per unit. Where duplicate determinations were carried out, both are given in the table.

TABLE 6

	Activity		Dry weight				Dry weight of 1 unit calculated on ash-free basis	
Solution	Units/cc.	Total units	Mg./cc.	Ash	Corrected mg.	Total mg.	Mg./unit	Mg./plant unit
Original Solution *A*	64 × 4.5° = 288	6180	0.52	2%	0.51	11.2	0.0018	$9 \cdot 10^{-6}$
Solution *A*, extracted at pH 7 = *B*	8 × 9.9° = 79.2	792	0.388	5%	0.370	3.9	0.0049	$24.5 \cdot 10^{-6}$
A then extracted at pH 4 = *C*	{32×13.2° = 423; 64×6.3° = 403}	4130	0.712	0	0.712	7.1	0.00172	$8.6 \cdot 10^{-6}$
Part of sol. *C* reëxtracted with ether = *D*	{8 × 10.0° = 80; 16×5.0° = 80}	800	{0.155; 0.156}	8%	0.142	..	0.00178	$8.9 \cdot 10^{-6}$
Part of *D* reëxtracted with chloroform	6 × 10.3° = 61.8	309	{0.089; 0.084}	0	0.0865	..	0.00139	$7.0 \cdot 10^{-6}$

Comparison of the total dry weights of solutions B and C with that of the initial solution A shows that all the solid material is accounted for within 2%, so that no loss has occurred. The total activities show, however, that inactivation to the extent of about 20% has occurred. Unless this inactivated material is quantitatively extracted at pH 7, which is not likely, some of it will be present in the pH 4 extract. Therefore, although some impurity has certainly been removed in the pH 7 extract, the net increase in purity is only slight. Some inactivation appears to be inseparable from each ether extraction, so that the highest purity could never be expected to be reached by reëxtracting with ether. Accordingly a final extraction of a part of the last extract was made with chloroform, using two equilibrations with an equal volume of chloroform. By this method the final dry weight per plant unit was found to be 7.0 millionths of a milligram, which represents only a slight improvement over the purity of the final ether extractions above, and also of that in table 4. It seems probable from the above data that we have here to deal with a

mixture of the growth substance and its oxidized form, the two substances being probably so closely related that even fractional extraction at different H-ion concentrations does not separate them.

For further progress in purification, larger quantities of material will be necessary in order to obtain sufficient for the preparation of crystalline derivatives and for analysis. Experiments along these lines are already in progress.

SUMMARY

1. A technique is described by which it is possible to obtain, from cultures of molds, considerable quantities of the substance which controls the elongation of plant cells.

2. The activity of this "growth substance" is tested by its action upon coleoptiles of Avena, and a biological system of units is suggested, whereby, using a standardized technique, it is possible to express the activities of the substance quantitatively.

3. The growth substance is proved to be an acid, which has a dissociation constant of $1.8 \cdot 10^{-5}$.

4. Some of the chemical properties, in regard to its oxidizability and stability to acids and alkalies have been determined.

5. A method for purification is given, by which extracts of approximately constant purity can be obtained.

6. The highest purity obtained corresponded to $7.0 \cdot 10^{-6}$ mg. dry material per plant unit.

* Unpublished work by Mr. Bonner, subsequently carried out in this laboratory, has shown that under suitable conditions *Rhizopus* does produce large amounts of the hormone in liquid media.

[1] Boysen-Jensen, *P. Bioch. Zeitschr.*, **205**, 236, 1931.

[2] Boysen-Jensen, *Bioch. Zeitschr.*, **1**, 238, 1931.

[3] Cholodny, N., *Biol. Zentralbl.*, **47**, 604, 1927.

[4] Dolk, H. E., *Proc. Kon. Akad. Wetensch., Amsterdam*, **29**, 1113, 1926.

[5] Dolk, H. E., *Geotropie en Groeistof*, Diss. Utrecht, 1930.

[6] Garbarini, *Chem. Zentralbl.*, 1126, 1909.

[7] Heyn, A. N. J., *Rec. Trav. bot. neérl.*, **28**, 113, 1930.

[8] Nielsen, N., *Jahrb. wiss. Bot.*, **73**, 125, 1930.

[9] Nielsen, N., *Bioch. Zeitschr.*, **23**, 244, 1931.

[10] Paal, A., *Jahrb. wiss. Bot.*, **58**, 406, 1918.

[11] Seubert, E., *Z. f. Bot.*, **17**, 49, 1925.

[12] Went, F. W., *Rec. trav. bot. neérl.*, **25**, 1, 1928.

[13] Wey, v. d. H. G., *Proc. Kon. Akad. Wetensch., Amsterdam*, **34**, 874, 1931.