THE EFFECT OF VARIOUS GROWTH-REGULATORS ON EMBRYOS OF CAPSELLA BURSA-PASTORIS GROWING IN VITRO

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ABBREVIATIONS

b = regression coefficient
s_b = standard deviation of regression coefficient
n = number of embryos
$\bar{x}$ = mean initial length
x_0 = critical initial length
y = length increment
GA = gibberellic acid
IAA = indole-3-acetic acid
INC = insoluble nitrogen content

CHAPTER 1

INTRODUCTION

Embryogenesis is the first step in the development of the higher plant. It is clearly distinct from the following stages. During embryogenesis a differentiation takes place of a single cell, the zygote, into a complex, still meristematic tissue system.

During recent years, new approaches to experimental research, and among them the embryo-culture technique, have been developed. In spite of this even in recent efforts (DEMAGGIO and WETMORE, 1961; NORSTOG, 1961) the cultivation of very young embryos remained in

general rather hazardous. This is certainly due to a lack of knowledge of the factors governing the successive internal changes which take place during embryogenesis. The application of physiologically-active substances to embryos cultivated in vitro may contribute to a better understanding of some problems of embryogenesis in plants.

The present author has tested the effect of three growth-regulators belonging to three different types, viz. indole-3-acetic acid, gibberellic acid and kinetin, on embryo growth in vitro. Indole-3-acetic acid may be regarded as a natural growth hormone in higher plants (WILDMAN and BONNER, 1948; REINERT, 1950; TERPSTRA, 1953). It plays a part in cell elongation, cell division, cambial activity, abscission, parthenocarpy, tumor formation, root initiation, dominance relationships between buds, nastic responses and tropisms in general (SINNOTT, 1960). The literature concerning the action of IAA on embryo growth is reviewed in chapter 5.

There is evidence that gibberellic acid is another natural growth hormone in higher plants (PHINNEY and WEST, 1960). The literature dealing with the action of GA on embryo growth is reviewed in chapter 4.

GOLDACRE and BOTTOMLEY (1959) isolated a kinetin-like substance from apple fruitlets, but they examined only its physiological activity, and not its chemical properties; so kinetin itself can not yet be regarded as a natural growth hormone. However, kinetin is known to be effective on meristems (LAETCH and BRIGGS, 1961). The literature on the action of kinetin on embryo growth is reviewed in chapter 6.

The effect of various combinations of IAA, GA, and kinetin on embryo growth in vitro will be discussed in chapter 7.

CHAPTER 2

MATERIAL AND METHODS

MATERIAL

The experiments were carried out with immature embryos of *Capsella bursa-pastoris* (L.) Med. RIJVEN (1952) described a suitable medium for the cultivation of these embryos and a method to cultivate embryos in culture cells, each capable to contain 25 embryos growing singly in a drop of the medium.

The plants were collected a few hours before the beginning of an experiment. In order to reduce the variability of the material, it was brought together in the following way.

1. For each experiment only one plant was used.
2. The number of inflorescences that were used, was limited as much as possible.
3. Care was taken that in each experiment the embryos with different initial length were homogeneously distributed over the various culture cells.

A diagrammatic representation of the development of an embryo that remained enclosed in the ovule, is given by RIJVEN (1952). See Fig. 1 and Table 1.

TABLE I

The stages of development of the embryo in the ovule (after RIJVEN, 1952).

Stage	Length*	Shape
I	18– 50 μ	globular.
II	50– 80 μ	reversed trapezium; height and breadth equal, third dimension somewhat smaller.
III	80– 150 μ	heart.
IV	150– 350 μ	intermediate between III and V, characterized by longitudinal growth of the cotyledons and of the hypocotyl to a slightly less extent.
V	350– 700 μ	"torpedo"; the cotyledons are flattened against each other and take half the length of the embryo.
VI	700– 900 μ	"walking-stick", caused by the cotyledons having turned back in the top of the ovule.
VII	900–1700 μ	upturned U, the one leg formed by the hypocotyl, the other by the flattened cotyledons.
VIII	1760 μ	full-grown embryo.

* without suspensor.

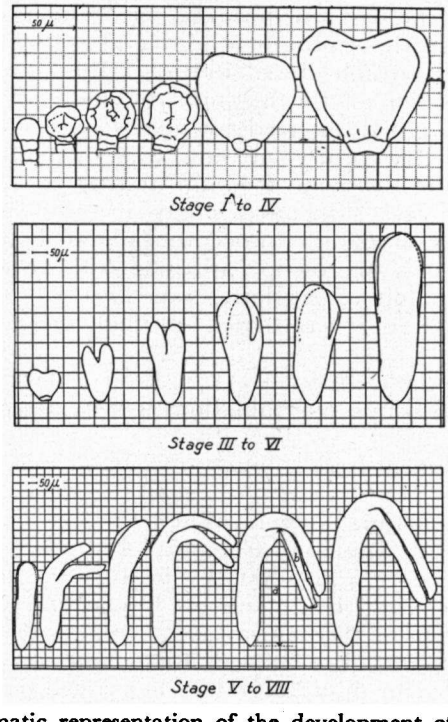

Fig. 1. Diagrammatic representation of the development of an embryo in the ovule (after RIJVEN, 1952).

METHODS

The procedure followed for the cultivation of the embryos, has been described in detail by RIJVEN (1952). The basal medium contained the following mineral nutrients:

	conc. in mg/l
$Ca(NO_3)_2$	128
$MgSO_4 \cdot 7\ H_2O$	77
KNO_3	133
$MnSO_4 \cdot 2\ H_2O$	0.12
H_3BO_3	0.12
$ZnSO_4 \cdot 7\ H_2O$	0.6
$CuSO_4 \cdot 5\ H_2O$	0.3
$(NH_4)_2MoO_4$	0.3
1 % Fe^{+++} citrate	2 ml

Apart from the salts the media contained sucrose in different concentrations. Glutamin was added to the final concentration of 780 mg/l.

The media were buffered with a phosphate buffer after Sørensen (2.5 mM) pH = 5.8.

To this basal medium the growth-regulator(s) was (were) added. The media were sterilized by filtration through sintered-glass filters (Schott G 5).

Embryos were excised from the ovule under a binocular microscope. This was done in the basal medium, i.e. before the growth-regulators were added. Next the embryos of different initial length were as homogeneously as possible distributed over the culture cells; in the latter the composition of the medium differed.

The embryos were transferred by means of a sterilized braking-pipette, first into the culture medium, and then into a culture-cell containing drops of the same medium and up to 25 embryos. The embryos were always suspended singly in drops of the medium between two glass slides; this procedure allows us to measure their length by means of a microscope provided with an eye-piece micrometer. The usual unit of the latter was 17.0 μ.

The whole preparation was carried out under conditions of absolute sterility.

The embryos were cultivated at 25° C in a dark incubator. The methods used for various determinations will be described along with the experiments.

For each experiment fresh solutions of 10^{-4} gr/ml growth-regulator were prepared.

The growth-regulators were furnished by:

1. gibberellic acid: Imperial Chemical Industries Ltd., London. It was dissolved in aqua dest. by heating the solution.
2. indole-3-acetic acid: Hoffmann-La Roche and Co. Ltd., Basle. It was dissolved in the same way.
3. kinetin: Nutritional Biochemicals Corporation, Cleveland, Ohio. It was dissolved in dilute KOH. When a solution of 10^{-5} gr/ml

was tested, the pH of the medium appeared to have shifted from 5.8 to 6.2. The pH of the medium of the controls therefore was adjusted also to 6.2 by adding 0.1 N KOH.

CHAPTER 3

STATISTICAL ANALYSIS OF EMBRYO GROWTH IN VITRO

The unavoidable variability in size of the embryos made it necessary to subject the experimental results to a statistical analysis. Here follows an example of the mathematical treatment of the material. In the following experiment the effect of GA in a concentration of 10^{-5} gr/ml was tested. Excised torpedo-shaped embryos were cultivated in the basal medium containing 12 % sucrose (control: group A). In group B the growth-regulator was added to the basal medium. Table 2 gives the values x (initial length) and y (growth in 24 hours) for these two series. The values are given in units of 10 μ.

TABLE 2

The original growth rates observed in one experiment, serving as basis for the statistical analysis. Group A (control): basal medium with 12 % sucrose; group B: basal medium (12 % sucrose) with gibberellic acid 10^{-5} gr/ml. The values are given in units of 10 μ. x = initial length, y = growth in 24 hours. $d_{y.x.}$ = deviations from regression.

Group A			Group B		
x	y	$d_{y.x.}$	x	y	$d_{y.x.}$
26	2	— 3.6	30	10	— 1.7
26	4	— 1.6	33	19	+ 4.3
26	6	+ 0.4	34	19	+ 3.3
27	7	+ 0.8	36	13	— 4.7
29	6	— 1.5	39	16	— 4.7
29	9	+ 1.5	43	23	— 1.7
37	13	+ 0.5	43	26	+ 1.3
39	16	+ 2.3	44	27	+ 1.3
40	13	— 1.4	46	20	— 7.7
42	16	+ 0.4	46	23	— 4.7
42	21	+ 5.4	46	26	— 1.7
43	16	— 0.2	46	26	— 1.7
46	20	+ 1.9	47	33	+ 4.3
47	19	+ 0.3	49	46	+ 15.3
50	13	— 7.6	50	32	+ 0.3
52	23	+ 1.2	52	31	— 2.7
54	24	+ 0.9	52	35	+ 1.3
60	31	+ 4.2	54	36	+ 0.3
67	27	— 4.2	60	44	+ 2.3
			69	47	— 3.7

In order to establish the relation between these two variables, a correlation diagram was composed (Fig. 2).

The values suggest a linear regression between initial length and growth which can be described as the function

$$y = a + b \cdot x \qquad (1)$$

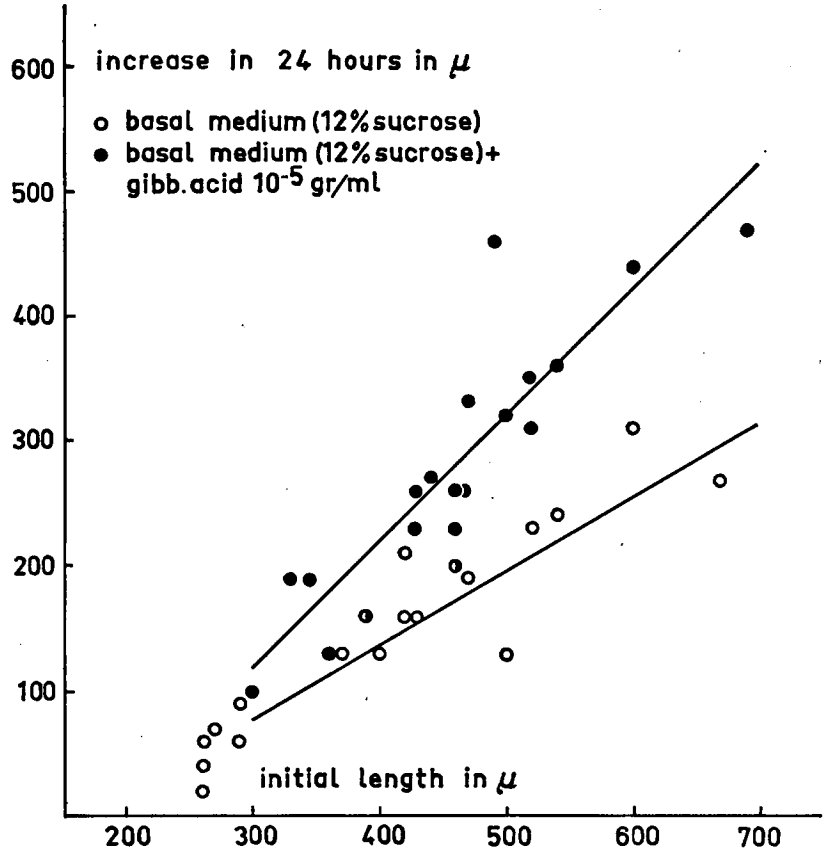

Fig. 2. Relation between initial length and the growth in 24 hours.

where y = length increment and x = initial length; b represents the slope of the regression line and is known as the regression coefficient; a is the ordinate of the point where the line crosses the y axis ($x = 0$). If the growth variances are equal for all initial lengths, the values a and b can be calculated by the aid of the equations:

$$b = \frac{n\sum xy - \sum x \sum y}{n\sum x^2 - (\sum x)^2} \tag{2}$$

$$a = \frac{\sum x^2 \sum y - \sum x \sum xy}{n\sum x^2 - (\sum x)^2} \tag{3}$$

By means of the values given in Table 3, the two constants a and b can be computed.

TABLE 3
Calculation of the values a and b

	Group A	Group B
n	19	20
$\sum x$	782	919
$\sum y$	286	552
$\sum x^2$	34840	43855
$\sum y^2$	5494	17318
$\sum xy$	13427	26994
a	—10.6	—18.4
b	0.624	1.002

The equation (1) may be written as

$$\text{Group A} \quad y = -10.6 + 0.624 \cdot x$$
$$\text{Group B} \quad y = -18.4 + 1.002 \cdot x$$

Of course, the empirical y-values deviate from the regression estimates.

These differences, $y - Y$, which are known as deviations from regression, are symbolized by $d_{y.x.}$. These values are given in Table 2. From the figures we may conclude that

1) there is no systematic course in the alternation of + and — values, and that there is therefore no reason to doubt of the linear relation between initial length and growth,
2) there is no systematic course in the absolute values of the deviations from regression. Therefore we assume that the growth variances are equal for all initial lengths.

The slope of the regression line, b, provides a measure of the increase of y for a specified increase of x. The value a is the ordinate of the point where the regression line crosses the Y axis, and has only a mathematical meaning ($x = 0$). On the other hand, the regression line crosses the X axis at a point where $y = 0$. If extrapolation outside the region of observation is allowed, we may conclude that there is a *critical initial length* (x_0) below which no growth occurs in the course of this particular experiment.

x_0 can be calculated by the aid of the equation

$$x_0 = -a/b \tag{4}$$

We find in this experiment the following values for x_0:

$$\text{Group A:} \quad x_0 = 17.0 = 170\ \mu$$
$$\text{Group B:} \quad x_0 = 18.4 = 184\ \mu$$

As will be seen in chapter 4, the difference between the two x_0 values may be neglected. The two regression lines are written now as follows:

$$y = b \cdot (x - x_0) \tag{5}$$

$$\text{Group A:} \quad y = 0.624 \cdot (x - 170)$$
$$\text{Group B:} \quad y = 1.002 \cdot (x - 184)$$

In group A as well as in group B growth (y) increases proportionately to the difference between the initial length (x) and its critical value (x_0).

The regression coefficient (b) is a measure for the growth rate. To check whether there is a significant difference between the two b values, the standard deviation of the regression coefficient (s_b) must be calculated.

The following equations were used:

$$q_{\text{tot.}} = \sum y^2 - \frac{(\sum y)^2}{n} \tag{6}$$

$$q_{\text{regression}} = b \cdot \frac{n \sum xy - \sum x \sum y}{n} \tag{7}$$

$$q_{\text{rest}} = q_{\text{tot.}} - q_{\text{regr.}}$$

$$s^2 = \frac{q_{\text{rest}}}{n - 2}$$

$$s_b^2 = \frac{n \cdot s^2}{n \sum x^2 - (\sum x)^2}$$

$$s_b = \sqrt{\frac{n \cdot s^2}{n \sum x^2 - (\sum x)^2}} \tag{8}$$

Application of (8) to the values listed in Table 3 results in:

Group A: $s_b = 0.059$
Group B: $s_b = 0.124$ (see Table 4).

TABLE 4
Calculation of the standard deviation of the regression coefficient.

	Group A	Group B
$q$total	1188.9474	2082.8000
$q$regression	1032.8822	1633.9446
$q$rest	156.0652	448.8554
s^2	9.1803	24.9364
s_b^2	0.003458	0.015327
s_b	0.059	0.124

It can be demonstrated by means of a t test that the regression coefficient of group B is significantly different ($P < 0.05$) from that of group A, which means that the growth rate in group B is higher than that in group A.

In experiments with globular and with heart-shaped embryos the linear relation between initial length and growth was less clear. Therefore we tested the significancy of this relation by calculating the t value by the aid of the equation $t = b/s_b$ with $n - 2$ degrees of freedom.

The probabilities belonging to the t values can be found in Fischer's t table.

In several experiments the differences between the x_0 values of the various series within one experiment were considerable. For that reason these x_0 values are mentioned in all tables.

For the reader's convenience we calculated the increase in length (y) at the mean initial length of the whole experiment ($\bar{x}$) by means of the regression equation. These calculations are justified if the mean initial lengths of the several series belonging to the same experiment are equal. As the differences remained within 10 % of the mean initial length, we believe that we did not introduce a serious error by calculating the increase in length in this way.

The results of the statistical analysis are given in Table 5.

TABLE 5

Explanation see text

Treatment	n	b	s_b	x_0 in μ	y in μ at $x = 436\ \mu$
control	19	0.624	0.059	170	166
GA 10^{-5} gr/ml	20	1.002	0.124	184	253

The number of the embryos in a culture cell which were used for statistical analysis, was often reduced by the following events, viz.

1) infection by micro-organisms,
2) abnormal growth values, which made it impossible to fit these measurements into the linear regression (these values were discarded; however, the number of these exceptions never exceeded 10 % of the total number), and
3) shifting of the embryo to the margin of the drop, where it cannot be measured (this inconvenience was reduced as much as possible by measuring the embryos already after 24 hours).

Summarizing the mathematical results we may conclude that

1) there is a linear relation between initial length and growth; this relation can be expressed by the equation (1): $y = a + b \cdot x$,
2) the regression coefficient and the critical initial length are important biological values,
3) a comparison of regression coefficients is a justified way for estimating differences in growth rate.

More information on the biological meaning of the linear relation between initial length and growth, and on the critical initial length will be provided later on (see chapter 8).

CHAPTER 4

THE EFFECT OF GIBBERELLIC ACID ON EMBRYO GROWTH IN VITRO

4.1. Survey of the literature

It has been shown in recent years that quite a number of processes in the plant are influenced by gibberellic acid. For a survey of the literature pertaining to the activities of this substance, the reader is referred to the reviews given by Stowe and Yamaki (1957) and by Brian (1959). It seemed worthwhile to investigate the effect of gibberellic acid on embryo growth, especially since gibberellins have been isolated and identified from immature seeds of *Phaseolus multiflorus* (McMillan and Suter, 1958). West and Phinney (1959) extracted a "bean factor 1" from immature seeds of *Phaseolus vulgaris*, which proved to be identical with gibberellin A 1. Further gibberellin A 1 was isolated by Kawarada and Sumiki (1959) from the elongated suckers of a bud variation of *Citrus unshiu*.

In each case the substance was identified by means of its infra-red spectrum and by its physiological activities.

As it is of special interest in connection with our own research, it is worthwhile to mention the discovery of gibberellin-like substances in coconut milk by Radley and Dear (1958).

Recently Corcoran and Phinney (1962) demonstrated in *Phaseolus*, *Lupinus* and *Echinocystis* a correlation between growth of the seed and of the embryo and the amounts of gibberellin-like substances that are present in the seed. These data suggest that these substances are involved in seed growth, but that there is no direct relation to fruit growth.

The observation of Nickel (1958) that gibberellin-like substances can be obtained from cotyledon tissue of *Phaseolus vulgaris* which had been cultured for several years on a standard medium, suggests that gibberellin-like substances are produced by the embryo itself.

Dure and Jensen (1957) tested the effect of GA on the growth of embryos of *Gossypium hirsutum*. They found that GA promoted both cell division and elongation of the axes, whereas cotyledon expansion, also caused by GA, apparently was the result of cell enlargement only.

In the experiments of Mauney (1961), who worked with excised cotton embryos, GA caused excessive elongation of the embryonic radicle and degeneration of the embryo into a callus.

As Dure and Jensen and later Mauney cultivated the immature cotton embryos in an agar medium containing sucrose at a concentration of only 2 %, it seems probable that they did not study normal embryo growth but what Dieterich (1924) called "künstliche Frühgeburt".

It seemed worthwhile to test the influence of GA on embryos in liquid media in which sucrose is present at a higher concentration.

According to Rijven (1952), a high sugar concentration would

prevent germination phenomena. A preliminary report on our own results was published in 1961 (VEEN, 1961). In the present study the different aspects of the effect exercised by GA on embryo growth in vitro were subjected to a more detailed analysis.

4.2 THE EFFECT OF GIBBERELLIC ACID ON GLOBULAR AND ON YOUNG HEART-SHAPED EMBRYOS

RIJVEN (1952) cultivated young heart-shaped embryos in 18 % sucrose because there were indications that a 12 % solution was hypotonic for these embryos. The growth of these excised embryos ($< 150\ \mu$) lagged behind as compared with that of embryos included in the ovule; this was undoubtedly due to some deficiency in the medium.

In our first experiments the effect of GA was tested on the growth of globular and of heart-shaped embryos, this substance being added in three concentrations. The results are given in Table 6.

TABLE 6

Effect of GA on growth of globular and of heart-shaped embryos. Embryos cultivated for 48 hours in basal medium with 18 % sucrose (control), to which GA was added in three concentrations. For the calculation we used all embryos, those that did not grow at all included. The regression coefficient denotes the linear regression between length increment and initial length.

Treatment	n	b	s_b	x_0 in μ	y in μ at $\bar{x} = 115\ \mu$
0	20	1.374	0.217	51	88
10^{-7} gr/ml	15	1.181	0.214	68	56
10^{-6} gr/ml	13	1.465	0.232	56	86
10^{-5} gr/ml	22	1.448	0.180	58	83

As the standard deviations are considerable, the differences in the regression coefficients may be neglected. From the results we may conclude that addition of GA to the medium did not exert any effect on the growth rate of these young embryos. Many of them grew neither in the control medium, nor in the presence of GA. There was no significant increase of the percentage of growing embryos in the presence of GA. The percentage of embryos which in one experiment grew more than 10 μ, is given in Table 7.

TABLE 7

Percentage of globular and of heart-shaped embryos which started growing.
initial size Group 1: from 49 μ to 99 μ
Group 2: from 100 μ to 149 μ
Group 3: from 150 μ to 199 μ

		gibberellic acid			
Group	control	10^{-5} gr/ml	10^{-6} gr/ml	10^{-7} gr/ml	total
1	16 % (1/6)	30 % (4/13)	25 % (1/4)	20 % (1/5)	25 % (7/28)
2	83 % (10/12)	83 % (5/6)	100 % (7/7)	25 % (1/4)	79 % (23/29)
3	100 % (4/4)	100 % (4/4)	100 % (2/2)	100 % (8/8)	100 % (18/18)

Although the number of embryos observed was small, we may conclude that GA does not increase the capacity to grow.

Only a few globular embryos (50–99 μ) survived the transfer from the ovule into the liquid medium. For heart-shaped embryos the chance of a resumption of growth is much better. In Fig. 3 we give a diagram which shows the relation between initial length and growth for those embryos with an initial length of 50 μ up to 150 μ that had grown 10 μ and more.

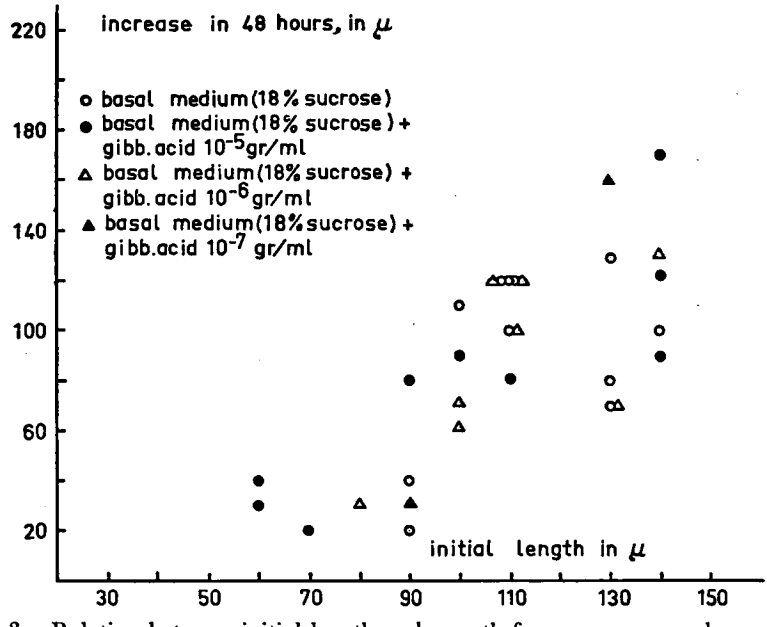

Fig. 3. Relation between initial length and growth for very young embryos that actually did grow, viz. in basal medium containing 18 % sucrose (control) as well as in this medium after GA had been added in three different concentrations.

From these experiments we conclude that the growth in vitro of globular and of young heart-shaped embryos is poor, and that addition of GA increased neither the percentage of growing embryos nor the growth rate of these embryos.

4.3. The effect of GA on embryo growth of "torpedoes"

Next the effect of GA on the growth of torpedo-shaped embryos was studied. From a series of experiments of which the results are given in Table 8, it appears that

1) the critical initial length varies, whether GA is present or absent, between 50 μ and 200 μ,
2) 10^{-5} gr/ml GA increases the growth rate of "torpedoes" considerably; even at the low concentration of 10^{-7} gr/ml GA appears to be active in these experiments. Figures 2,4 and 5 demonstrate the effect of GA.

TABLE 8

Effect of GA on the growth of "torpedoes". Embryos cultivated for 24 hours in basal medium containing 12 % sucrose (control) and in similar media to which GA had been added. The regression coefficient denotes the linear regression between length increment and initial length. The length increment (y) at the mean initial ($\bar{x}$) was calculated.

Exp.	Treatment	n	b	s_b	x_0 in μ	y in μ at $\bar{x} = 500\ \mu$
1	0	19	0.624	0.059	170	206
	10^{-5} gr/ml	20	1.002	0.124	184	317
2	0	20	0.707	0.111	127	264
	10^{-5} gr/ml	14	1.227	0.156	153	426
3	0	14	0.711	0.114	148	250
	10^{-5} gr/ml	14	1.695	0.140	171	557
	10^{-6} gr/ml	18	1.544	0.136	126	577
	10^{-7} gr/ml	11	0.821	0.134	190	254
4	0	21	0.438	0.160	—	318
	10^{-5} gr/ml	25	1.575	0.155	106	619
	10^{-6} gr/ml	23	1.182	0.199	13	576
	10^{-7} gr/ml	23	0.712	0.128	—	439

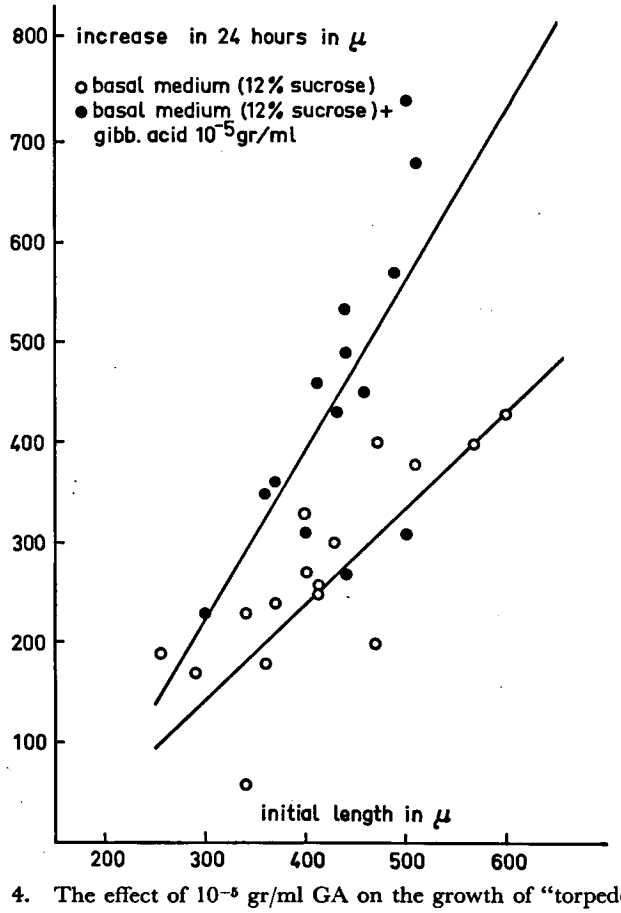

Fig. 4. The effect of 10^{-5} gr/ml GA on the growth of "torpedoes".

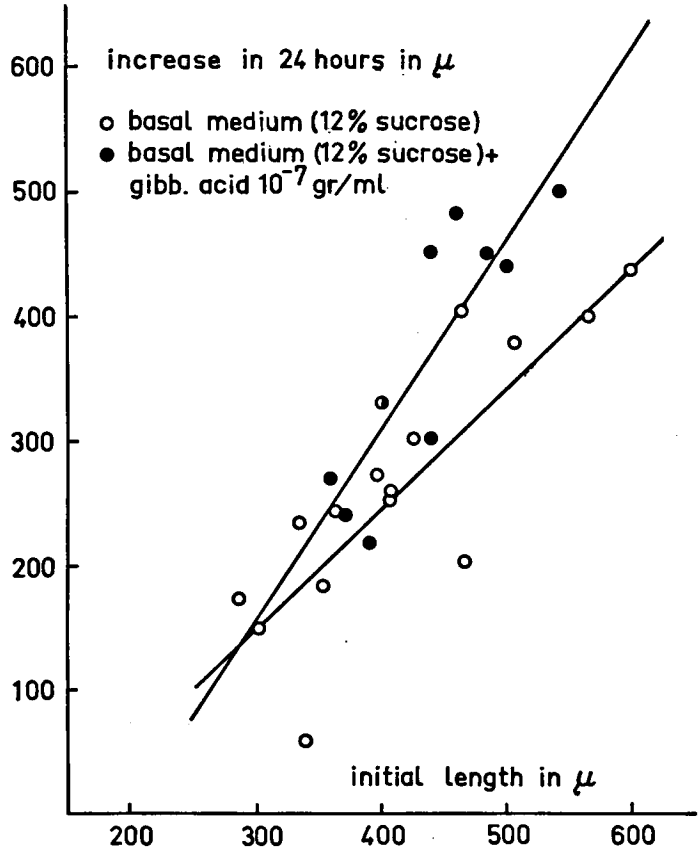

Fig. 5. The effect of 10^{-7} gr/ml GA on the growth of "torpedoes".

TABLE 9

Some characteristic features of the growth stimulated by GA.

	basal medium	basal medium with GA 10^{-5} gr/ml
initial length	700 μ	700 μ
length after 7 days	2200 μ	4200 μ
epinastic curves of cotyledons	—	+
length cotyledons/length axis	1100/1100	1600/2600
diameter basal part axis/length root	300/325	400/657
length cells of axis	20 μ	30 μ
fresh weight in mg of embryo	0.123 mg.	0.180 mg.

The characteristic features of the growth stimulation by GA, are reflected in the following observations made on 50 embryos in the course of a 7 days' cultivation period. See Table 9 and Fig. 6 and 7.

The increase of the root-meristem activity is remarkable as well as

the epinastic growth of the cotyledons. These two phenomena are typical manifestations of "germination". Germination is described by RIJVEN (1952) as "the moment that the embryonic tissue — in a state of cell division and plasmatic growth — becomes separated by an intercalated section of incipient cell-elongation". As this process of cell elongation is correlated with an uptake of water, a hypotonic medium is the first requirement for germination. In media with 12 % sucrose (without GA) cell elongation was observed neither by Rijven, nor by us.

In experiments with mature embryos of *Capsella bursa-pastoris* enclosed within the seed coat, it was found that GA may stimulate cell elongation. The seeds were put on moist filter paper in Petri dishes. GA was added in a concentration of 10^{-5} gr/ml. After 96 hours in the controls an elongation of the axes was observed. This elongation was strongly stimulated by GA (ratio length hypocotyl of control/length hypocotyl of embryos treated with GA = 100/177).

So if there is cell elongation, GA may increase it considerably.

Therefore it seemed to be of interest to test the effect of GA on embryo growth in more diluted sucrose solutions in which, according to RIJVEN (1952), germination phenomena, including cell elongation, are starting.

4.4. THE EFFECT OF THE SUCROSE CONCENTRATION ON EMBRYO GROWTH

The results of a typical experiment are given in Table 10.

TABLE 10

The effect of the sucrose concentration on the growth shown by "torpedoes" in 24 hours. The regression coefficient denotes the linear regression between length increment and initial length. The length increment (y) at the mean initial length ($\bar{x}$) was calculated.

Treatment	n	b	s_b	x_0 in μ	y in μ at $\bar{x} = 500\ \mu$
3 % sucrose	16	1.033	0.191	208	320
12 % ,,	23	0.603	0.032	124	227

The individual values of this experiment are plotted in Fig. 8.

There is a strong indication ($P < 0.05$) that already after 24 hours the growth in 3 % sucrose exceeds that in 12 % sucrose. After 2 and 4 days this effect is much more pronounced (see 4.5).

We further notice a development of the root meristem as well as epinastic curvatures of the cotyledons. Contrary to the expectation, in the 3 % sucrose solution cell elongation was observed only in 6 out of 150 embryos. The length attained by the hypocotyl cells of these embryos was 70 μ, which is still far below that of full-grown hypocotyl cells during normal germination (120 μ).

GA appeared to increase the number of embryos showing such elongated hypocotyl cells. However, this effect was observable only in experiments of a few days' duration and after the embryo had attained

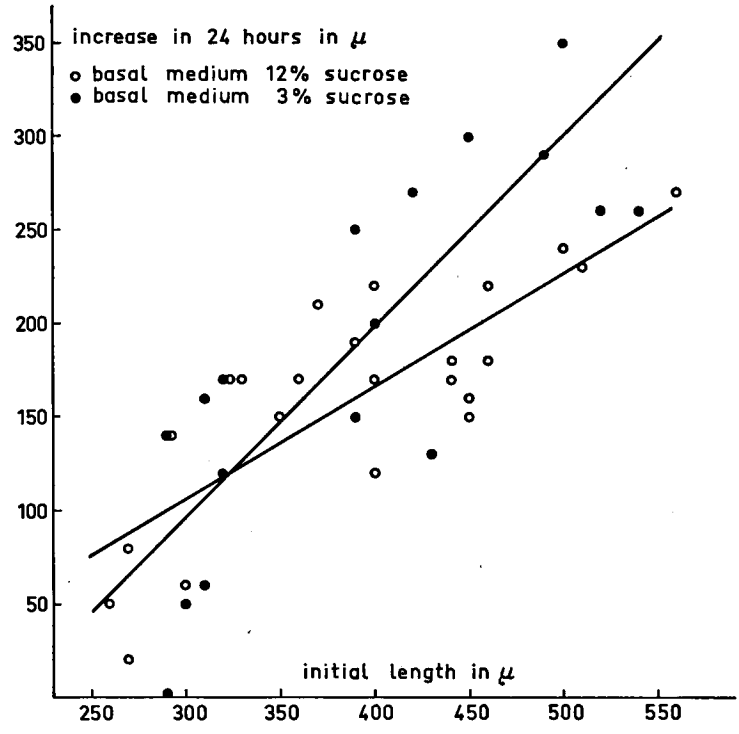

Fig. 8. Effect of the sucrose concentration on embryo growth in "torpedoes".

a length of ca. 6000 μ. This effect of GA was accompanied by the development of root hairs.

The increase of the cell length in 12 % and 3 % sucrose solution, however, is not due to cell elongation in the strict sense of the word, because the cells increase in all directions. We will call this way of growth "cell enlargement", a phenomenon which according to LINSKENS (1961) is common in embryo cultures: "Die Entwicklungsprozesse in vitro stimmen nicht in alle Punkten mit denjenigen in vivo überein, zum Beispiel werden die Zellen des Embryos in künstlichem Medium ansehnlich grösser".

This statement is confirmed by the following figures. The cells of the meristem of the hypocotyl in an embryo included in the ovule never show a diameter of more then 12 μ. In the basal medium with 12 % sucrose the average cell diameter after a weeks cultivation is 20 μ, and in the presence of GA an average diameter of 30 μ is attained at the end of this period. Moreover, the growth of the cells in vitro is accompanied by a change of their shape. The cells become more or less isodiametric, and this transformation results in the appearance of intercellular spaces, which are absent under natural circumstances, i.e. in an embryo which is included in the ovule.

A further analysis of the effect of GA on the growth of "torpedoes"

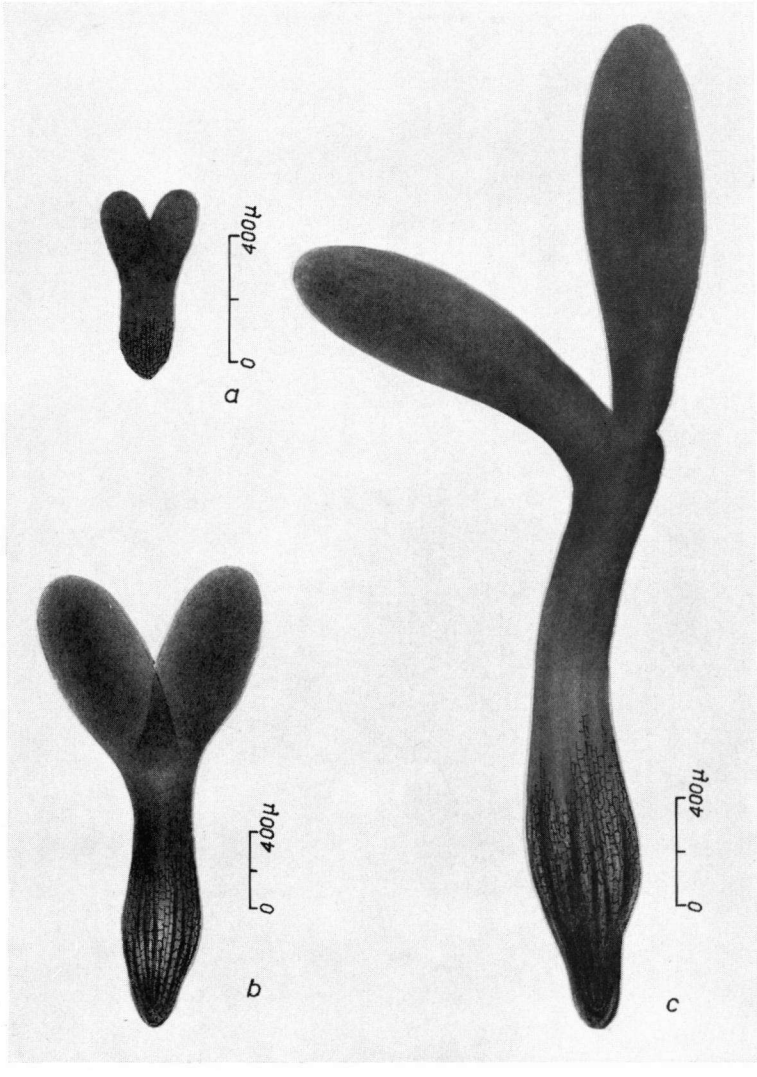

Fig. 6. Effect of GA on embryo growth of Capsella bursa-pastoris. a: initial length; b: embryo after 7 days' cultivation in basal medium containing 12 % sucrose; c: embryo after 7 days' cultivation in basal medium containing 12 % sucrose with GA 10^{-5} gr/ml.

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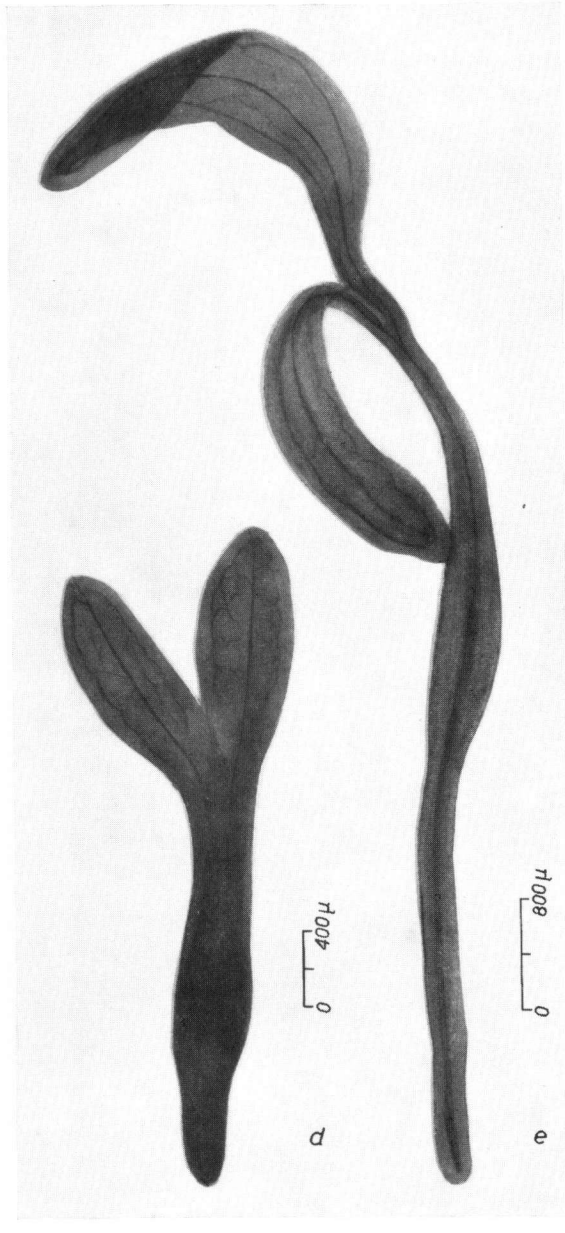

Fig. 7. Effect of GA on embryo growth of Capsella bursa-pastoris. d: embryo after three weeks' cultivation in basal medium containing 12 % sucrose; e: embryo after three weeks' cultivation in basal medium containing 12 % sucrose with GA 10^{-5} gr/ml.

is described in the following section. In order to answer the question whether the GA-induced growth is due to increased water uptake only, determinations were made of the insoluble nitrogen content. An actual increase of this N-fraction would point to an increased synthesis of proteins and, possibly, to an increased rate of cell division.

4.5. The effect of GA on protein synthesis

Method

For the determination of the *insoluble nitrogen content* (INC), embryos that had developed in media with or without GA, were fixed in a mixture of formaldehyde (33 %), acetic acid and aethanol (70 %) (5:5:90). In this fixation liquid the embryos were kept for at least 48 hours. We applied the method of Nesslerisation after Levy (1936) with a few modifications after Rijven (1958). The Nessler reagent was prepared according to the directions of Folin and Wu (cf. Glick, 1949). For the estimation of the INC per embryo a standard was needed. To this purpose a series of NH_4Cl solutions were prepared containing from 0.5 μg up to 10 μg N per sample. Between these two limits a linear ratio was found between the extinction and the concentration. As at higher N-concentrations colloidal turbidity disturbs the determination, the number of embryos per determination was varied (from 1 up to 7). The length of these embryos was as uniform as possible.

First the embryos were washed twice in 96 % aethanol before the determination of the nitrogen. Then they were transferred into digestion vessels by means of braking-pipettes. These vessels were made from insulin ampullae by pulling a constriction approximately in the middle of them (after Brüel, et al., 1946), their size being 1.60 × 5 cm. The alcohol was evaporated, after which Levy's digestion mixture was added.

The vessels were placed in 14 mm deep cylindrical holes bored in a brass block, in which they fitted tightly. The block was heated on an electric heating plate, which was provided with a three-steps heating switch. The vessels were heated for $1^1/_2$ hours at ca. 150° C in order to remove the water. Then they were heated at 295° C for $^1/_2$ hour, during which period the vessels were closed with glass beads after Brüel.

After about three hours the vessels were removed from the block, and cooled, and then 1.4 ml of distilled water was added by means of a micro-burette. Next, under strong aeration, 0.6 ml Nessler reagent was added, and 20 minutes later the extinction of the colour which had developed, was measured by means of a spectrophotometer (Zeiss PMQ 11). The transmission of the reaction medium was compared with that of the standard solutions at the wave length of 420 mμ.

Results

The results of 4 experiments in which not only the effect of GA was studied, but in which also the sucrose concentration was varied, are given in Table 11. The measurements were made with a two days' interval.

TABLE 11

The effect of GA on embryo length and on INC per embryo in basal media with 3 % and with 12 % sucrose.

	3 % sucrose		12 % sucrose	
	without GA	with GA	without GA	with GA
mean initial length . . .	590 μ		590 μ	
mean initial INC	0.14 μg		0.14 μg	
after two days				
length	1567 μ	2867 μ	1091 μ	2065 μ
N-content	0.72 μg	1.25 μg	0.43 μg	0.73 μg
after four days				
length	2172 μ	5910 μ	1507 μ	3154 μ
N-content	1.06 μg	2.71 μg	0.74 μg.	1.33 μg
after six days				
length	—	—	2062 μ	4205 μ
N-content	—	—	1.11 μg	1.66 μg

The figures of Table 11 are graphically represented in Figures 9 and 10.

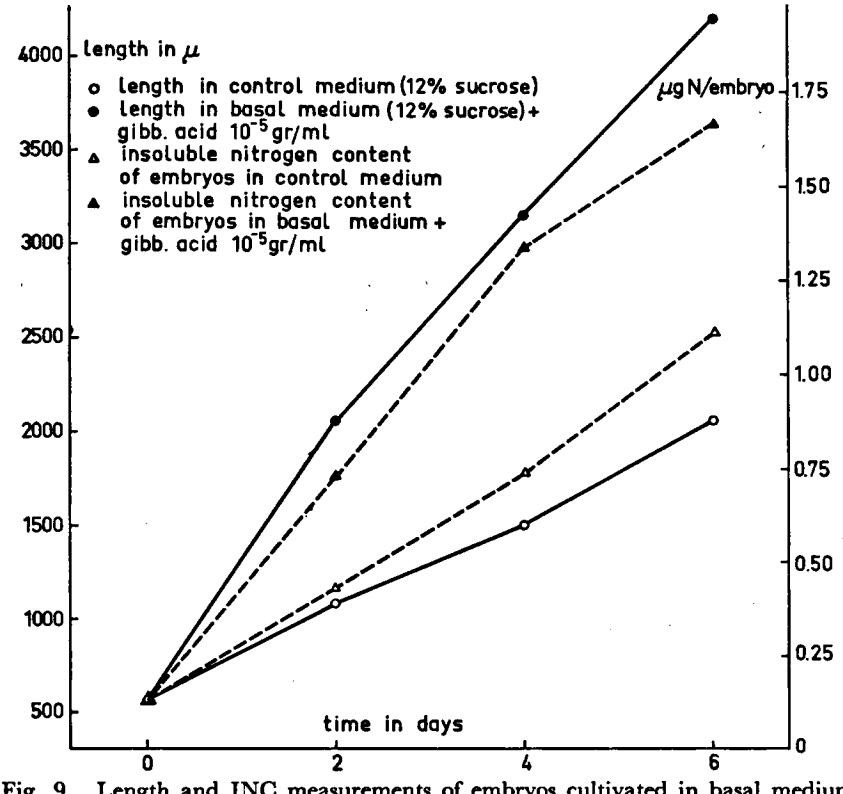

Fig. 9. Length and INC measurements of embryos cultivated in basal medium (12 % sucrose) with or without GA

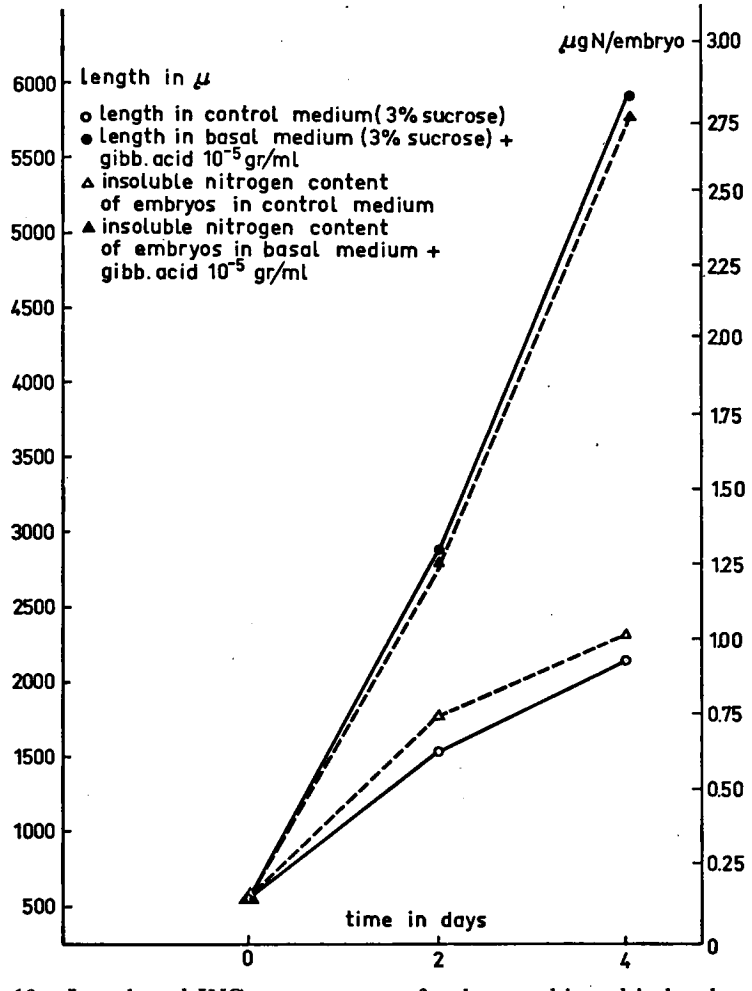

Fig. 10. Length and INC measurements of embryos cultivated in basal medium (3 % sucrose) with or without GA.

From the results we may conclude that in growing embryos the INC increases. This increase is influenced by GA as well as by the concentration of sucrose in the solution. It is very difficult to estimate the effect of GA on the INC, because we do not possess exact determinations of the volume, or of the fresh or dry weight of the embryos, i.e. a standard to which the INC could be related. The embryos are too small to determine their dry weight, therefore RIETSEMA, et al. (1955), working with *Datura* embryos, made volume determinations in vivo. In our case, with embryos cultivated in vitro, there is unfortunately so much variability in the shape of the embryos that it is impossible to calculate their volume in an exact way. Considering the

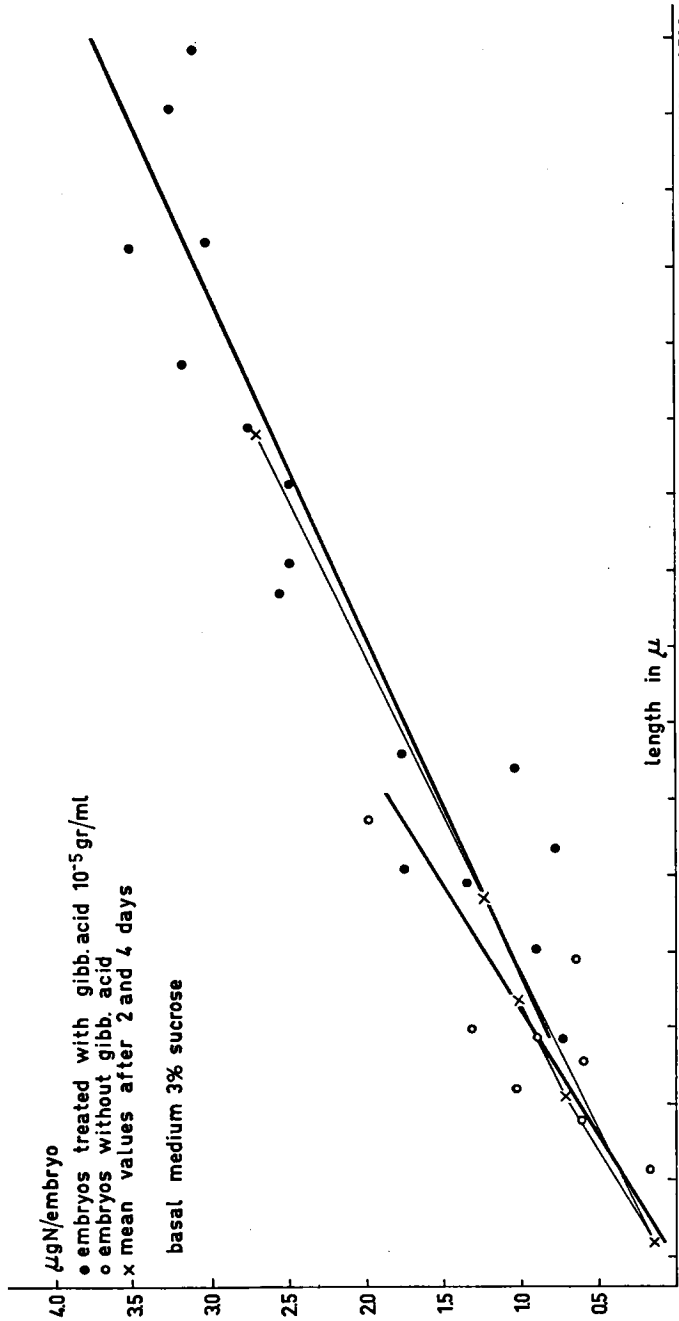

Fig. 11. Relation between embryo length and INC of the embryo in the presence or absence of 10^{-5} gr/ml GA. Embryos cultivated in 3 % sucrose (basal medium).

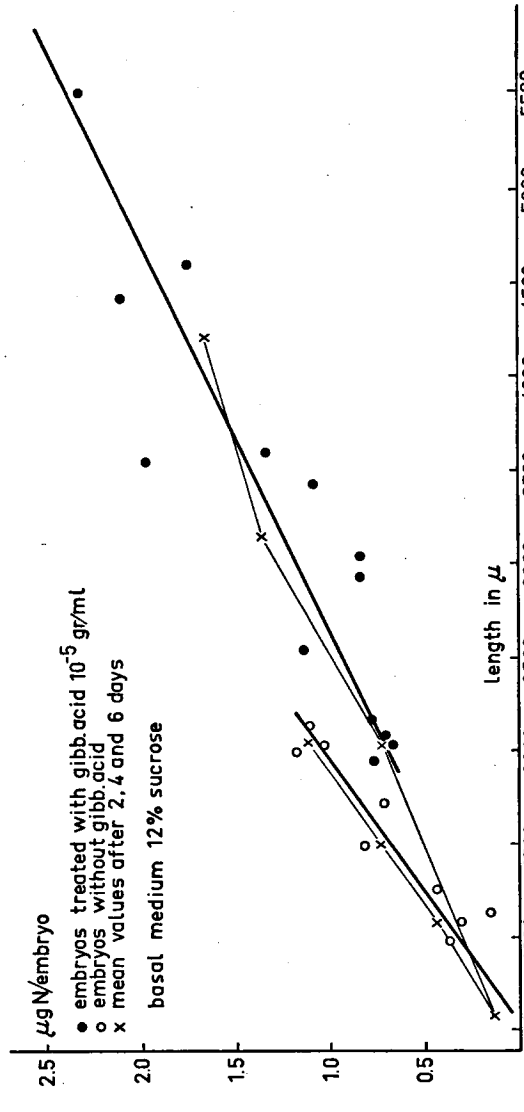

Fig. 12. Relation between embryo length and INC of the embryo in the presence or absence of 10^{-5} gr/ml GA. Embryos cultivated in 12 % sucrose (basal medium).

observations described in chapter 4.3 on the characteristic features of embryo development in this stage, we believe that it is allowed to relate the INC to the length of the embryos. Therefore the individual measurements of N-content and the corresponding lengths are reproduced in Figs. 11 and 12.

Fig. 12 shows that the absolute INC reached by GA-treated embryos in 2, 4 and 6 days is higher than that reached by the controls. However, the amount of insoluble nitrogen per unit of length increase is less (see Table 12). This is true for embryos cultivated in the basal medium with 12 % sucrose. The effect of GA on the INC in a basal medium with only 3 % sucrose is less clear (Fig. 11).

A possible difference between the INC in 12 % sucrose with or without GA is endorsed by the values of the function $y = a + b \cdot x$, which were computed from the figures given in Fig. 12. The same computation was carried out with the figures given in Fig. 11 (see Table 12).

TABLE 12

Correlation between embryo length and INC of embryos cultivated in media with 3 % and with 12 % sucrose in the presence or absence of GA.

	3 % sucrose		12 % sucrose	
	without GA	with GA	without GA	with GA
n	9	17	11	14
b	0.620	0.441	0.716	0.482
s_b	0.140	0.040	0.088	0.055
P_{CORR}	< 0.01	< 0.01	< 0.01	< 0.01
$P_{GA\text{-effect}}$	$P > 0.2$		$P < 0.05$	
increase in insoluble N-content at a length increase of 200 μ	0.124 μg	0.088 μg	0.143 μg	0.096 μg

So our conclusion with regard to the effect of GA on the nitrogen content of embryos must take into account the sugar concentration of the medium. In the presence of 12 % sucrose there is a high increase of protein synthesis, which is stimulated disproportionally by GA, whereas in a medium containing only 3 % sucrose, this stimulation of the protein synthesis by GA is proportional to the increase in length of the embryo.

Our conclusion that the effect of a lowering of the sucrose concentration to 3 %, has much in common with the effect exercised by GA in a 12 % sucrose solution, is supported by observations of ALLSOP (1962). Working with sporelings of *Marsilea drummondii*, he observed that addition of GA to a medium with 4 % glucose resulted in a transformation of "land" to "water" forms. The same effect was produced after the transfer from a 4 % to a 2 % glucose solution.

This is therefore another instance of a development in which the effect of GA is comparable with that of a lowering of the sugar

concentration in the medium. The question whether the decrease of the osmotic value of the surrounding solution is to be regarded as the decisive factor, or whether a possible change of the internal sugar concentration caused by GA, is of paramount importance and will be discussed in section 4.9.

4.6. The relation embryo length/protein content in embryos developing in the ovule

Since the cells of embryos developing in the ovule are considerably smaller than those of embryos cultivated in vitro, we may expect also a different relation between their length and their protein content. This point was investigated by the aid of embryos that were fixed immediately after they had been excised from the ovule. The results are shown in Fig. 13.

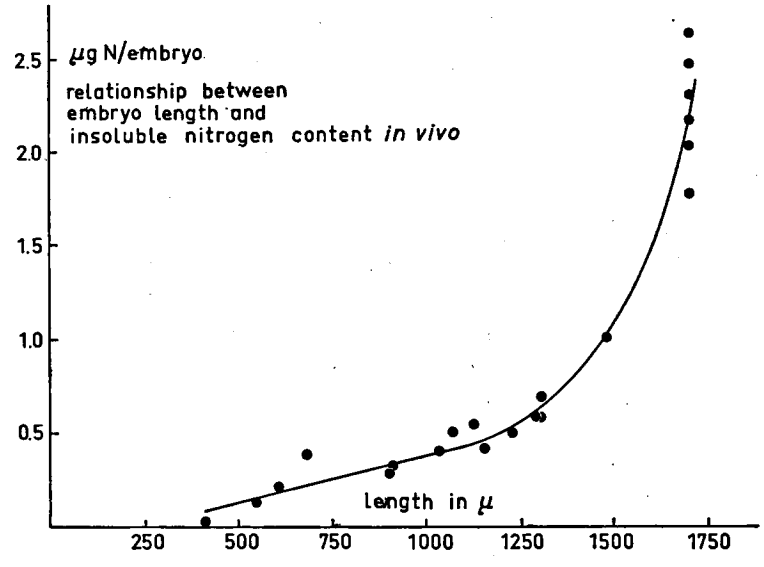

Fig. 13. Relation between embryo length and INC of embryos developing in the ovule.

If we compare these results, which were obtained with a great variety of embryo lengths, with those obtained by Rijven (1958), we notice in both cases a linear relation between length and INC for "torpedoes". On the other hand above a length of 1300 μ the increase of the INC is much higher than that in length.

A mature embryo contains ca. 2.3 μg N.

These results correspond with those obtained by Rietsema and Blondel (1959), who worked with *Datura stramonium* embryos. They found that the period in which the synthesis of reserve protein reaches its highest rate, starts when the embryo stops growing in size.

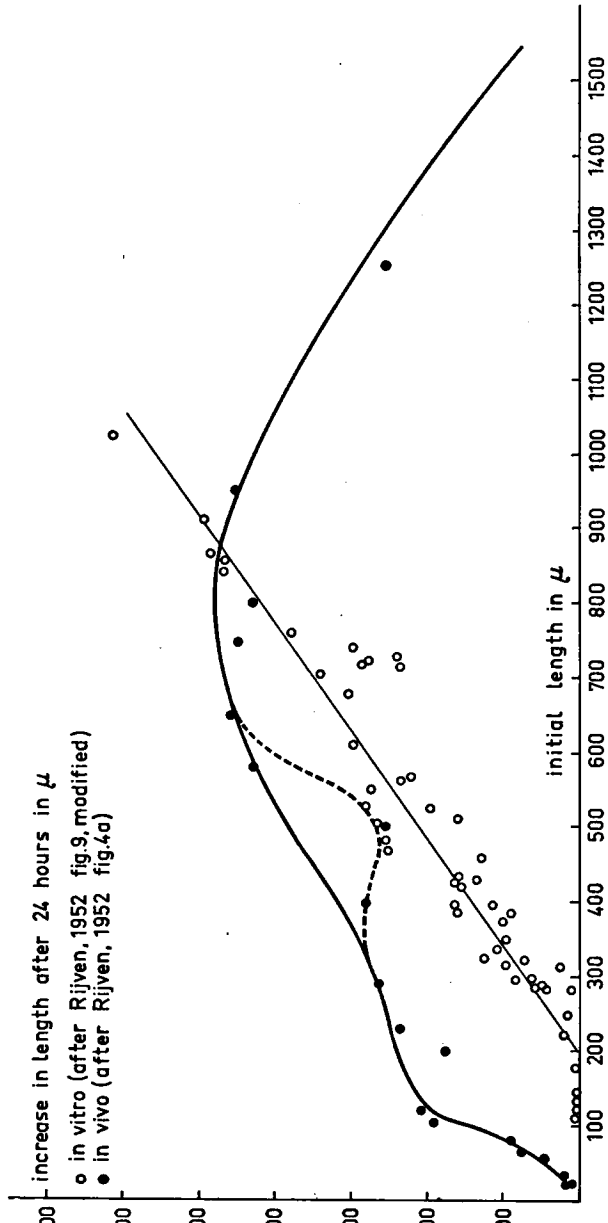

Fig. 14. Growth of embryos at different lengths in the ovule and in vitro (after RIJVEN, 1952).

RIJVEN (1952) observed with *Capsella* embryos which were left in the ovule, a decrease of the growth rate. For the reader's convenience we have included Fig. 14, which illustrates the characteristics of embryo growth rates in the ovule and in vitro (after RIJVEN, 1952).

As to our own experiment mentioned in this section, we suppose that the sequence is the same as in the *Datura* embryo: as soon as the growth rate of the embryo in the ovule begins to decrease, the rate of protein synthesis increases.

4.7. VARIATION OF THE DURATION OF THE GA TREATMENT

We have tried to obtain some information with regard to the time required for a temporary GA treatment which causes about the same effect on the growth of embryos in vitro as is found in embryos that are continuously submersed in a medium with GA. It is not improbable that this time is influenced by the GA concentration. We applied GA at a concentration of 10^{-5} gr/ml. We proceeded as follows: torpedo-shaped embryos were submersed for a definite period in the basal medium containing GA. Then they were carefully washed in the basal medium without GA, and cultivated in it during a period of 24 hours, at the end of which the length was determined.

TABLE 13

Effect of the duration of GA-treatment on the growth of "torpedoes". The regression coefficient denotes the linear regression between length increment and initial length. The length increment (y) at the mean initial length ($\bar{x}$) was calculated.

treatment time in min.	n	b	y in μ at $\bar{x} = 650\ \mu$
0	24	0.448	179
15	25	0.910	455
60	17	0.904	470
continuous submersion	24	1.178	589

The results show that more than 75 % of the maximum effect was already attained after a 15 minutes period of submersion.

We have not determined the minimum submersion time required for a measurable increase of the growth rate. GALSTON and WARBURG (1959) found that an exposure to GA as short as 3 seconds was sufficient to produce a marked effect on the subsequent growth of epicotyl sections of the pea. In pea sections the uptake will be facilitated by the wound surface and by the presence of a vascular system, factors that are absent in the embryo. At any rate, we are convinced that an exposure to GA far shorter than 15 minutes would be sufficient to obtain a clear effect on the embryo growth in vitro.

4.8. THE EFFECT OF GA ON IMMATURE EMBRYOS ENCLOSED IN THE OVULE

In recent years many investigations were carried out in India on the influence of growth-regulators on the growth of isolated ovules,

with special reference to the development of the embryo. RANGA SWAMY (1961) cultivated ovules of *Citrus microcarpa* in vitro. After a few weeks outgrowths developed abundantly over the entire surface of the nucellus. These structures have been termed "pseudobulbils". Addition of GA did not favour proliferation, but it promoted root formation on the pseudobulbils. Growth as well as differentiation of the embryo in excised ovules of *Papaver somniferum* (MAHESHWARI and LAL, 1961) were largely inhibited by the application of GA. For example after 20 days, embryos of 60 μ length, treated with GA, were 280 μ long, whereas the controls had reached more than twice that length. This inhibition later on was followed by an abnormal increase in length of the GA-treated embryos, so that a final length of 1100 μ was observed (whereas the untreated ones stopped growth at 650 μ).

SACHAR and KANTA (1958) tested the effect of GA on the growth of isolated ovaries of *Tropaeolum majus*, however without any positive result.

The results of these investigations induced us to compare the effect exercised by GA on embryos that are still enclosed in the ovule to that exercised on isolated embryos.

The torpedo-shaped embryos used for this purpose, were isolated out of ovules belonging to one valve of a silicle, whereas the ovules were taken from the other valve of this silicle; these ovules contained embryos of exactly the same length. Both, ovules and isolated embryos, were cultivated in media with different sucrose concentrations, viz. 1, 6 and 12 %, and with or without 10^{-5} gr/ml GA.

In Table 14 the increase in length of isolated embryos and of embryos enclosed in the ovule is expressed as a percentage of the initial length.

TABLE 14

Effect of GA on the growth of isolated embryos and of embryos enclosed in the ovule. Growth expressed as a percentage of the initial langth. Initial length = 600 μ. Measurements were made after 48 hours.

Treatment	growth in the ovule		growth of excised embryos	
	n	%-growth	n	%-growth
12 % sucrose				
without GA	9	0	11	81
with GA	7	43	12	181
6 % sucrose				
without GA	10	32	8	129
with GA	9	44	13	278
1 % sucrose				
without GA	10	10	14	76
with GA	8	0	12	209

It appears from Table 14 that the growth of embryos in ovules is in all media strongly inhibited. Addition of GA does not remove the disastrous effect which cultivating ovules in vitro exercises on the embryo growth.

We do not know whether this inhibition is due to a natural inhibitor

present in the endosperm, or to substances formed in the ovules as a consequence of the unnatural medium. In the latter case, opening of the ovule might improve the embryo growth. We therefore opened the ovule at the top without injuring the embryo itself. We observed not only that the growth remained slow but also that the growth was qualitatively different. Whereas the hypocotyl did not grow at all, the cotyledons increased in length, which gave rise to the abnormal shape shown in Fig. 15.

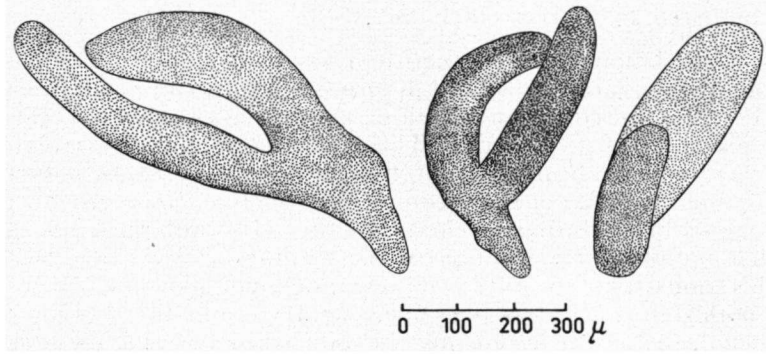

Fig. 15. Shape of embryos in opened ovules cultivated in basal medium with 12 % sucrose.

This result will be discussed in connection with the effect of kinetin on embryo growth (see chapter 6).

4.9. Discussion

We know from investigations by Rijven (1952), Rietsema et al. (1953), Norstog (1961) and DeMaggio and Wetmore (1961) that it is extremely difficult to grow very young embryos in vitro. As to the composition of the medium, larger embryos are less exigent. In all cases sugar must be added, in the first place to serve as a substrate for the carbohydrate metabolism, secondly to maintain the osmotic value of the medium. Rijven (1952) stated that the osmotic value of the ovule sap is isotonic with a solution of 12 % sucrose. In preliminary experiments we compared the effect of a 12 % sucrose solution to that of a 2 % sucrose solution supplied with 10 % lactose. In the latter case the osmotic value of the solution is equal to that of 12 % sucrose and, as lactose does not act as a carbohydrate source, the substrate for the carbohydrate metabolism was less well provided for. The results showed that there was neither a difference in growth rate nor in morphological respect. From this experiment we conclude that the effect observed in a 3 % solution is due only to a diminished osmotic value of the solution.

In highly concentrated sucrose solutions (in accordance with Rijven we used 18 % sucrose to cultivate very young embryos) the growth of globular (so called pro-embryos) and of heart-shaped embryos

remains limited. This situation cannot be improved by the use of GA. The absence of a response to GA observed in these particular experiments in this very early stage of development, however, does not imply that the action of GA is limited to germination phenomena, since other factors may have inhibited the response, e.g.

1) shortage of other growth factors,
2) an unfavourable osmotic value, and
3) the more pronounced heterotrophy of very young embryos, as compared to that of older ones.

The stimulation of the root meristem activity as well as the induction of epinastic curvatures observed in torpedo-shaped embryos cultivated in a 12 % sucrose containing medium, proves that GA may be regarded as a promotor of germination. These manifestations of germination, however, may also be produced in the absence of GA, viz. by reduction of the osmotic value of the medium (3 % sucrose). The growth rate of these embryos during the first day after GA addition is the same as that found after a dilution of the medium.

This similarity of the effects, however, does not allow the conclusion that both factors act upon the same system. Germination is a complex of phenomena of which some may be induced by lowering the osmotic value of the environment, while others may be induced even in highly concentrated sucrose solutions. In the latter GA appeared to be active.

From a review by KOLLER et al. (1962) it appears that several authors assume an action of GA on the germination process.

The promotion of cell elongation by GA noticed by DURE and JENSEN (1957), working with embryos of *Gossypium hirsutum*, was reproduced by us in experiments with mature embryos which remained enclosed in the seed. Cell elongation is independent of the presence of GA, but it is promoted by this growth-regulator.

It is not unlikely that substances like IAA or IAN (indole aceto nitrile), which were shown to be present in adult plants of *Capsella bursa-pastoris* (KIERMAYER, 1957) are involved in this process of cell elongation.

The promoting effect of GA on the INC of immature embryos will consist for the greater part of an enhanced synthesis of proteins and not of nucleic acids. This view is based on results obtained by HEYES (1960), who analysed pea roots. He found that the growing zone of the pea root contains 5 to 6 times as much protein as nucleic acids.

The similarity of the effect on the protein synthesis exercised by GA and that exercised by a decrease of the sucrose concentration is compatible with the hypothesis that GA is a natural growth hormone in plants.

CHAPTER 5

THE EFFECT OF INDOLE-3-ACETIC ACID ON EMBRYO GROWTH IN VITRO

5.1. Survey of the literature

The question discussed in this section is whether we must consider IAA to be a natural growth-regulator in embryogenesis. In the literature we find different answers to this question. Van Overbeek (1942), working with *Datura* embryos, believed that auxin keeps the embryo in the embryonic stage. Working with the same embryos, Sanders (1950) could not detect any stimulating influence at all of IAA on the growth, while Rietsema et al. (1953) observed a strongly inhibiting action on these embryos. As *Datura* ovules were found to contain auxin, the latter authors conclude that auxin must have a function in the regulation of embryo growth. Some factor in the endosperm would keep the balance between inhibiting and promoting regulators.

With embryos of other plants similar observations were made. Hatcher (1945) points to the fact that an increase of the auxin content of the embryo of *Secale* is accompanied by a decrease of its growth rate. Though the results with ovules of *Papaver somniferum* were variable, Maheshwari and Lal (1961) claim that IAA has a tendency to inhibit the growth of the embryo.

Dure and Jensen (1957), working with *Gossypium*, observed an inhibition of cell elongation and cell division in the axis of the embryo after addition of IAA to the culture medium.

The above-mentioned studies were all made with young, immature embryos. The effect of IAA on the growth of mature embryos of *Pisum* was investigated by Kruyt (1954), who found that low concentrations of IAA (10^{-8} Mol.) stimulate the growth of both sprout and root, whereas IAA inhibits their growth at a concentration of 10^{-5} Mol.

When we finally come to our own object, the *Capsella* embryo, the only available data on the effect of IAA on embryo growth are those of Rijven (1952). He observed that the growth of immature embryos is not notably influenced by IAA. At a concentration of 10^{-10} and 10^{-9} gr/ml there is a small increase of growth, at higher concentrations (10^{-5} and 10^{-6} gr/ml) growth is slightly inhibited.

In the next section our own data on the effect of IAA on the embryo growth will be reported. The reader who is interested in the effect of all kinds of growth substances on embryo growth may be referred to the review given by Rappaport (1954).

5.2. The effect of IAA on globular, heart-shaped and torpedo-shaped embryos

Our observations match well those made by Rijven (1952). We applied IAA at two concentrations (10^{-9} and 10^{-5} gr/ml) to globular

and to heart-shaped embryos in basal medium with 18 % sucrose, and to "torpedoes" in the basal medium with 12 % sucrose. As has been mentioned in a previous chapter (chapter 4.2), the cultivation of young embryos in vitro is a hazardous procedure. Many of them do not grow at all; there is a strong indication that they do not survive the treatment. The chance of survival is not considerably influenced by the presence of IAA in the medium, as we may hesitatingly conclude (because of the small number of embryos) from Table 15.

TABLE 15

Chance of survival of globular and young heart-shaped embryos in basal medium (with 18 % sucrose) with and without IAA. Measurements were made after 72 hours cultivation.

Initial size: Group 1 49μ 99 μ
Group 2 100 μ–149 μ
Group 3 150 μ–199 μ

Group	control	IAA		total
		10^{-5} gr/ml	10^{-9} gr/ml	
1	21 % (3/14)	0 % (0/2)	67 % (2/3)	26 % (5/19)
2	54 % (7/13)	36 % (4/11)	67 % (6/9)	52 % (17/33)
3	67 % (4/6)	75 % (6/8)	67 % (2/3)	71 % (12/17)

The values obtained with surviving globular and heart-shaped embryos are plotted in Fig. 16.

The growth of the young embryos as well as that of the "torpedoes" is always inhibited by IAA at the highest applied concentration of 10^{-5} gr/ml. Continuation of the cultivation of "torpedoes" for 7 days in a basal medium with 12 % sucrose with 10^{-5} gr/ml IAA did not

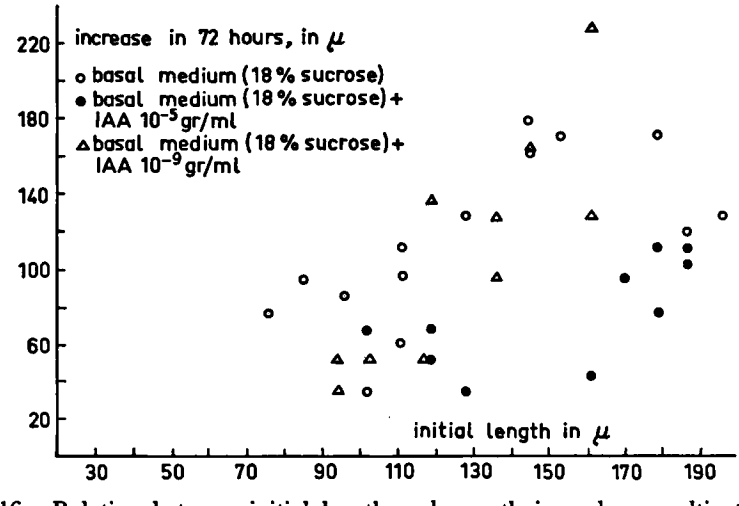

Fig. 16. Relation between initial length and growth in embryos cultivated in basal medium with 18 % sucrose (control) and in two similar media to which IAA had been added.

cause morphological changes as compared with the non-IAA-treated embryos. Their final length (2000 μ) was only 10 % less than that of the controls. This is completely in accordance with the findings of Rijven.

The slight stimulation by the low concentration of 10^{-9} gr/ml IAA on the growth of "torpedoes" could be confirmed too. The values are given in Table 16.

TABLE 16

Effect of IAA on the embryo growth of globular and of young heart-shaped embryos cultivated for 72 hours in basal medium (with 18 % sucrose) and of "torpedoes" cultivated for 24 hours in basal medium (with 12 % sucrose). For our calculations we used all embryos, those that did not grow at all included. The regression coefficient denotes the linear regression between length increment and initial length. The length increment (y) at the mean initial length ($\bar{x}$) was calculated.

Exp.	Treatment	n	b	s_b	x_0 in μ	y in μ at $\bar{x}$ =
			"torpedoes" $\bar{x}$ = 584 μ			
1	0	24	0.532	0.051	80	268
	10^{-9} gr/ml	18	0.638	0.074	92	313
2	0	15	0.719	0.070	179	291
	10^{-5} gr/ml	16	0.616	0.084	228	219
			globular and heart-shaped embryos $\bar{x}$ = 121 μ			
3	0	11	1.098	0.392	48	80
	10^{-9} gr/ml	12	1.982	0.394	76	89
4	0	17	1.418	0.232	68	75
	10^{-5} gr/ml	18	0.850	0.160	77	37

From the results of Rijven and those obtained in our own experiments the answer to the question put at the beginning of this chapter, must be in a negative sense; although IAA may be involved in the growth and development of the *Capsella* plant, it is not obvious that it acts as a natural growth-regulator during embryogenesis.

The combined action of IAA and GA on embryo growth in vitro will be described and discussed in chapter 7.

CHAPTER 6

THE EFFECT OF KINETIN ON EMBRYO GROWTH IN VITRO

6.1. Introduction

Kinetin was first prepared from commercial samples of desoxyribose-nucleic acid by Skoog and collaborators. It is an adenine derivative, viz. 6 furfuryl-amino purine. Though kinetin is highly active in inducing cell-division in excised pith tissue of tobacco, its effect is only exerted in the presence of IAA (Das et al., 1956; Skoog

and MILLER, 1957). Its natural occurrence in plants has not yet been established. GOLDACRE and BOTTOMLEY (1959) obtained an extract from young apple fruits with a kinetin-like activity; MILLER (1962) isolated and partially purified a compound from young maize kernels, which resembled kinetin in several chemical and physiological properties, but which obviously was not identical with kinetin itself. FOX (1962), moreover, isolated a kinetin-like substance from tobacco tissue.

A survey of all effects caused by kinetin has been written by MILLER (1961). Confining ourselves to the action of kinetin on embryo growth we may mention the following observations. MAHESHWARI and LAL (1961) noticed an acceleration of growth and of differentiation of the pro-embryos of *Papaver somniferum* after treatment with kinetin. Ten days after inoculation the kinetin-treated embryos were as long as 450 μ, a length not even attained by embryos enclosed in the ovule. In addition, the cotyledons were already well developed and the stem tip was observable. This initial start, however, was followed by a comparatively poor growth, so that the final length of the mature embryo (540 μ) was less than that of embryos which developed under natural conditions (650 μ).

SACHAR and KAPOOR (1959) tried to stimulate the growth in vitro of *Zephyranthes* ovules by means of kinetin. These attempts were not successful.

A modified pattern of growth was observed by RANGA SWAMY (1961) cultivating pseudobulbils of *Citrus microcarpa* in media containing kinetin.

The next sections will deal with the effect of kinetin on growth and development of globular, heart-shaped and torpedo-shaped embryos cultivated in vitro. A preliminary report on the first results has been published in 1962 (VEEN, 1962). In Chapter 7 the results of the combined action of kinetin and IAA will be reported.

6.2. THE EFFECT OF KINETIN ON THE GROWTH OF GLOBULAR AND OF HEART-SHAPED EMBRYOS

We tested several concentrations of kinetin on the growth of very young embryos cultivated in basal medium with 18 % sucrose. First we determined the percentage of growing embryos in three experiments in which kinetin at a concentration of 10^{-8} gr/ml was tested (Table 17).

The data of Table 17 show a considerable increase of the survival chance for embryos cultivated in basal medium with kinetin. It may be noticed that the survival chance of the control plants was small if compared with the observations made in earlier experiments (see Table 7 and 15). This difference is certainly due to a seasonal fluctuation which is known to occur in more processes in embryogenesis.

In spite of the favourable effect exercised by kinetin on embryos of less than 100 μ, the chance to grow actually in a culture in vitro remains small. The reasons mentioned in Chapter 4 hold true here also.

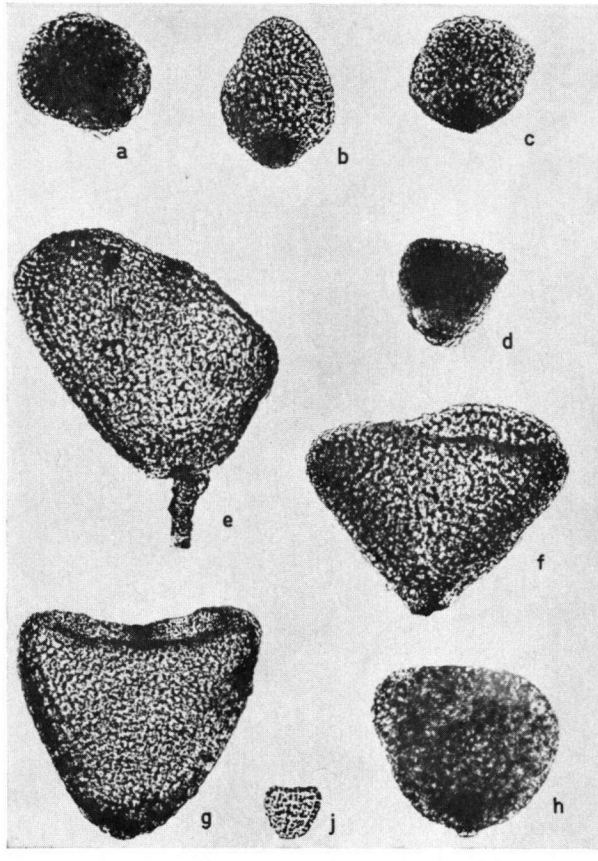

Fig. 18. Embryos cultivated for 10–12 days in basal medium containing 18 % sucrose with different concentrations of kinetin. Magnification 95 ×.

No.	kinetin conc. gr/ml	initial length	final length	after .. days
a	10^{-8}	60 μ	187 μ	10
b	10^{-8}	68 μ	196 μ	10
c	10^{-8}	68 μ	153 μ	12
d	10^{-7}	85 μ	136 μ	10
e	10^{-8}	94 μ	323 μ	12
f	10^{-9}	77 μ	323 μ	10
g	10^{-8}	119 μ	340 μ	12
h	10^{-8}	85 μ	221 μ	12
j	0	85 μ	85 μ	10

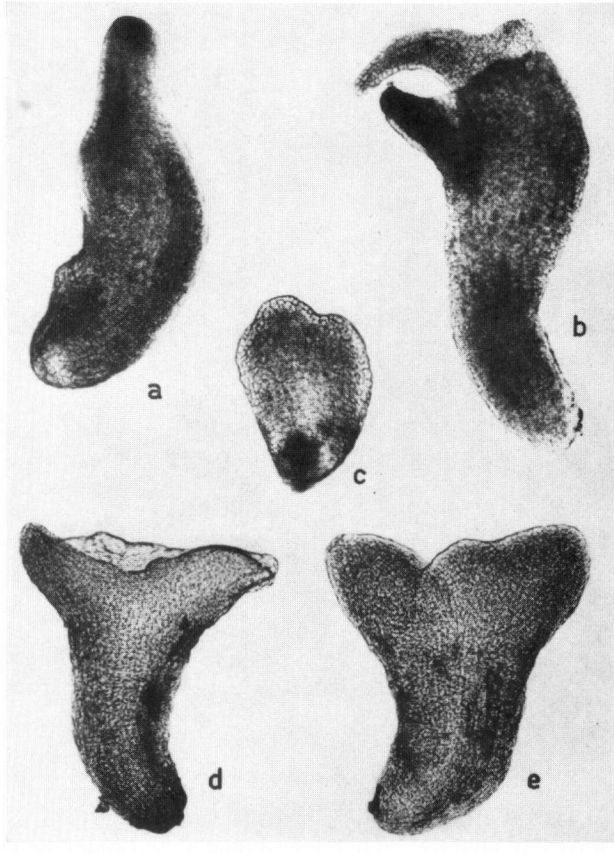

Fig. 19. Embryos cultivated for 8 days in basal medium containing 18 % sucrose with kinetin 10^{-8} gr/ml, followed by cultivation during 4 days in basal medium containing 12 % sucrose with GA in a concentration of 10^{-5} gr/ml. Magnification 63 ×, photographed after 12 days.

No.	initial length	length after 8 days	length after 12 days
a	119 μ	374 μ	790 μ
b	127 μ	416 μ	884 μ
c	85 μ	297 μ	374 μ
d	136 μ	425 μ	654 μ
e	93 μ	433 μ	697 μ

TABLE 17

Percentage of growing embryos cultivated in basal medium containing 18 % sucrose and in the same medium with kinetin (10^{-8} gr/ml). Measurements were made after 120 hours (Exp. 1) and after 72 hours (Exp. 2 and 3).

Initial size Group 1: 49– 99 μ
Group 2: 100–149 μ
Group 3: 150–199 μ

Exp.	Group	Control	Kinetin 10^{-8} gr/ml
1	1	0 % (0/5)	18 % (4/22)
	2	23 % (3/13)	80 % (4/5)
	3	50 % (3/6)	87 % (7/8)
2	1	50 % (4/8)	67 % (6/9)
	2	50 % (5/10)	90 % (9/10)
	3	33 % (1/3)	—
3	1	12 % (1/8)	25 % (2/8)
	2	14 % (1/7)	100 % (10/10)
	3	25 % (2/8)	100 % (5/5)
Total	1	24 % (5/21)	31 % (12/19)
	2	30 % (9/30)	92 % (23/25)
	3	35 % (6/17)	91 % (12/13)

Next we estimated the growth rate of embryos cultivated in basal medium with 18 % sucrose to which kinetin in several concentrations had been added. From our experiments (Table 18) it appears that the growth rate of globular and of heart-shaped embryos is increased by kinetin at a low concentration.

TABLE 18

Effect of kinetin on the growth of globular and of heart-shaped embryos cultivated either for 120 hours (Exp. 1) or for 72 hours (Exp. 2, 3, 4 and 5) in basal medium containing 18 % sucrose and in the same medium with kinetin. For the calculation we used all embryos, those that did not grow at all included. The regression coefficient denotes the linear regression between growth increment and initial length. The length increment (y) was calculated at the mean initial length ($\bar{x}$).

Exp.	Treatment	n	b	s_b	x_0 in μ	y in μ at $\bar{x} = 110\ \mu$
1	0	6	1.716	—	80	51
	10^{-8} gr/ml	10	1.866	0.171	63	87
	10^{-5} gr/ml	8	0.283	0.109	59	14
2	0	19	0.179	0.087	53	10
	10^{-9} gr/ml	15	2.353	0.760	65	106
3	0	22	0.181	0.143	54	10
	10^{-8} gr/ml	24	1.901	0.196	67	82
4	0	19	0.413	0.114	91	8
	10^{-8} gr/ml	25	1.271	0.167	52	78
	10^{-5} gr/ml	26	0.418	0.071	69	18
5	0	21	0.218	0.216	40	15
	10^{-8} gr/ml	19	1.408	0.324	43	94

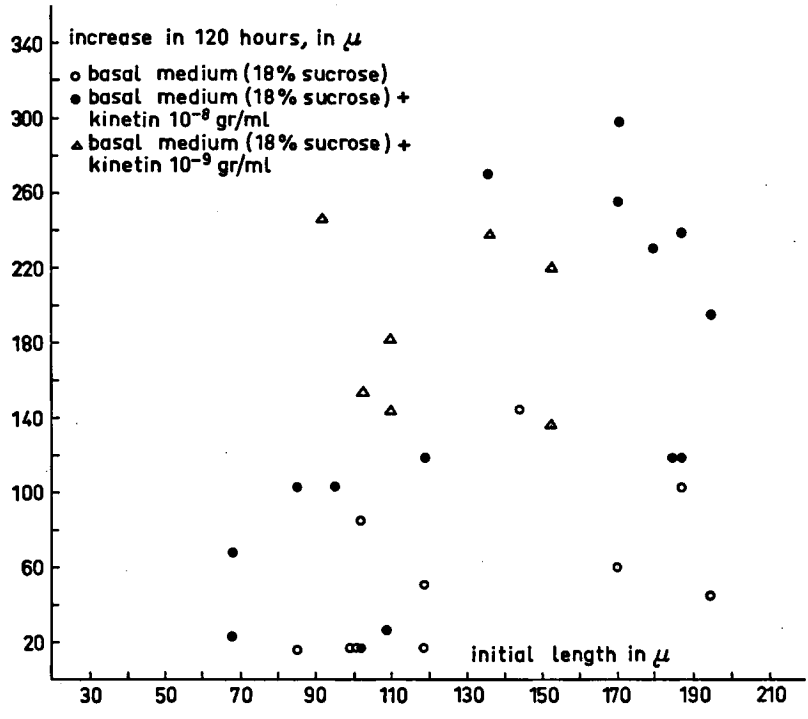

Fig. 17. Relation between initial length and growth for embryos cultivated in basal medium containing 18 % sucrose (control) and in similar media to which kinetin was added in a concentration of 10^{-8} and 10^{-9} gr/ml

The individual values of a typical experiment with low concentrations of kinetin are graphically represented in Fig. 17.

We further noticed in these experiments with kinetin that the growth pattern of globular and of young heart-shaped embryos differs from the normal development of embryos that remain enclosed in the ovule. They developed into abnormal shapes, and consisted entirely of cells of the original size. Photographs of several embryos cultivated for 10–12 days in basal medium with kinetin are shown in Fig. 18. In Fig. 19 the embryos are shown after 12 days of cultivation, first during 8 days in basal medium (18 % sucrose) + kinetin and then during 4 days in the basal medium with 12 % sucrose + GA.

In the presence of kinetin globular embryos developed without differentiation of cotyledons, i.e. they remained pro-embryos, whereas the root meristem became brownish discoloured (Fig. 18 a, b, c and d). Embryos with a length of 100 μ at the moment of excision from the ovule, are flattened by the initial differentiation of the cotyledons.

According to POLLOCK and JENSEN (1962), in *Capsella* the change from a globular to a heart-shaped embryo is brought about simply by an increased mitotic activity. This increased activity in definite regions was obviously disturbed in our experiments, as we observed

a meristematic zone in the upper part, which gives rise to a cup-shaped embryo (Fig. 18 f and g). Embryos with an initial length of more than 150 μ are not influenced in such a drastic way by kinetin. However, an inhibition of the development of the root meristem is always noticeable.

With the kinetin-treated embryos that had been transferred after 8 days into a basal medium with 12 % sucrose and GA in a concentration of 10^{-5} gr/ml, a resumption of growth was observed, which manifested itself mainly as an increase in the length of the hypocotyl. The root meristem was never regenerated (Fig. 19 b, d and e), nor was the bilaterally symmetric structure restored.

Modified ontogenetic patterns were observed by several other investigators (VAN OVERBEEK, 1942; TUKEY, 1937; SANDERS, 1950; RIJVEN 1952).

From all those experiments as well as from our own observations on untreated embryos it is obvious that kinetin is not the direct inducer of the abnormal growth. RIJVEN (1952) observed a few young embryos that developed a crown of cotyledons (after the addition of coconut milk, personal information).

In the ovule a deviating growth pattern of the embryo is very rare. In the course of our investigation we excised ca. 6000 embryos from the ovule. Among these embryos only four had three cotyledons. Their further growth in vitro, however, did not show any abnormality.

6.3. THE EFFECT OF KINETIN ON THE GROWTH OF "TORPEDOES"

In studying the effect of kinetin on embryo growth, we next tested it on "torpedoes", using a range of concentrations. The results of several experiments are given in Table 19.

TABLE 19

Effect of kinetin on the growth of "torpedoes" during a 24 hours' cultivation period in basal medium containing 12 % sucrose (control) or in the same medium with various concentrations of kinetin. The regression coefficient denotes the linear regression between length increment and initial length. The length increment (y) at the mean initial length ($\bar{x}$) was calculated.

Exp.	Treatment	n	b	s_b	x_0 in μ	y in μ at $\bar{x} = 513\ \mu$
1	0	9	0.655	0.036	112	263
	10^{-9} gr/ml	10	0.564	0.046	—	322
	10^{-8} gr/ml	7	0.694	0.138	105	283
	10^{-7} gr/ml	9	0.463	0.099	—	306
	10^{-6} gr/ml	10	0.315	0.120	208	97
	10^{-5} gr/ml	8	0.364	0.092	299	79
2	0	19	0.653	0.045	55	299
	10^{-5} gr/ml	10	0.327	0.055	283	75
	10^{-9} gr/ml	15	0.570	0.093	74	250
3	0	17	0.704	0.125	61	318
	10^{-5} gr/ml	21	0.479	0.135	317	94
	10^{-9} gr/ml	22	0.526	0.054	53	241

It appears from Table 19 that the growth of "torpedoes" is strongly inhibited in the more concentrated kinetin solutions. Further data on kinetin inhibited growth at the end of a 7 days'cultivation period are given in Table 20.

TABLE 20

Some characteristic features of the growth of embryos inhibited by 10^{-5} gr/ml kinetin during a 7 days cultivation. (The controls are those of Table 9).

	basal medium (from Table 9)	basal medium + kinetin
initial length	700 μ	534 μ
length after 7 days	2200 μ	1079 μ
length cotyledon/length axis	1100/1100	596/483

Table 20 shows that the growth of the cotyledons as well as that of the axis of the embryo is inhibited by kinetin. The latter part is most strongly affected, as is also apparent by a brown discoloration of the basis and by a necrosis of the root meristem. As to the effect of kinetin in a lower concentration (10^{-8} — 10^{-9} gr/ml), here too a similar discoloration was often observed; however, the strong growth inhibition caused by the higher concentrations was absent. The slight inhibition or, sometimes, a stimulation that actually were measured, cannot be considered significant.

6.4. Discussion

The effect of kinetin on embryo growth in vitro can be separated in two clearly distinct actions, viz.

1) an increase of cell-divisions at the apical end of the embryo, which is not followed by a differentiation of cotyledons,
2) an inhibition and even damage of the root meristem in all stages of development.

In this respect kinetin action differs from IAA action. If we compare the actions of kinetin and of IAA on the growth of "torpedoes", it appears that kinetin interferes strongly with the embryo growth, while the inhibiting action of IAA remains more limited. The critical initial length (x_0) shifts from ca. 175 μ to a value of ca. 300 μ if the medium contains a high concentration of kinetin, a phenomenon which is not observed with IAA. Finally, in the presence of IAA the hypocotyl and the cotyledons are equally inhibited, whereas in the case of kinetin the hypocotyl appears to be the most sensitive part of the embryo. In this case the effect of kinetin on an excised embryo causes a development which resembles that of an embryo in an opened ovule (chapter 4.8).

The specific inhibition of the hypocotyl by kinetin might give us some indication of the function exercised by kinetin-like substances as natural regulators in plant embryogenesis. In the next chapter some information is given on interactions of IAA, GA and kinetin in the regulation of embryo growth.

CHAPTER 7

THE EFFECT OF VARIOUS COMBINATIONS OF GIBBERELLIC ACID, INDOLE-3-ACETIC ACID AND KINETIN ON EMBRYO GROWTH IN VITRO

This chapter deals with the following questions,

1) are the effects of GA antagonized by IAA or by kinetin, and
2) can an antagonized or synergistic effect be demonstrated between kinetin and IAA?

In the literature we find many indications that GA is a natural growth-regulator, and that its action is influenced by the presence of natural stimulators and inhibitors. With regard to the nature of those substances we are ignorant, but we would not be surprised if IAA or kinetin or kinetin-like substances would turn out to be natural regulators of the action of GA.

We have added GA, IAA and kinetin to the culture medium of "torpedoes" in the combinations given in Table 21.

In Experiment 1 the concentration of GA was sub-optimal whereas the concentrations of kinetin and of IAA were physiologically high. A diagrammatic representation of the data of Exp. 1 is given in Fig. 20.

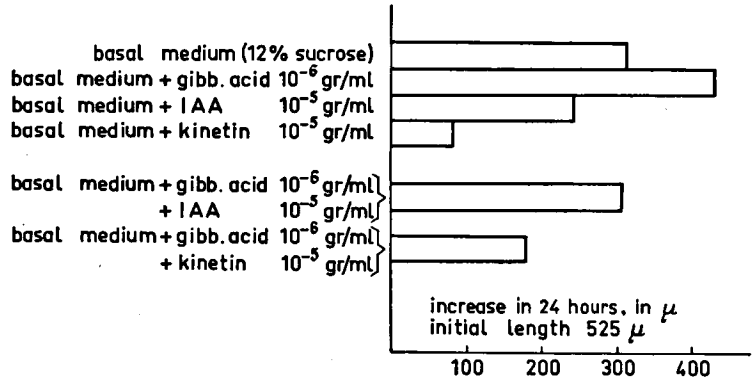

Fig. 20. The antagonizing effects on the growth of "torpedoes" exercised by IAA and by kinetin on the growth-promoting effect of GA. Data from Exp. 1 (see Table 21).

It appears that kinetin as well as IAA antagonize the GA-effect. In basal medium containing 10^{-6} gr/ml GA as well as 10^{-5} gr/ml kinetin after some days a brownish discoloration of the root meristem became visible. This phenomenon had been observed in earlier experiments after a treatment with kinetin at the same high concentration. Epinasty of the cotyledons induced by GA, was not antagonized. In basal medium containing 10^{-6} gr/ml GA as well as

TABLE 21

Effect of different combinations of IAA, GA and kinetin on the growth of "torpedoes". Embryos cultivated for 24 hours in basal medium (+ 12 % sucrose) and in similar media in which growth-regulators were included. The regression coefficient denotes the linear regression between length and growth. Concentrations are expressed in gr/ml.

Exp.	Treatment	n	b	s_b	x_0 in μ	y in μ at $\bar{x} = 525\ \mu$
1	0	11	0.640	0.095	37	312
	10^{-6} GA	19	0.829	0.079	5	431
	10^{-5} IAA	22	0.331	0.084	—	243
	10^{-5} kinetin	10	0.332	0.082	285	80
	10^{-6} GA + 10^{-5} IAA	23	0.726	0.089	102	307
	10^{-6} GA + 10^{-5} kinetin	16	0.519	0.045	180	179
2	0	19	0.562	0.128	71	255
	10^{-5} IAA	21	0.513	0.160	98	219
	10^{-5} kinetin	16	0.316	0.065	221	96
	10^{-8} kinetin	22	0.698	0.055	114	287
	10^{-5} GA	19	0.819	0.109	109	341
	10^{-5} GA + 10^{-8} kinetin	19	1.166	0.134	183	399
	10^{-5} GA + 10^{-5} IAA + 10^{-5} kinetin	17	0.406	0.098	253	110
	10^{-5} IAA + 10^{-5} kinetin	24	0.273	0.074	245	76
3	0	27	0.565	0.165	100	240
	10^{-10} IAA	18	0.708	0.099	98	302
	10^{-8} kinetin	15	0.822	0.159	69	375
	10^{-10} IAA + 10^{-8} kinetin	14	0.633	0.081	81	272

10^{-5} gr/ml IAA the inhibition of the growth-promoting effect of GA was accompanied by morphological changes such as appeared with GA alone.

Our second experiment shows that kinetin at a low concentration of 10^{-8} gr/ml is unable to affect significantly the increased growth rate induced by GA (10^{-5} gr/ml). This experiment demonstrates further that the growth is strongly inhibited when all three substances are present in a high concentration.

Finally it appears that the inhibiting action of 10^{-5} gr/ml kinetin is not significantly influenced by 10^{-5} gr/ml IAA.

In Experiment 3 we tested the effect of the combination of 10^{-10} gr/ml IAA with 10^{-8} gr/ml kinetin. Synergistic action of these substances was clearly absent.

The two questions posed in the beginning of this chapter may be answered as follows:

The increased growth rate, induced by GA, may be antagonized by IAA and by kinetin. Since the occurrence of IAA in seeds has often been demonstrated, we prefer IAA to kinetin as a possible candidate for the position of a natural inhibitor of GA. In this regard the results reported in Chapter 5 were disappointing, as the growth of immature embryos was not significantly influenced by IAA. In general the estimation of IAA present in seeds was carried out by the aid of a physiological test, i.c. an *Avena* one, and not by a chemical method.

As long as the natural growth-regulators present within embryo tissues are not identified, one can only speculate by supposing some interaction.

Finally we have found no arguments in favour either of an IAA-dependent action of GA or of a kinetin-dependent one. Such a dependency has been often observed in tissue cultures (GAUTHERET, 1961).

We therefore conclude that the three regulators investigated by us influence the growth of embryos in vitro through different mechanisms.

As the isolation and cultivation of young embryos ($< 150\ \mu$) is difficult, we did not make an attempt to investigate possible synergistic or antagonistic actions exercised by the three growth-regulators on these small embryos.

CHAPTER 8

GENERAL DISCUSSION

In the first place we have to discuss embryo growth in the ovule to make clear that embryo growth in vitro differs considerably from that in the ovule. The development of the pro-embryo has been described by several authors (HANSTEIN, 1870; SOUÈGES, 1919). Comparatively little attention has been given to the subsequent changes which appear during embryogenesis. Such an investigation with embryos of *Phlox drummondii* was carried out by MILLER and WETMORE (1945).

WARDLAW (1955) describes the embryogenesis of *Capsella bursa-pastoris* from the spherical stage onward, in the following way: "the spherical embryo continues to enlarge with further divisions of its constituent cells. As the cotyledons begin to develop in the distal region, the spherical embryo becomes transformed into a somewhat flattened cordate body. Both the cotyledons and the hypocotyl now begin to elongate, chiefly by transverse divisions of their cells; the nascent shoot apex consists of a small-celled region, situated in the depression between the cotyledons. The root apex is meanwhile becoming organised, and incipient vascular tissue can be seen in the hypocotyl between the shoot and root apices. The enlarging embryo begins to bend as it adapts itself to the curved embryo sac."

To this description we may add the following observations. The procambium bundle shows no further differentiation into phloem or xylem elements. This is at variance with the findings of MILLER and WETMORE in the case of *Phlox* embryos (1945). During embryogenesis the shoot meristem remains at rest. We did not succeed in activating this shoot meristem in embryo cultures either with or without growth-regulators in the medium. SENGHAS (1957) investigated the histogenetic development of the shoot apex in *Capsella*. A distinct epicotyl is lacking. The visible elongation of the shoot apex starts when the plant passes into the flowering phase. Though, on the other hand, the root meristem is already differentiated during embryogenesis, it is unlikely that the name "radicle" is correct. This meristem becomes highly active during the germination process.

RIJVEN (1952) investigated the differences in growth-rate during various stages of the embryogenesis within the ovule; see Fig. 14.

In several regards embryo growth within the ovule differs distinctly from that in vitro. Earlier we mentioned the differences in cell diameter. In other experiments we have found a linear ratio between embryo length and the INC of embryos grown in vitro. Such a relation seems to exist also for "torpedoes" within the ovule (Fig. 13). We have calculated the increase in INC for an increase in length of 200 μ for "torpedoes" developing in the ovule. After such an increase 0.101 μg N has been incorporated which is considerably less than the amount found in in vitro cultures with 12 % sucrose (0.143 μg N; see Table 12).

Finally we did observe that in "torpedoes", after 7 days' cultivation in vitro in basal medium with 12 % sucrose (without growth-regulators), the procambium bundle differentiates into xylem elements, starting at the inner side of the cotyledons.

So we have to conclude that growth in vitro differs considerably from embryo growth in the ovule. If morphogenetic differences are caused by endogenous differences of growth-regulators or metabolites, one has to be careful when applying results obtained in vitro on the embryogenesis within the ovule.

According to RIJVEN (1956), the presence of a linear ratio between embryo length and growth, which was found in our own experiments too, means that the dividing cells of the embryo are equally spread over the whole length of the embryo. With embryos of *Anagallis arvensis* Rijven could not find such a relation. Therefore he concludes that the growth of these embryos "is already restricted to certain well-defined meristematic zones which produce new, non-dividing cells at a constant rate".

For all experiments we calculated the x_0-values. Unfortunately, the variability of this value proved to be considerable, so we could draw only one conclusion from these figures: when kinetin is added in a high concentration to the basal medium containing 12 % sucrose, the critical initial length shifts to a higher value.

We did not find arguments in favour of an embryogenesis-controling function of GA and IAA. Kinetin, on the other hand, increases the

growth rate of very young embryos. However, the growth pattern is modified too, and radially cup-shaped embryos are formed. Since the same thing happened with the few embryos that were grown in the basal medium without kinetin, this abnormality can not be considered to be a direct result of the treatment with kinetin. Further, the root meristem is always inhibited by kinetin. So the action of this substance is different in different parts of the embryo.

If one assumes a patterned distribution of growth-regulators or metabolites, preceding morphogenetic and histological developments, one can postulate one or more centres in the embryo where these substances are produced. Dependent on the concentration gradients of these substances in the embryo tissue, morphogenetic changes would be induced.

Recently TURING (1952) advocated such a system on absolutely theoretical and mathematical arguments only.

HACCIUS (1956) too assumes a gradient in the concentration of growth factors in the embryo tissue at the moment the development of the cotyledons starts.

As kinetin causes a concentration-dependent reaction in different parts of the embryo, kinetin, or eventually a chemically related substance, would well fit in the suggested system. The differences in growth pattern in the ovule and in vitro, observed with globular and heart-shaped embryos, could be attributed in that case to the circumstance that in vitro an all-round action of this growth-factor is exerted.

On the other hand, the view of RIJVEN (1952) that within the ovule the change of the radially symmetrical embryo into a bilaterally symmetrical one, would be due to bilateral gradients in the ovular environment, can be maintained.

Conclusive arguments for such a gradient system can only be obtained by the isolation and chemical identification of these substances from the embryo tissue. The smallness of the immature embryos will make this work extremely difficult.

Recently VAN OVERBEEK (1962) gives a survey of endogenous regulators of fruit growth. Van Overbeek assumes that a sequence of growth-factor mixtures determines the growth pattern of the fruit. These mixtures are composed of three types of hormones, viz. auxins, gibberellins and kinins, but the ratio between them is continuously varying. A sequence of responses to these three types of hormones was earlier demonstrated by WRIGHT (1961), working with wheat coleoptiles.

In our own investigations we have found some evidence for the presence of such a sequence of responses to hormones. Kinetin brings about cell division in very young embryos. It is likely that gibberellic acid, on the other hand, is involved in the germination process. As indole-3-acetic acid has been isolated from mature plants of *Capsella*, it is probable that IAA plays its part in the growth of the full-grown *Capsella* plant.

SUMMARY

1. The effect of three growth-regulators on the growth in vitro of *Capsella bursa-pastoris* embryos was studied.
2. Gibberellic acid increases the growth rate of "torpedoes" considerably. It also stimulates the root-meristem activity, and induces epinastic curvatures of the cotyledons. As these phenomena are part of the process of germination, we may classify GA as a promotor of germination. It is unlikely that GA functions as an agent controling embryogenesis.
3. Our experiments with indole-3-acetic acid make it improbable that IAA acts as a natural growth-regulator during embryogenesis.
4. The effect of kinetin on embryo growth in vitro is twofold: it causes an inhibition of the root meristem and an increased cell division in the very young embryo. The latter leads to a modified growth pattern.
5. From experiments with combinations of the three growth-regulators we conclude that these substances influence the growth of the embryo through different mechanisms.
6. In the general discussion attention was paid to the fundamental differences between embryo growth in vitro and embryo growth in the ovule. One has to be careful in applying results obtained with embryo growth in vitro on embryo growth in the ovule.

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