Quite analogically, we have extended the discussions on the single six-ring to the double six-ring.

We have, however, entirely neglected the steric forces and the dipole forces. If we could consider these, we should be able to point out which of the configurations and formations found here are the most probable ones. Next time we hope to come back on this question.

Anticipating the facts which we shall have to state then, we only wish to point out that the spatial formation which is always being given for the cis-dekalin, the cis-bed-bed $\gamma\gamma$ formation (see fig. 18) is certainly not the most probable one; we shall rather have to choose the cis-chair-chair configuration (see fig. 22).

We equally want to point to the fact that perhaps some of the isomerism cases of the six-rings, which are being recorded as cis-trans-isomerism, must be reduced to the configuration isomerisms which we have traced here; for there is still too little attention being paid to the latter isomerism possibilities.

In connection with these problems of isomerism, we want to draw attention to a systematical classification of isomerism given by Ir. F. TELLEGEN in Part IV of his dissertation. Delft 1934 (yet to appear).

Lastly I want to express my thanks to Prof. BÖESEKEN, Prof. ZWIKKER and Ir. TELLEGEN for the discussions which I might have with them about the questions treated in this paper.

Delft, April 1934.

Physical Laboratory of the Technical High School Delft.

Botany. — *On the pea test method for auxin, the plant growth hormone.* By F. W. WENT. (From the WILLIAM G. KERCKHOFF Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California).

(Communicated at the meeting of September 29, 1934).

Introduction.

For experiments on auxin the test method which has been used almost exclusively is the *Avena* method, described by me (1928) and modified by VAN DER WEY (1931). This test is quantitative and relatively easy, but it requires a constant and high humidity and constant temperature, while all operations have to be carried out in red or orange light. To

those unfamiliar with it, the technique gives the impression of being unnecessarily complicated. Thus LOEWE (1933) writes about the different test methods (l.c. p. 1016); "Keine ist daraufhin geprüft wieweit sie für Routinezwecke zur rasch unterrichtenden Erkennung oder zur schnellen Ausführung von Reihenmessungen vereinfacht und von allzu aufwandreichem und zeitraubendem Beiwerk befreit werden kann, am wenigsten die Wentsche Methoden." This may seem to be the case because most of the preliminary work leading to the elaborated method has not been published as being without importance. But since about half a million plants have already been treated in the prescribed way in the various laboratories, it is improbable that any obvious simplifications have been overlooked. It is possible for one person to analyse quantitatively 20—30 solutions in one day (KÖGL, HAAGEN—SMIT and ERXLEBEN, 1933). The method of NIELSEN is in general no simpler.

A second statement of LOEWE (1933, p. 1016); "Eine möglichst vereinfachte Potometeranordnung dürfte sich vielleicht am besten als Testreaktion eignen", disregards the principal advantages of curvatures in growth measurements. As all test objects have a residual growth, the effect of auxin must be measured with a differential method. In straight-growth determinations (as with a potometer) the growth effect must be calculated by subtracting the growth of untreated from treated plants, which decreases the accuracy considerably because of the individual differences in growth. If the auxin action is superimposed on the residual growth only in one longitudinal section of an organ, a curvature appears *independent* of its residual growth. This makes superfluous the determination of growth on a set of control plants and increases the accuracy of the actual auxin determination.

Of all the described methods for the demonstration of auxin, perhaps the lanolin method, as used and described by LAIBACH (1933, 1934) is the most convenient one. However, it is not quantitative and requires rather concentrated auxin solutions. Instead of applying it dissolved in agar blocks or any other gelatinous material which necessitates a high humidity, the solutions are mixed with wool fat (*lanum anhydrum*) and applied as such, making use of the solubility of auxin in fat.

Still simpler and more convenient, while also semi-quantitative, is the pea test method for auxin. This presented itself in the course of investigations on root-formation in etiolated Pisum shoots, for which the apical parts are split and placed in solutions. It was observed that if these solutions contain more than 6 units of growth substance (= ca 480 AE) per cc, easily recognised inward curvatures of the split ends occur. These curvatures proved an easy and reliable test for auxin under relatively simple experimental conditions. Only a few of the many possible variations in the conditions of the test have been tried out; a closer investigation may increase the accuracy, and a standardisation might prove relatively simple and useful.

Method.

The peas used in the following experiments were "Alaska 89", obtained through the courtesy of the Gallatin Valley Seed Co. The peas are grown in a dark room. Small amounts of white light do not decrease the sensitivity of the plants so long as the growth of the seedlings does not decrease appreciably, and so long as the shoots are straight. They can be used at a length of from 5 to 20 cm.

Pieces of stem 2 to 20 cm long are cut about 5 mm below the terminal bud, and are used either directly or 4 to 8 hours after cutting. Immediately before application of the auxin solutions the top of the cut piece of stem is split lengthwise by an exactly median cut with a razor blade over a length of 1—3 cm. If the cut is very far from median the curvatures are smaller. Upon subsequent immersion in the solutions the two halves will bend out because of the tissue tension. If no auxin is present in the solution these original outward curvatures do not change any more and are fixed, since they do not go back upon plasmolysis. In the presence of auxin, however, about an hour after immersion (at 25°) the free ends of the two halves will start to bend inwards; the higher the concentration the further they bend. After about 6 hours the final state is reached and the originally most rapidly growing zone will be convex at the outside. If the median slit is long enough, the halves will each show an *S*-shape. Also these curvatures are not changed by plasmolysis. For the appearance of the curvatures it is only necessary to have the apical cut surface in contact with the auxin containing solution. So either the shoots are reversed and placed upside down in a small amount of solution (5 plants together in 0.2—1 cc) or they are left floating on the surface of the solution in a shallow dish. If the auxin can enter only through the surface of the radial cut no curvatures appear.

The simplest quantitative auxin determination with the pea test is to place a number of pea shoots, apically slit lengthwise over a distance of $\pm$ 10 mm, in a whole range of dilutions of the solution to be tested; 5 plants per dilution will be enough. After 12 hours it is determined in which concentration a well recognizable reaction occurs. In this way either directly or by intrapolation the critical concentration may be calculated with an accuracy of 50 %. This concentration corresponds with $\pm$ 6 units of growth substance per cc (DOLK and THIMANN, 1932) or 480 A. E. per cc or 1.4×10^{-5} mg auxin *A* per cc (KÖGL, HAAGEN SMITH and ERXLEBEN, 1933). The only work involved in this method is the making up of the dilutions and the splitting of the peas; the estimation of the reaction is done at a glance. In table I a number of determinations of auxin with the pea test are compared with the results of the *Avena* test, proving that there is an excellent correlation between the two. In the course of the last few months it has been found that as a qualitative method this pea test is somewhat more useful than the

TABLE I.

Activities of solutions and products, expressed in units of growth substance per mg, as tested with the *Avena* method and with the pea test, if the activity of the K V solution in both cases is assumed to be the same (activity for auxin A).

Test substance	Avena test	Pea test
"Br. F." extract of Rhizopus	6.5×10^3	6×10^3
"Gallen B"	2.7	2 — 4
Pollen of Hicoria	65	60
Pollen of Quercus alba	0.7	<1
Auxin B crystalline	2.3×10^5	2.5×10^5
Hetero-auxin crystalline	2.8×10^5	4×10^5

Avena test because a greater range of concentrations may be observed. The *Avena* test, however, is preferable in a quantitative sense.

More quantitative expression of the auxin action can be obtained by classifying the curvatures, as the example of table II shows. The plants

TABLE II.

Auxin curvatures estimated according to valuation given in text. Each figure mean of 20 plants. 5 plants per bottle.

	32 units/cc	8 units/cc	2 units/cc
1 cc per bottle	3.5	0.8	0
0.5 cc per bottle	1.9	0.4	0
0.25 cc per bottle	1.6	0.4	0

were slit over a length of 15 mm and placed upside down in 1.0, 0.5, or 0.25 cc of solution in bottles with an inside diameter of 1 cm. The curvature of each plant was valued according to the following description:

0 = no auxin reaction, 2 halves concave at the outside over their whole length (see figure).
1 = slight auxin reaction, trace of convexity in limited region.
2 = definite auxin reaction, ends of both halves approximately parallel.
3 = fair auxin reaction, ends bent inwards (see figure).
4 = strong auxin reaction, ends just touching.
5 = very strong auxin reaction, both halves crossing at end.
6 = very strong auxin reaction, halves crossing midway or at base (see figure).

In table II the curvatures are given as means of 20 plants each. It can be seen that the curvatures in the 8 u/cc solution are just $^1/_4$ of

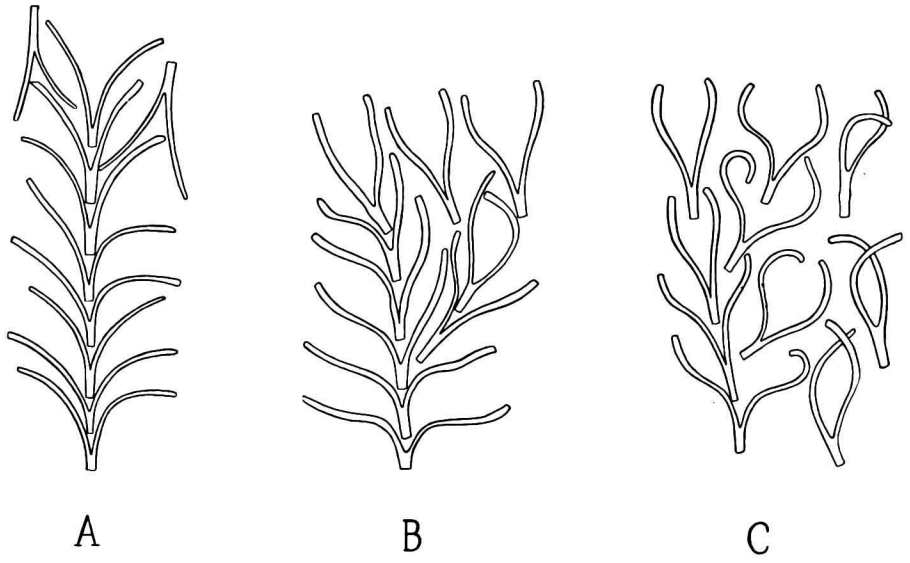

Fig. 1. Curvatures in split sections of pea stems, immersed in
a. water.
b. solution containing 30 units per cc, (curvature about no. 3).
c. solution containing 150 units per cc, (curvature about no. 6).

those in the 32 u/cc, whereas the amount of solution has a smaller effect. It is clear that good quantitative determinations can be made with this method of estimation. Also the method is quick and easy.

As the estimations mentioned in the foregoing paragraph have the drawback of being subjective, especially since the value of the numbers given is completely arbitrary, some experiments were carried out in which the curvatures were measured with a graduated arc, as in the *Avena* test. The measured angle was comprised between the tangents to the extreme tip and to the region where the concave curvature due to tissue tension changes into the concave auxin curvature. This method, however, takes much more time and is not recommended as a substitute for the *Avena* method. The peas were split apically over a length of 2.5 cm and placed in petri dishes filled with 20 cc of solution. Most dishes were placed in the dark room at 25° C., some at 25° C. in white light (incandescent lamps) of approximately 1000 meter-candle intensity, and one in subdued daylight in an ordinary room at 16—20° C. The results are given in table III. It is clear that there is no difference between the reactivity of short plants (ca 6 cm) and long ones (ca 12 cm), just as in the *Avena* test. It is best to cut off the upper 5 mm of the stem, as this part does not curve in the described way with auxin; its presence even slightly decreases the amount of the curvature. If a longer

TABLE III.

Auxin curvatures in degrees. Split pea shoots floating in solutions. Measurement 20 hours after beginning. Each figure is the mean of at least 20 curvatures; variation indicated as mean error.

units/cc	150	60		30	15	0
length of plants	long	long	short	long	long	long
Dark, extreme tip cut off		62	67.5			
Dark, 5 mm tip cut off	87 ± 13	70 ± 7	74	37 ± 5	21 ± 3	ca – 25
Dark, 10 mm tip cut off		45				
Dark, short incision		44				
Light, 25° C., 5 mm tip cut off	69 ± 6	26 ± 4				ca — 25
Light, 18° C., 5 mm tip cut off		75 ± 7				

part of the stem has been cut, part of the reacting tissue is removed, so that the curvatures are considerably smaller. The latter is also the case if the median incision is not deep enough. Most interesting, however, are the experiments in the light. The plants in the subdued daylight, at a considerably lower temperature, gave essentially the same curvatures as the plants in the dark. However, they were exposed to the light only for the first 2 hours, since after that the evening fell, so that it is possible that the reaction took place in the dark. Nevertheless in this experiment it is proven that the reaction is not affected by temporary illumination with subdued light, and does not require a constant temperature in order to give almost quantitative response. The experiments at constant high light intensity prove that too much light decreases the sensitivity of the plants (cf. VAN OVERBEEK 1933). It is clear that this test is quantitative even under uncontrolled conditions, and that no special equipment is necessary at all. The plants can be grown in an ordinary room under a dark cover, treated in subdued daylight and the reactions can take place in the same room under dark cover, without taking any special precautions to insure a constant temperature. A second interesting general conclusion can be drawn from the quantitative relations between concentration of auxin and rate of curvature. In this case the latter is a linear function of the log of the concentration, at least in the dark. In the light the relation seems to be a different one, which makes it questionable whether too much stress should be laid on the logarithmic relation. In *Avena*, the curvature, and also the straight growth, is a direct linear function of the auxin concentration (WENT 1928, VAN DER WEY 1931, THIMANN and BONNER 1933). Hence it is likely that the curvature in *Avena* has a completely different mechanism from that in *Pisum*. The pea test has the practical advantage that it is applicable over a

larger range of concentration. In the experiment of table III it was possible to distinguish quantitatively differences over the range from 6 to 150 growth substance units per cc.

Specificity and reliability of the test.

Hundreds of solutions have been tested already with the pea test; in most cases *Avena* tests were run parallel for comparison. For the latter determinations I am indebted to Dr. K. V. THIMANN and Mrs. F. DOLK. Without exception both tests gave exactly the same qualitative results, and also the quantitative agreement was remarkable. In the few cases of seeming discrepancy the pea test was the more reliable as duplicate determinations with the *Avena* test showed.

A number of pure organic and inorganic substances from very different chemical groups are completely inactive in the pea test; thus it is unnecessary to mention them. FITTING's objection (1933) to the *Avena* test, that not enough different pure chemicals have been tried to prove the absolute specificity of the method is unfounded; in Utrecht, Pasadena, and many other laboratories hundreds of chemically pure substances have been tried. In the very few positive cases the presence of minute amounts of auxin as impurity could be proven. Because of the complete specificity to auxin these negative results have not been published.

To be completely certain that the pea test is a response to auxins only, the activity of crystalline auxin preparations was tested. Hereby I express my sincere thanks to Prof. F. KÖGL for sending these preparations. The auxin *A* was inactive upon receipt; neither *Avena* or peas reacted at all. Auxin *B* and hetero-auxin both were completely active, giving the same relation of activity in peas and *Avena* as impure auxin *A* preparations do (see table I).

There are some cases in which the higher concentrations of certain solutions do not give an auxin curvature, whereas further dilutions do. In *Avena* this is a well known effect in very high concentrations and due to sideways leakage of auxin. In peas it may be due to:

1. the presence of toxic substances, directly injuring the reacting cells,
2. inhibition of the auxin reaction by special chemicals.

In the first case the toxicity mostly causes loss of turgidity, so that it is easily recognizable. In many crude extracts the concentration of toxic substances sometimes is so high, that there is but a narrow range of concentrations giving positive reactions, but in most cases the active range is from 10—100 times the critical concentration (i. e. the concentration which just gives a visible reaction).

As examples of the second case may be mentioned Cu-acetate and Ni-acetate. If added to auxin solutions in concentrations of 0.001 M ($MnSO_4$ in 0.01 M) no curvatures occur. A concentration of 0.0001 M (0.001 $MnSO_4$) has no detectable effect. With the *Avena* test 0.002 M

Cu-acetate gave a decrease in the auxin curvature of about 60 %. So it seems that the *Avena* test is somewhat less sensitive to traces of copper than the pea test. Inositol in 0.5 and 0.1 % solutions inhibits the auxin curvatures in peas completely, and 0.02 and 0.004 % partially; lower concentrations not at all.

High concentrations of auxin themselves do not decrease the degree of curvature at all in peas, as contrasted with the *Avena* test; e. g. a concentration of 0.02 mg/l of auxin *B* gives a visible curvature, 9 mg/l a much stronger one. With hetero-auxin the corresponding figures are 0.015 and 15 mg/l, i. e. a concentration range of 1000 times. Higher concentrations have not been used, but it is unlikely that with them the rate of curvature will decrease again.

Mechanism of the curvatures.

The following experiments and considerations may serve partially to elucidate the problem of the mechanism of the auxin curvatures in split pea shoots.

1. The curvatures are caused by auxins, so most likely they have to do with cell wall plasticity and growth. However, the logarithmic relation between curvatures and auxin concentration suggests that this is not a phenomenon due to straight growth.

2. The growth of the apical 25 mm of 4 sets of 15 etiolated pea shoots was measured with a horizontal microscope. They had been decapitated 7 hours before. In 2 sets a median cut was made not reaching either end so that the halves could not spread. Then they were inverted and placed with their tips in water or in auxin solution (about 100 units/cc).

Growth in water, not split: 0.26, split: 0.19 mm.
Growth in auxin, not split: 0.64, split: 0.62 ± 0.057 mm.

This means that a wound as made in splitting the peas does not appreciably change the growth rate of the whole shoot nor does it affect the reaction on auxin.

3. Hence we must conclude that in the auxin curvatures either the growth of the epidermis is increased or that of the wound side is decreased.

4. Even the most rapidly growing shoots have a tissue tension causing the halves to bend outwards, so that even with a generous auxin supply the epidermis (with the neighbouring cells) grows slower than the rest of the shoot. Strips of epidermis do not grow in auxin after their initial shortening, while shoots with epidermis removed grow but slightly faster than intact shoots. So, when supplied with auxin, the epidermis does not change its properties markedly, at least not enough to explain the curvatures. BONNER (1934), in explaining the acid curvatures, reached the same conclusion with the epidermis of coleoptiles of *Avena*.

5. The only possible explanation remaining is an active shortening reaction of the cells bordering the cut surface. This can only be a reaction of one or a few cell layers as otherwise the growth rate of the split shoot ought to be influenced (cf. 2). It is supposed that the increased plasticity of the cell wall (cf. 1) freed of the tissue tension at the cut side, makes the cell extend more or less in the radial direction, thereby shortening its longitudinal dimension, like a muscle. Such a thickening as is here supposed in single cells has, in fact, been observed to some degree in the whole shoot treated with auxin after a strip of epidermis has been removed. The growing region will then increase its diameter from 10—25 %.

6. If the thickening reaction of the wound surface cells is the primary auxin reaction, the secondary shortening reaction is not likely to have a linear relation to the primary reaction, but the relation will probably be more or less logarithmic, as a rough mathematical consideration shows; the relation found experimentally is just of this type.

7. If the epidermis is first peeled off, and the shoots are subsequently split, the tissue tension curvatures in water are somewhat stronger, but no auxin curvatures occur at all. This is easily explained by the symmetrical wound surfaces, at both sides of the split halves.

8. At first it was thought that the primary reaction taking place was a partial inactivation of auxin near the wound surface. However, in that case auxin curvatures should not occur in plants lacking the oxidase system necessary for the oxidative destruction of auxin (THIMANN, 1934). But leaf stalks of *Malva parviflora*, if split and treated like pea shoots, give similar auxin curvatures. Moreover it might then be expected that curvatures with auxin *B* and heteroauxin should differ quantitatively, compared with *Avena*, since these two compounds differ in the ease with which they are oxidised. This is not the case, as table I shows.

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