IN VITRO STUDIES ON THE EMBRYO OF CAPSELLA BURSA-PASTORIS

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With plates V—VI

INTRODUCTION

The fertilized egg-cell or zygote must often be considered pre-eminently omnipotent. With many animals and also with lower plants the zygote develops independently in the external medium and all factors, needed for the growth and differentiation of the embryo, must be assumed to be present in the egg. The whole problem of morphogenesis is presumably comprised in the zygote itself, even though external factors may affect the development to a certain extent.

With higher plants as well as with viviparous animals things may be different. In Spermatophytes the zygote and later the embryo are embedded in the embryo-sack, the nucellus or the endosperm, and it seems likely that the surrounding tissue supplies the zygote and later the embryo with substances, needed for its development and differentiation. It may be presumed that this happens in a special pattern and according to a definite schedule. Even a very young embryo (globular stage) displays some differentiation in its basal-apical polarity. In later stages (heart-shaped stage) further important differentiation can be stated at the apical end: biradiate symmetry superseding the original polyradiate symmetry.

In this condition the fertilized egg-cell and the young embryo appear exceptionally suitable for the study of the differentiation and morphogenesis in dependency on the milieu by applying an in-vitro technique. The realization of such a technique probably would create a field of new possibilities for morphogenetic studies in various directions.

On the other hand, not only the problem of morphogenesis, but also that of the metabolism of the embryo has increased the interest of physiologists. Organisms that in later stages are completely autotrophic, start their life heterotrophically. Already a casual survey of the literature shows that the heterotrophism increases and that the medium has to comply with higher demands, the younger the embryos are. The knowledge of these demands is still very deficient. One of the reasons why very young embryos still cannot be cultivated is our lack of knowledge of the chemo-differentiation, protein-synthesis being one of the main problems of biochemistry.

A second reason why the cultivation of young embryos did not succeed satisfactorily, is that the physico-chemical conditions of the natural medium inside the ovule have not yet been studied, though this medium could yield valuable data, such as pH and osmotic value. As yet no one has deliberately tried to imitate the embryogenesis in the ovule. Several investigators mention a premature germination of the embryos in the culture; this abnormality is due to an insufficient knowledge of the factors controling this phenomenon.

Germination is defined here to start at 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. The appearance of cell-elongation marks off a new phase

of the life-cycle, as it affects the nature of growth and structure essentially. So we emphasize the necessity of distinguishing between pregerminal and postgerminal cultures, both being quite different objects of investigation.

The purpose of our study was, to collect more information on the requirements for growing young embryos according to normal embryogenesis.

Our experiments have been conducted on *Capsella bursa-pastoris* (L) Med.. This choice was based on the following considerations: 1. The embryo of *Capsella* is the classic example of the dicotyledonous embryo, the development of which has often been described. 2. The plants are flowering for six months at a stretch and during this time yield all embryonic stages. 3. In the inflorescence these stages are arranged along the axis in silicles, the younger ones at the top, the older ones near the base, thus facilitating the search for specific stages. 4. Each silicle contains about 20—25 embryos of approximately equal lengths (so the application of statistical analysis would be possible). 5. Capsella embryos can easily be isolated.

We started by gathering some information about the embryo and its environment in the ovule. Chapter II contains these data.

Next we tried the culture of young embryos, making good use of the obtained information. A culture-method was developed that allowed daily examination and measuring of the growth, this being necessary for the analysis of several factors, especially of the nitrogen-source. These experiments have all been made on "torpedoes" and heart-shaped embryos, and will be recorded in Chapter III.

Finally, the viability of cultured embryos was tested in a post-germinal cultivation. The findings in this field are reported in Chapter IV.

The methods, used for the various purposes, will be described along with the experiments.

The disappointing fact that we did not yet succeed in cultivating globular embryos postponed our aspirations for the experimental-morphological range. However, this negative result may have its significance as will be shown in the discussion at the end of this paper.

CHAPTER I

SURVEY OF THE LITERATURE

In this survey we will confine ourselves to the discussion of those papers that deal with the cultivation of plant-embryos excised from the ovule during their embryogenesis.

Thus far in literature the name "embryo-culture" has mainly been used in the case of the cultivation of mature or even germinating embryos, which in order to make an analysis of their heterotrophy

are deprived of their natural food-supply. In spite of the above-mentioned difference between pre- and post-germinal embryo cultures we cannot entirely omit the discussion of the latter as information gained by its study largely contributes to the successful pregerminal culture.

The first to realize the idea of cultivating immature plant-embryos *in vitro* was HANNIG (1904). He succeeded in making a sterile culture of embryos of Crucifers (mainly *Raphanus sativus*) when using TOLLENS' nutrient solution with 10 % sucrose as a medium for embryos of about 2 mm in length (8 mm when mature). The embryos were kept in this medium for 29 days and during that time they grew 1—2 mm. Then they were transferred to cotton-wool in a salt-solution and, having been planted out in earth after germination, they developed into flowering plants. Since he stated, that the growth-rate of the embryos in his medium decreased, he tried to improve the growth by changing the nitrogen-source; he tested peptone, aparagine, leucine, glycocoll and tyrosine. But this part of HANNIGS investigation, carried out on *Cochlearia*, cannot be considered sufficiently exact for a quantitative analysis. The number of cultures in one series was very small; the original lengths of the embryos were not the same and there was also a variation in the duration of the experiments. These results were all negative and are not conclusive. His finding of amino acids inhibiting the growth in concentrations of more than 0.1 % raises the surmise, that the medium had been altered unfavourably during the sterilization by heat. Moreover, his choice of amino-acids was rather inadequate, as will be learned from our results. HANNIG, however, could state the need of a high oxygen-tension in an experiment with two series of embryos; in one of the series the embryos were scarcely covered by the medium, while in the other series one or two cm sunken under its surface. He found an appreciable difference between the two series. Interesting from a morphological point of view is HANNIGS observation that the embryos do not bend when being cultivated, but remain straight.

A second paper on plant-embryo-culture did not appear until twenty years later: DIETERICH (1924). He worked on representatives of about fifteen plant-families, trying to find out whether embryos are able to germinate without having passed a resting-period. DIETERICH excised the embryos when nearly mature and cultivated them on KNOP solution with 1.5 % agar. Younger embryos could mostly be brought to germination also, when 2.5—5 % sucrose had been added. In the case of these younger embryos a difference in development could be stated between embryos, cultivated on top of the agar and others, cultivated under the agar-surface, the latter continuing the embryonic growth until mature length, whereas the former started germination precociously. This phenomenon, called "künstliche Frühgeburt" by DIETERICH, appeared later also in several other investigations, and gained special attention as a deviation of normal development.

The embryo-culture was used by LAIBACH (1925, 1929) as an expedient for growing hybrids of *Linum*-species that normally abort

in the ovule. The embryos (1.5 mm) were excised and cultivated on cotton-wool in a solution with 15 % sucrose. This culture-period lasted for 18 days, at the end of which time the originally green embryos had become ivory in colour. Then they were transplanted on filterpaper and germination followed.

This may be considered a starting point for many later investigations. By means of the embryo-culture special hybrids were grown by: JÖRGENSEN (1928); WERCKMEISTER (1934); SKORSTEDT (1935); BENSLEY (1940); SKIRM (1942); LAMMERTS (1942); BLAKESLEE and SATINA (1944); SMITH (1944); BRINK, COOPER and AUSHERMAN (1944); CUMMINGS (1945); MAC LEAN (1946); SANDERS (1948, 1950); STONE and DUFFIELD (1950).

TUKEY (1933, 1934, 1938, 1944) brought in culture thousands of embryos of early-ripening species and varieties of stone-fruits that otherwise would have aborted in the seed.

An investigation of LAMMERTS (1942) is in the same line.

The embryo-culture served another purpose in the work of RANDOLPH (1945). He succeeded in shortening the life cycle of *Iris*-species by passing over the two years resting-period in the seed.

A further step in this direction was taken by COX, MUNGER and SMITH (1945), who excised embryos of *Brassica*, thus withdrawing them from the influence of inhibiting substances in the seed-coat.

The method of the embryo-culture has also been applied for the benefit of vernalisation-research, mainly by PURVIS. FOR a review of the literature in this field we refer to MURNEEK and WHYTE (1948).

TUKEY cultivated his embryos in small bottles on a nutrient-solution with 0.65 % agar and 0.5—2 % dextrose. Like DIETERICH he observed an aberrant embryo-development. This phenomenon roused his interest and in 1938 he published a study of it on peach-embryos. He found, that dwarfism in the seedlings was stronger the younger the embryos were when excised. Thus he distinguished a number of different "growth-patterns". After having been subdued to a thirty-days period of reduced illumination, all forms could grow into mature plants when brought back to the greenhouse.

Besides TUKEY several other investigators mentioned an abnormal morphogenesis of the cultivated embryos, and invariably the phenomenon turned out to be accompanied by precocious cell-elongation: LI (1934) on *Ginkgo*-embryos; LA RUE (1936) and LA RUE and AVERY (1938) on embryos of *Gramineae*. MERRY (1942) found "growth-patterns" for *Hordeum sativum*.

VAN OVERBEEK (1942 b) held the opinion, that a high "auxin"-concentration would keep the embryos in embryonic condition; he based this view a.o. on an observation by THIMANN (1937), who noted an inhibiting action of "auxin" on bud-development.

KENT and BRINK (1947) reported that tomato-juice and hydrolysate of casein prevented germination when added to a control-medium on which the embryos germinated readily. Continuing this investigation, ZIEBUR, BRINK, GRAF and STAHMANN in 1950 published

the results of an analysis of this inhibition, that appeared to be due to inorganic substances in the casein-hydrolysate and they found that the same results could be obtained by using isotonic solutions of sucrose or mannitol.

In this connection it is worth noting that, whenever in the literature phenomena of the "künstliche Frühgeburt" are mentioned, investigators are sure to have been working with low sugar-concentrations, i.e. low osmotic values of the medium.

The view that the osmotic value of the medium is one of the factors determining the nature of the growth, is likewise supported by some findings of DUYM, KOMEN, ULTEE and V. D. WEIDE (1947) in this laboratory, viz. that the inhibition of germination by extract from *Beta*-seeds at least partly is due to the presence of osmotically active material.

Returning to the problem of the requirements of the medium we must state, that no notable progress was made in the pre-germinal embryo-culture until 1940.

Interesting and epoch-making in the field of plant-tissue-culture was a paper by WHITE (1932); he was the first who added growth-factors to the medium though it was, conformably to the time, in the form of an undefined yeast-extract. On a medium consisting of a salt solution with only 2 % glucose and added yeast-extract he managed to grow, by way of random-test, a small (0.2 mm) heart-shaped embryo of *Portulaca oleracea* to a length of 1.84 mm in three weeks.

There is only one other publication of some importance to be mentioned, viz. that from LI and SHEN (1934), who found a growth-promoting action on yeast-cells by extracts of rice and of wheat-bran; according to WILLIAMS these extracts contain pantothenic acid. On the growth of *Ginkgo*-embryos, however, they had an inhibiting effect, in this case growth-stimulation could only be obtained by adding low concentrations of an extract of *Ginkgo*-seed-endosperm.

During those years the post-germinal embryo-culture yielded more valuable results. In a series of investigations on mature or germinating decotylated *Pisum*-embryos, the favourable influence of several growth-factors was shown. Thus it became clear that SCHOPFERS concept: "auxo-heterotrophy" could very well be applied to embryos (RYTZ, 1939). Parts of this research were carried out by:

VON HAUSEN (1935, 1936), who noted the influence of ascorbic acid on the growth.

KÖGL and HAAGEN SMIT (1936), who demonstrated aneurin (thiamin) and biotin as required growth-factors, whereas ascorbic acid did not promote growth;

J. BONNER and AXTMAN (1937), who stated a positive effect for pantothenic acid;

J. BONNER (1938), who did so for niacin;

J. and D. BONNER (1938), who once again mentioned ascorbic acid as an active growth-factor.

According to RYTZ (1939) the discrepancy of these results is due

to the fact, that different *Pisum*-varieties had been used. The probability of this view was endorsed by RYTZ himself by showing a difference in the activity of aneurin on different varieties. Moreover, SAUBERT–VON HAUSEN (1948) could actually confirm this opinion for ascorbic acid. She also tested other growth-factors, viz. vitamins B_1, B_2 and B_6, biotin and pantothenic acid, adding them to a salt-solution with 4 % sucrose, together with ascorbic acid. By simultaneous administration of these growth-substances an additional growth-response was obtained, but there still was a marked difference with control-seedlings with cotyledons.

It is only after 1940 that the experience, gathered from the experiments mentioned, has been employed in behalf of the pre-germinal embryo-culture.

Progress was made by an analysis of various growth-factors by VAN OVERBEEK, CONKLIN and BLAKESLEE (1942 a) on *Datura*. This was the first more extensive study on the culture of young embryos. Viable seedlings could not be obtained without adding growth factors to the medium that consisted of a nutrient solution with agar and 1 % glucose. Only when the cultivation was started with already bent embryos growth factors could be omitted, but younger embryos ("torpedo"-stage) needed growth substances such as glycine, aneurin, ascorbic acid, niacin, vitamin B_6, adenine, succinic acid and pantothenic acid, but the necessity of each of these factors apart was not demonstrated. Embryos smaller than 0.5 mm, however, did not thrive even on such a medium; addition of coconut-milk in this case gave rise to an enormous growth, e.g. from 0.15 mm to 6 mm in 7 days. Casually VAN OVERBEEK (1942 b) claims to have grown even pro-embryos (0.10 mm).

This strong growth-promoting effect of coconut-milk was accredited to an unknown "embryo-factor", supposed to be present in it. As a remarkable detail must be mentioned that the younger embryos started growing callus-like when the coconut-milk had been autoclaved. According to VAN OVERBEEK this abnormality might be due to an increase in the "auxin"-content, that could have resulted from the heating.[1] In the same paper he indicated also the presence of inhibiting substances in coconut-milk, e.g. a "root-inhibitor" that could be removed by shaking with alcohol.

Continuing the research on the optimal conditions for growing embryos, VAN OVERBEEK, SIU and HAAGEN SMIT (1944) not only tested several pH's and temperatures, but once more they investigated the composition of the medium. Sucrose was shown to be a far better source of carbon than glucose; as an explanation for this fact they suggest the difference in liability of the two substances to phosphorylation. Coconut-milk proved to be superior to natural extracts, such as those from yeast, from wheat-germs or from ovules of *Datura*, though

[1] In this connection we may refer the work by CURTIS (1947), who likewise achieved a noteworthy undifferentiated growth of *Vanda*-embryos, unto 1.5 ml, by adding barbiturates (10 p.p.m.) to the medium.

these extracts did promote the growth in young embryos. Next the authors subjected coconut-milk to chemical purifications and finally acquired a product 170 times more active than the original sap. This preparation promoted the growth of *Datura*-embryos when added to the basal medium, containing 2 % sucrose, in a dilution of 1 : 19000, indicating hormone-activity of the "embryo-factor". The argument, however, is not conclusive, since a quantitative test, e.g. a concentration-series, is lacking.

The coconut-milk was soon to lose its exclusive reputation as an exceptional growth-stimulant, for BLAKESLEE and SATINA (1944) demonstrated, again on *Datura*, that it could be replaced quite well by malt-extract, provided the latter had not been sterilized by heat, but by filtration. Heat-sterilization gave rise to inhibiting substances, but these could be dissolved in and removed with ether, as was shown by SOLOMON (1950).

For corn-embryos coconut-milk proved to be useless: HAAGEN SMIT, SIU and WILSON (1945) cultivated these embryos in order to find out the most adequate source of nitrogen. Asparagine worked very favourably and could bring down the limit of cultivability to 0.3 mm initial length, but addition of coconut-milk could not lower it further. This may be due to the low sugar-concentration.

SANDERS and BURKHOLDER (1948 a, b) were strongly interested in the problem of the nitrogen-supply. They first stated a notable growth and normal development of *Datura*-embryos when casein-hydrolysate had been added to the medium. The same results could be obtained when using a combination of all pure amino-acids instead, but this favourable effect could not be matched by incomplete mixtures or by single amino-acids. With respect to different species, viz. *D. stramonium* and *D. inoxia*, small differences in activity of the amino-acids were noted. Besides, the authors noticed some diversity of development of embryos, cultivated in various incomplete mixtures, the ratio between cotyledons and hypocotyl being affected. The conclusion from these results was as follows: "The growth-stimulus of the concentration of 20 acids results from the physiological interaction rather than from the summation of effects of individual acids".

Perhaps this conclusion also includes the explanation of the negative results of SPOERL (1948); he tested 19 single amino-acids as a source of nitrogen for Orchid-embryos, mostly seeds of *Cattleya*. At most some amino-acids equalled the effect of ammonium nitrate. The autoclaving of his media, however, raises some doubt as to the reliability of these results.

RAPPAPORT, SATINA and BLAKESLEE (1950) tried the influence of ribonucleic acid and desoxyribonucleic acid on *Datura*-embryos. The effect was rather an inhibition than a growth-promotion, even in a concentration of 0.0001 p.p.m..

Finally we must mention two publications, dealing with the differences in reaction between species of one genus or between hybrids. The investigations were carried out again on *Datura*-species.

DOERPINGHAUS (1947) cultivated ten different species on various

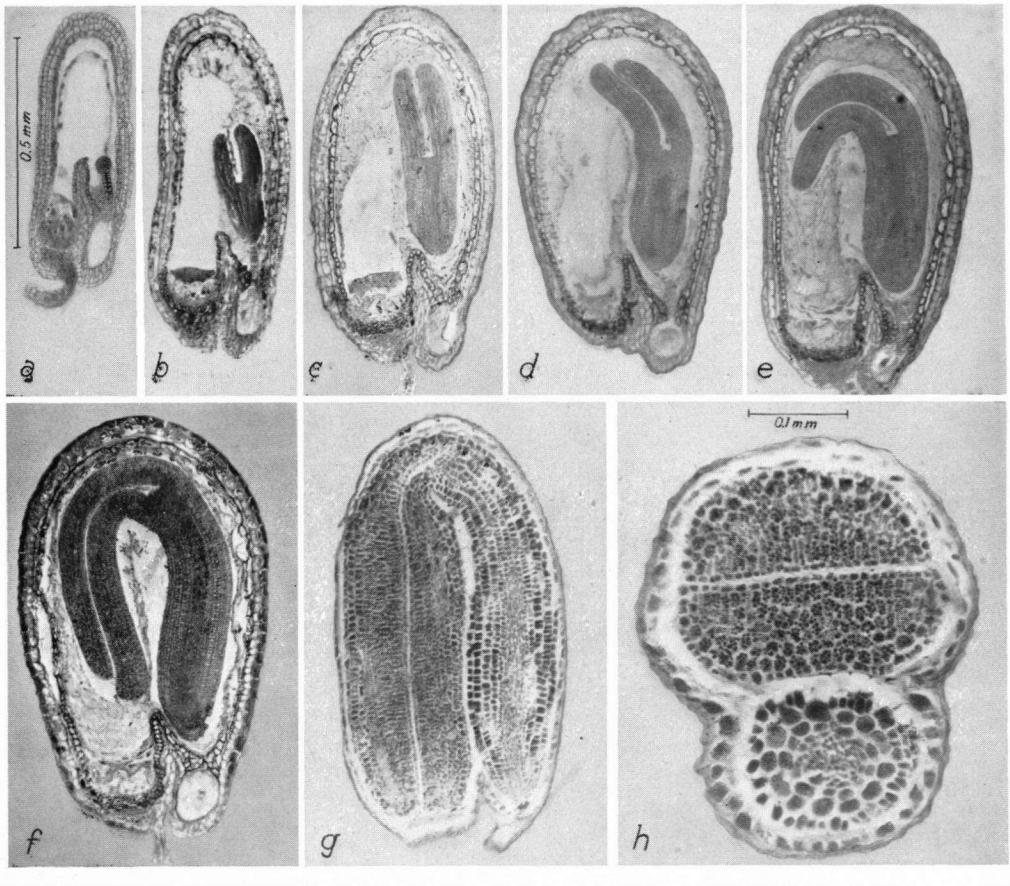

Fig. 1. Microphotographs of histological sections through ovules of different stages. a—f: developing ovules; g and h mature seeds. All reproductions at the same scale except h, which shows the transsection of the seed with abundant aleurone grains in the embryo. Fixation after Land; staining with gentianaviolet.

sugars. Though sucrose proved unalterably superior, other sources of carbon (mannose, dextrose, laevulose, glycerol, a.o.) did not act equally on the different species.

A similar fact could be stated by SANDERS (1950). The optimal sucrose-concentration appeared to be different for *D. stramonium* and three other species. Likewise those species did not show the same dependence on $(NaPO_3)_n$ (= "Calgon") concentration. In this paper also tests with growth substances are reported: various synthetical "auxins" (10 p.p.m.) did not stimulate the growth, nor did the growth substances mentioned by VAN OVERBEEK, CONKLIN and BLAKESLEE (1942). The growth-promoting action of malt extract would be due to soluble nitrogen containing substances, this extract being rich in amino-acids (DICKSON and BURKHART, 1942), that are known to promote growth.

CHAPTER II

THE EMBRYO IN OVULO

A. THE EMBRYO

§ 1. *Classification of the stages in embryogenesis*

The embryogenesis of *Capsella bursa-pastoris* has been studied regularly, since HANSTEIN for the first time did so in 1870. As for the development up to the globular stage, the most exact description is that by SOUÈGES (1916,1919).

Our own observations led to a classification of the whole development, which is given in table I and figure 2. The position of the embryo in the campylotropic ovule at various stages may be seen from the micro-photographs of histological sections (figure 1). Even in the younger stages, the nucellus is failing; the endosperm is of the nuclear type; in the mature seed only a layer of aleuron-filled cells is left of it, like in other Crucifers (GUIGNARD, 1893). According to the terminology of A. P. DE CANDOLLE the situation of the embryo in the seed is notorrhiz.

When freshly excised from the ovules, the embryos are green up from stage IV. As the embryo grows older, the colouring becomes stronger. Yet embryos from mature seeds are hard and ivory-white. No starch can be found in their cells then, while aleuron-grains and fats are abundant. In younger stages starch occurs, but not earlier than in stage IV. In this "intermediate" stage not more then a few cells contain very small grains, visible only when magnified 400 ×. Up from the torpedo-stage, starch grains are big and numerous enough to be noticed when magnified only 100 × ; most of them are located in the hypocotyl. It is certain, that fats are present in the embryos in stage V (torpedo) and in older stages; they quite probably are synthesized from the starch.

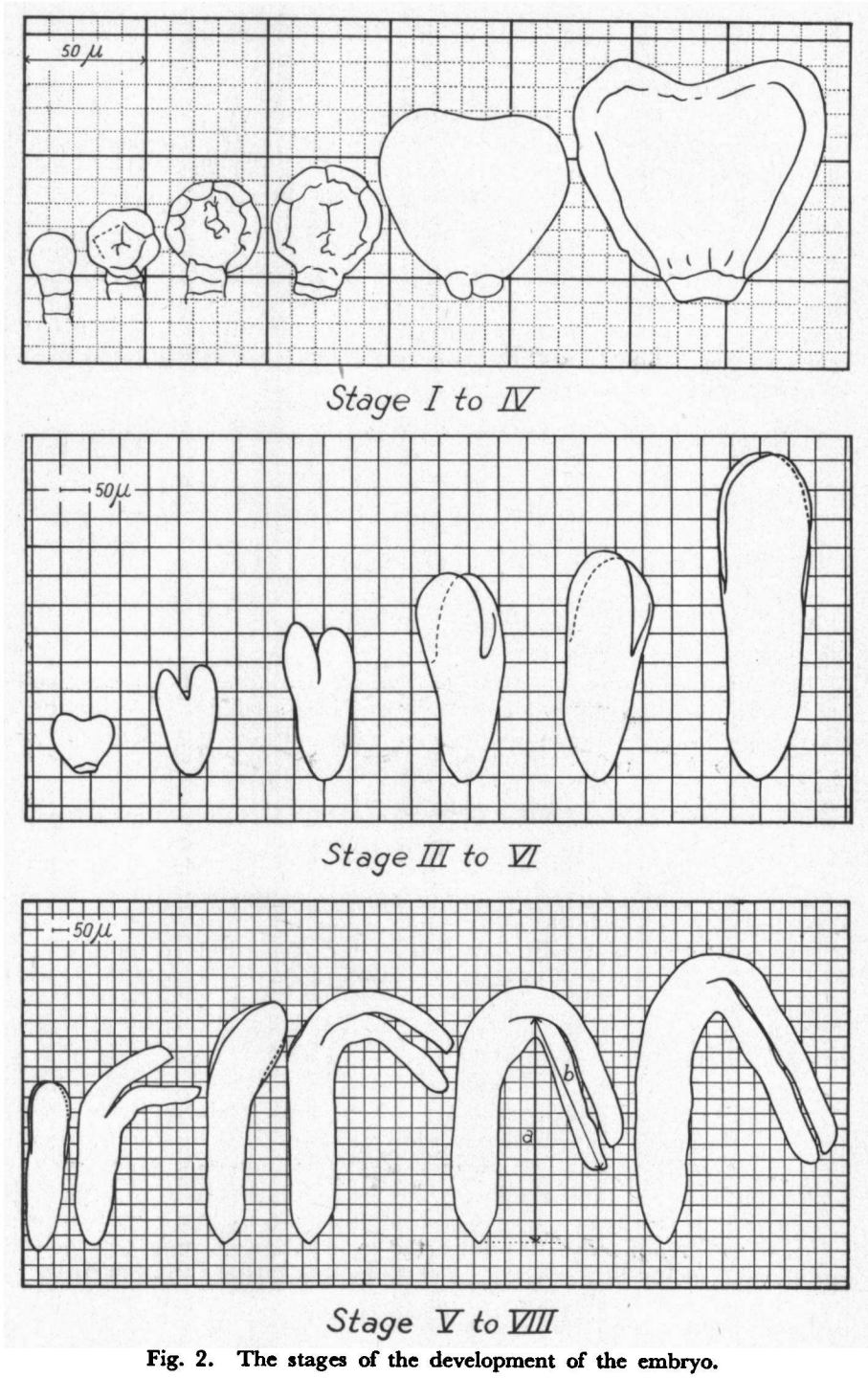

Fig. 2. The stages of the development of the embryo.

TABLE I

The stages of development of the embryo *in ovulo*

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.

§ 2. *The variation in length of the embryos from one silicle*

As it was necessary to get an idea of the variability of the embryos, contained in one silicle, we gathered 10 silicles from 2 well-grown plants and measured the embryos. The mean number of ovules per silicle was 24.1 (with standard deviation 3.3).

As the different silicles contain embryos of different stages and sizes, the standard deviations of the embryo length per silicle in their absolute values would only yield incomparable data. In order to obtain a uniform measure each standard deviation was expressed in a percentage of the mean embryo length of the silicle in question. After this the 10 thus computed standard deviations were averaged. From these calculations a mean value (14.8 %) resulted with a small standard error (0.6 %). As appears from these data the variability is constant; this is mainly due to the presence in many silicles of an odd small embryo.

Comparison of the mean lengths of embryos, derived from the two separate halves of one and the same silicle, however, gave no greater difference than 3.8 % of the lower value.

§ 3. *The correlation between the lengths of silicles, ovules and embryos*

In order to avoid a laborious search for definite stages when starting a culture, we tried to estimate beforehand the relation between the lengths of silicles, ovules and embryos on racemes of several plants.

Though there is some difference between the plants, they all show the same characteristics that are presented in figure 3a; this graph gives the data from one well-grown plant. On the abscis, the numbering of the silicles is plotted, the numbers 1, 2, 3a etc. indicating the first,

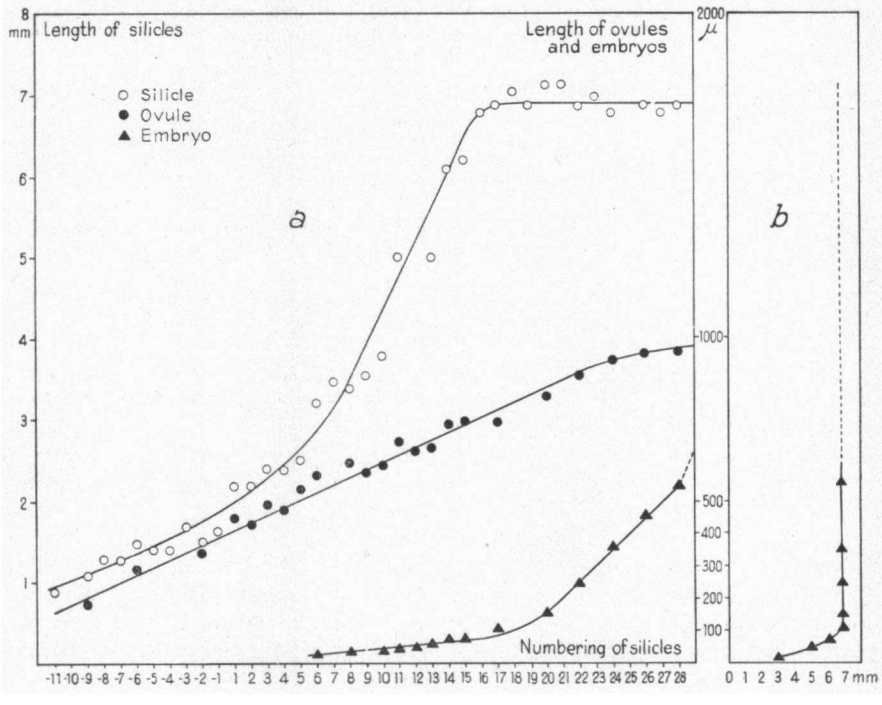

Fig. 3. Explanation see tex..

second, third etc. silicle from top to base, starting with the youngest silicle without petals. The numbering — 1, — 2, — 3 etc. therefore goes upward from the undermost silicle with petals. Thus the abscis may be understood as a time scale. On the left ordinate, the lengths of the silicles are set off, and on the right ordinate on a larger scale those of ovules and embryos. In figure 3b the embryo length is plotted directly against the size of the silicle.

From the data we may conclude that the silicle is the first to grow after pollination, even so that it has already reached mature length when the embryo is still very small, e.g. heart-shaped, as it happens to be in this case; in most other plants the embryos appear to be one stage older. In the course of our investigation we came to the conclusion that our example is representative for young vigorous plants. When the inflorescence passes out of flower, older embryos may be found in the top of the raceme.

§ 4. *The growth of the embryo in the ovule*

As will be clear the embryos cannot be measured without being excised. So an indirect method had to be applied to find out the rate of growth of the embryos in their natural environment. The method was based on our experience, described in the previous section.

A fine plant was selected and the silicles were numbered consecutively from top to base. We planned to measure all embryos, contained in the *odd-numbered* silicles *at a time t*, and the embryos in the *even* silicles *at a time t + 24 hours*.

Now it would be possible to compute the mean lengths of the even silicles also at time *t* by interpolation between the corresponding data from the measurements of the odd silicles at time *t*. This interpolation is facilitated by the arrangement of the silicles, but can only be reliable if applied on a regularly flowering plant, such as the one we had chosen. This plant had been grown in a pot in the open air; before starting the experiment it was placed in a room with a constant temperature of 25° C, and illuminated with a fluorescent tube (Philips T.L.) at a distance of 1.5 m until the end of the test. At a time *t* 30 odd-numbered silicles were removed; each silicle was put in a small numbered tube and kept in a refrigerator until measurement on the same day. Excision and measuring were carried out in a 12 % sucrose solution. 24 hours later we repeated this procedure with the even-numbered silicles. The total number of embryos measured for this experiment came up to ca. 1000.

The results of this experiment are presented in three graphs (figure 4 a, b and c) and will be discussed below.

In some more experiments another method for measuring the growth in the ovule was used. In those cases, the halves of several silicles were removed from the raceme at time *t*, and the other remaining halves 24 hours later; since the two halves of one silicle contain almost equal embryos (cf. section 2), the growth in 24 hours could be found by substraction of the means. The issues thus obtained confirmed the characteristics of the graphs given in figure 4, the discussion of which follows here.

Figure 4a shows the increase in length in 24 hours plotted against the initial length at time *t*. The sagging of the curve, given as a dotted line in the neighbourhood of 500 μ on the abscis is quite probably real. It may be understood as resulting from the fact that during the 24 hours of the experiment, the torpedo-shaped embryos were forced into the walking-stick shape.

From the same data the graph of fig. 4b is derived. Here the abscis is the same as for *a* but the increase in length on the ordinate is expressed as a percentage of the initial length. The reality of the peak at 100—150 μ is highly probable, as it resulted from the other experiments too. Embryos with this initial length start heart-shaped, and during the next 24 hours exhibit mainly longitudinal growth, unlike the preceding globular stages. One may expect that in the latter embryos, growth proceeds with at least equal intensity, but so as to escape for the major part from our longitudinal measurement.

In figure 4c embryo length is plotted against time. The "empirical

points" in this graph are derived from figure 4a, as is elucidated there by constructional lines; to begin with the smallest embryo the increase in length of 24 hours is added, which operation is repeated for the sum etc.. The curve drawn through the points, has a strong resemblance to a mathematical sigmoid curve; so we subjected it to a test according to LE HEUX (1947), to find out whether T. B. ROBERTSONS growth formula could be applied to it. The formula is:

$$\ln \frac{\mathcal{N}}{A-\mathcal{N}} = b\,(t-t_1)$$

in which

the variables $\mathcal{N}$ length of the embryo in μ
t age of the embryo in hours
and the parameters A maximal length of the embryo: 1760 μ
t age of the embryo when N = ½A, 141 hours
b modulus of the growth

By means of LE HEUX' "modulus chart for growth scale" we could establish the value of 1/b at 24 hours. Hence it follows, that in this case the length of the embryo must fit the function:

$$\ln \frac{\mathcal{N}}{1760-\mathcal{N}} = (1/24)\,(t-141)$$

In figure 4c some points give embryo lengths according to the theoretical suppositions. These "theoretical points" appear almost conterminous with the empirical curve.

B. THE ENDOSPERM

When the embryos are still immature, their ovules are highly turgescent, so that they release some sap in case they are wounded, e.g. with a needle. This sap can be sucked up into a capillary. When studied under the microscope, it appears to contain spherical nude protoplasts, that constitute the endosperm. The impression of a poorly developed endosperm is confirmed by the microphotographs of sections through ovules (figure 1).

Considering the sap to be the direct medium of the embryo, we tried to gather some facts of its chemical and physico-chemical properties.

§ 5. *Microchemical reactions on the sap from the ovule*

As an implement in this part of the investigation we used a modification of the braking-pipette. The ordinary braking-pipette has been described on page 176 and illustrated in figure 8, but its modified form is provided with an acuminate free tip, making it suitable for pricking into the ovules and sucking up the sap. In most cases, several ovules were drained in sequence, after which the reagent was taken in. Next the point of the capillary was closed by melting it in a micro-flame. If heating was required for the reaction, the same happened at the

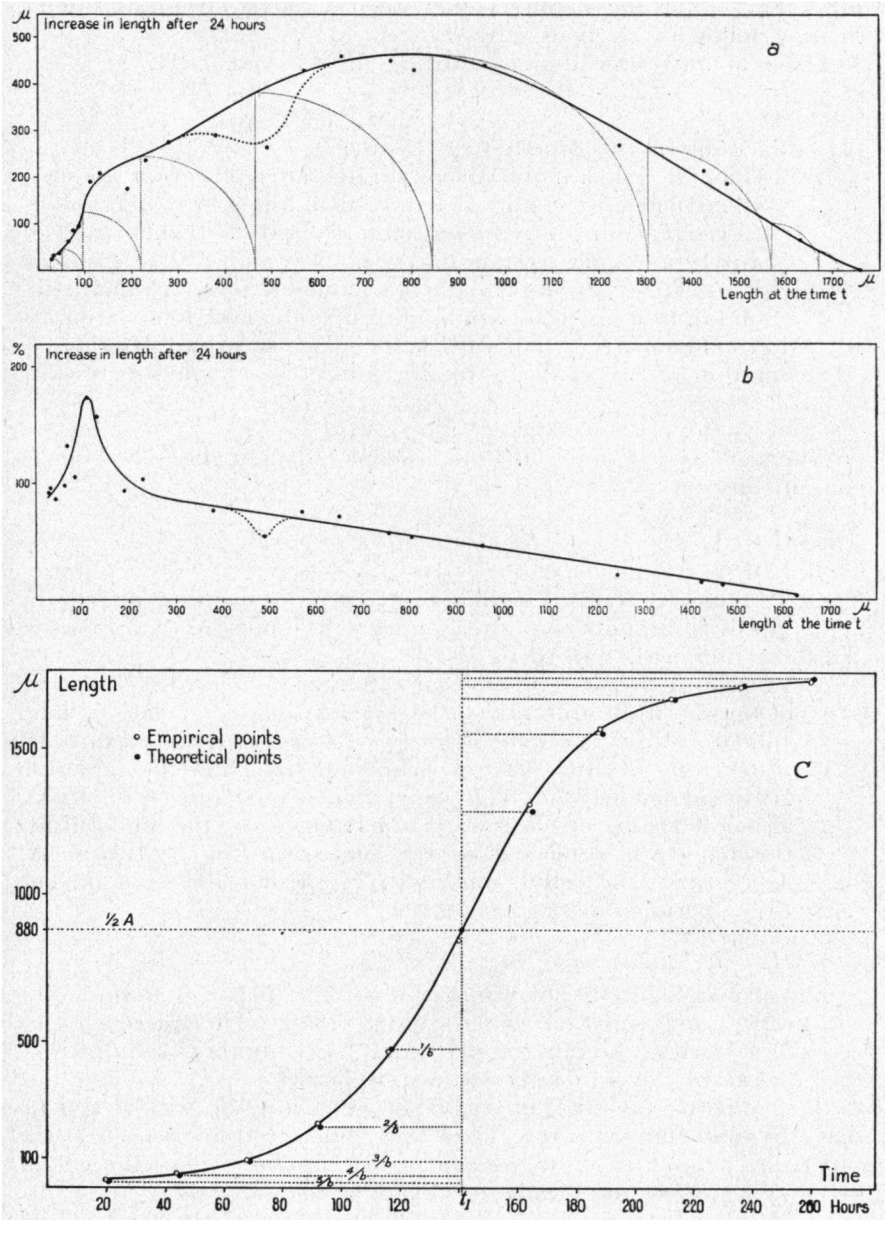

Fig. 4. Explanation see text.

other end, after the capillary had been removed from its holder, heating following in boiling water.

The reactions were done according to SCHOORL (1937).

Carbohydrates:

A. Reactions in the pipette.
   1. General reaction on carbohydrates after MOLISCH: positive when tried on sap from ovules containing 600 μ embryos.
   2. LUFFS reaction on reducing monoses: positive when tried on sap from ovules containing resp. 500 and 250 μ embryos.
   3. BARFOEDS reaction on reducing monoses: positive when tried on sap from ovules containing resp. 500 μ and 300 μ embryos.

B. Micro-chemical reaction with I–KI solution; in sap from ovules containing 200 μ embryos, small grains of starch could be shown.

Fats:

Whenever tested under the microscope, the sap did not seem to contain any fats.

Amino acids:

Ninhydrin reaction.

1. 4 Ovules containing embryos of 250 μ were drained and the sap was brought on filter paper: the spots gave a positive reaction with ninhydrin.
2. In order to ascertain the presence of free amino acids, a dialysate of the sap from a number of ovules was made as follows: small collodion bags (content 0.5 ml) were filled with 0.4 ml distilled water; one of them, the experimental bag, had the sap from 20 ovules added. All bags were then placed into 1 ml flasks, also filled with distilled water. 24 Hours later, the fluid outside the bags was subjected to the ninhydrin test (by Miss W. TERPSTRA); the result was positive only for the fluid outside the experimental bag.

§ 6. *pH of the ovular sap*

The principle of the determination of the pH was that of the "Indicator range method" after SMALL (1928). The quested value is enclosed within a narrow pH-range by a number of indicators, the critical pH of which being exactly known. Again the modified braking pipette was used, by means of which the sap was sucked up first, the indicator following. Then both fluids were blown out of the pipette to a small drop at the end of the capillary. Thus the colour of the drop was quite easily to be noted (cf. table II).

From these data we may conclude that the pH of the sap is about 6.0, both for ovules with small and with larger embryos.

When making these estimations we were struck by the fact that the pH of the integuments appeared to be much lower, as their cells became clearly red in colour when in touch with methyl red as indicator.

TABLE II

The pH of the ovular sap

Indicator	Embryo length in the ovule	Colour	pH
Bromthymol blue	70 μ	yellow	< 6.2
„ „	700 μ	„	< 6.2
Bromcresol purple. . . .	100 μ	yellow-light green	< 6.2
„ „	250 μ	dirty brown	> 5.9
„ „	700 μ	„ „	> 5.9
Diaethyl red	100 μ	yellow-salmon pinky. . .	± 5.9
Methyl red.	50 μ	yellow	> 5.6
„ „	250 μ	„	> 5.6
„ „	700 μ	„	> 5.6
Benzo-azo-naphtyl-amine.	100 μ	yellow	> 4.8
Bromcresol green	100 μ	blue	> 4.4
„ „	300 μ	„	> 4.4

§ 7. *The osmotic value of the ovular sap*

In nature the embryo is embedded in the ovular sap and may be assumed to be in osmotic balance with it. When excised and transferred into a solution, differing in osmotic value, the embryo will become either longer or shorter in dependence. On this principle our method of determining the osmotic value of the sap was based.

Embryos were excised and measured immediately (within 20 seconds). This value was put equal to 100. A series of mannitol solutions of various concentrations had been prepared; after being transferred to one of these solutions, each embryo was measured several times in the course of an hour. The values, gathered by those measurements, were expressed as a percentage of the initial value. In this experiment we used only "torpedoes", these being most easily prepared and measured.

In figure 5 we resume the results in a diagram, each curve being the average of 4 observations. Though there exists a slight permeability for mannitol, this does not interfere with our conclusion, that 3/8 mol is nearest to the osmotic balance with the embryo; thus the osmotic value of the sap must be isotonic to 3/8 mol (8.4 atm).

The shrinkage of the embryo after a strong initial swelling in pure water indicates the lethal effect of the latter.

The same, somewhat modified method proved to be suitable for the determination of the type of permeability of the embryo too.

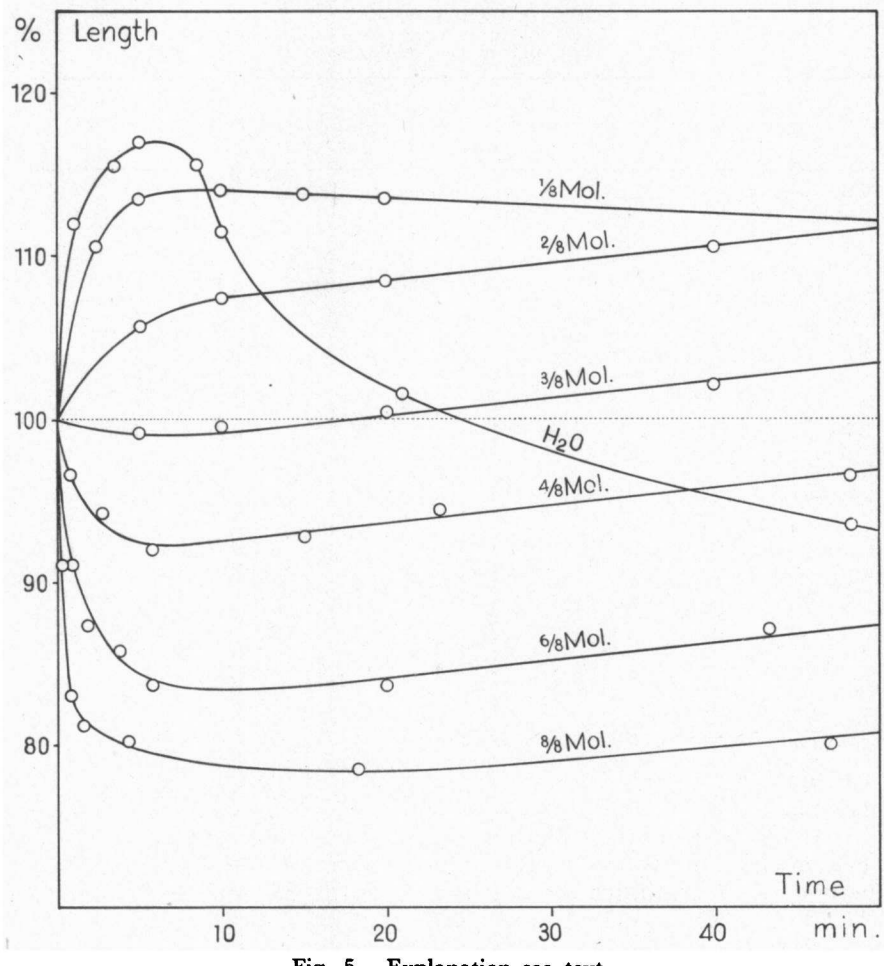

Fig. 5. Explanation see text.

After a 15 minutes stay in $\frac{1}{2}$ mol dextrose, "torpedoes" were transferred to an aequimolar solution of the substance in question. The procentual increase in length after 5 minutes was:

urea	9.6	(with s.e. ± 1.15)
methyl urea	16.1	(,, ,, ± 0.9)
glycerol	16.5	(,, ,, ± 1.5)

So, in respect of permeability, lipophily of the permeating substance seems to predominate over its molecular size.

CHAPTER III

THE PREGERMINAL EMBRYO CULTURE

A. MATERIAL AND METHODS

§ 1. *Material*

1. The plants. In most of the preliminary experiments (chapter III B) plants were used that had grown wild and were collected beforehand. Later *Capsella* plants were grown in the botanical garden of the laboratory, but these had not been selected genetically.
2. Culture cells.
   a. Small VAN TIEGHEM cells: on a slide a glass ring was glued by means of heat resistant resin. The height of the ring was 4 mm and its diameter 20 mm. The cell was covered by a square piece of a slide. Three such cells were kept in a petri dish; thus they were less subtle and more easily sterilized.
   b. Large culture cells, modification of the mentioned VAN TIEGHEM cell, consisting of a small petri dish (diam. 7.5 cm) covered by a square lantern slide (8.2 × 8.2 cm). The centre of this cover glass had been divided into 25 small squares (1 cm^2) by means of glass-pencil lines before sterilization. So one cell could hold 25 hanging culture drops.
   c. Large culture cells, modification of GAUTHERETS cell for the cultivation of a few plant cells (1942). The dimensions of the cell were equal to those of the cell sub *b*. The drops, however, were held between two parallel glass panels. The upper one, the cover glass, was divided in small squares as described sub *b* (cf. figure 6). The lower glass was round and smaller than the petri dish. The two glasses were kept apart by two small pieces of a slide. More details are found in figure 7. In our later experiments we always used this culture cell, because the "hanging drop" caused much trouble in measuring, as the embryos often stuck to the surface and then deviated from the horizontal site.
3. Braking pipette after ZEUTHEN (1943), in use for transferring embryos from one medium to another and for making the drops. The capillary (diam. 1 or 2 mm) was fastened in its holder (a glass tube, diam. 4 mm) by means of resin instead of paraffin, in order to make heat-sterilization possible. Cotton wool was plugged into the holder (figure 8) to prevent infection of the capillary from the operators mouth when in use. A number of pipettes (abt. 25) were kept together in a glass cylinder, in which they had been sterilized before the experiment.
4. Watch glasses, diam. 8 cm, covered by watch glasses, diam. 10 cm.

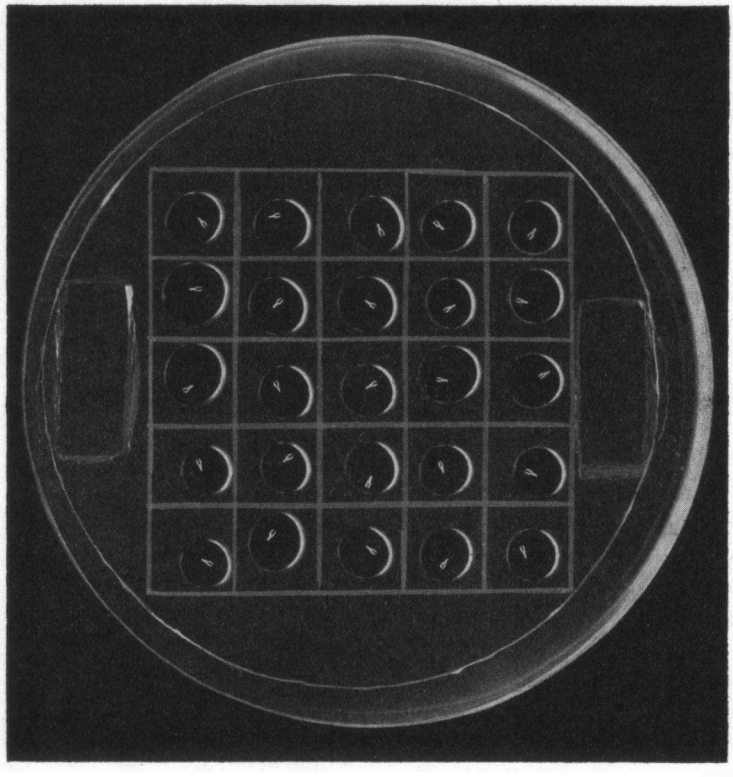

Fig. 6. Explanation see text.

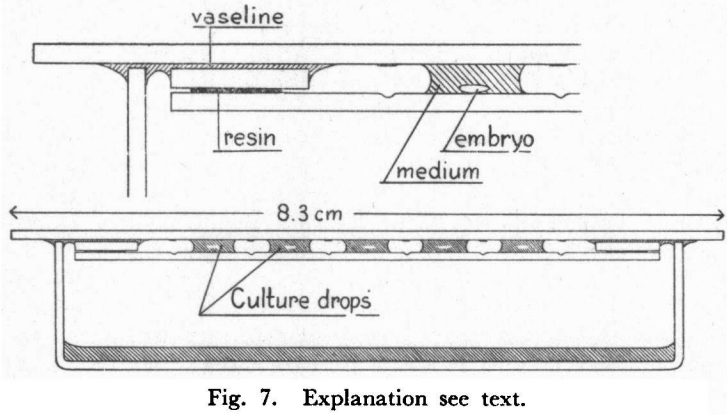

Fig. 7. Explanation see text.

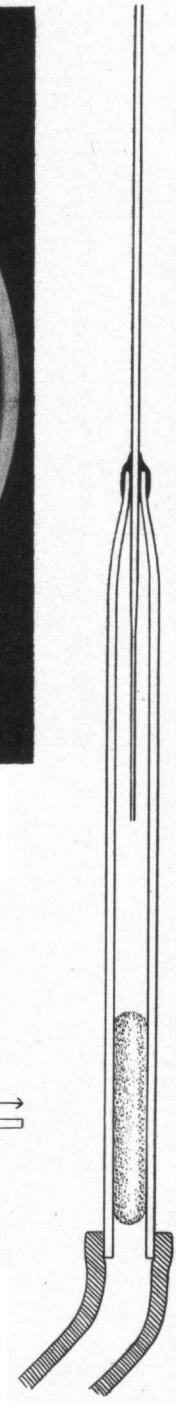

Fig. 8. Explanation see text.

5. Dissecting needles, straight and curved ones.
6. Cover glass tweezers after KÜHNE and CORNET.

§ 2. *Media*

As the finding of a suitable medium was one of the objects of our investigation, the medium was changed again and again, according to the demands of the experiments.

Our starting point was the salt solution of TUKEY (1933), supplied with a number of growth factors after VAN OVERBEEK c.s. (1942). As this solution contains some precipitates of salts that proved inconvenient, we always filtered the salt solution before adding the other components. Another improvement of this first medium was the replacement of KCl by 0.01 mol phosphate buffer after SØRENSEN, which gave the additional advantage, that the pH could be fixed at the desired value.

In all our later experiments we used a salt solution after OLSEN (1950) that did not show any precipitates and contained some trace elements. Also some more growth factors were administered viz. para-aminobenzoic acid, inositol and later lactoflavin, folic acid and biotin in addition.

Sugars were used in a much higher concentration than prescribed by VAN OVERBEEK c.s., viz. at least 80 g per l medium.

In a number of experiments amino acids were added according to their occurrence in the best known plant protein "edestin", as mentioned by TRISTRAM (1949).

Amino acids and growth factors were furnished by HOFFMANN–LA ROCHE, Basel.

The components of the media, and the compositions used, can be found in table III.

§ 3. *Sterilization*

Media in the beginning were sterilized by heating at 100° C during one hour in a KOCH sterilizer. Media containing amino acids, however, became unfavourably altered by this procedure; so we filtered them to free them from germs, in the earlier experiments through a SEITZ-filter, but later on always through glass filters (SCHOTT 11, G 5 auf 3).

Glassware was sterilized, wrapped up in paper, in a muffle at a temperature of 140° C during one hour.

Metal implements (dissecting needles and tweezers) were repeatedly sterilized in the flame when in use.

§ 4. *Excision of the embryos and starting the cultivation*

The first thing to be done was to arrange all implements and appliances in working order on the table. Amongst these was a glass of water in which the cut inflorescences of *Capsella*. A number of silicles, containing embryos in the desired stage, was selected and opened successively. Some practice was required for this operation, as the walls of the silicles had to be removed without severing the ovules from the placenta, in order to prevent their touching any

TABLE III
COMPONENTS OF THE MEDIUM
(* = omitted)

A) 1 Liter 0.01 mol phosphate buffer according to SØRENSEN.

B) Salts:	1) TUKEY (1933)	2) OLSEN (1950) modified
$CaSO_4$	185 mg	
$Ca_3(PO_4)_2$	185 „	
$MgSO_4.7H_2O$	185 „	101 mg
$Fe_3(PO_4)_2$	185 „	
*KCl	680 „	
KNO_3	135 „	149 mg
$Ca(NO_3)_2.4H_2O$	—	168 „
*KH_2PO_4	—	23 „
$MnSO_4.4H_2O$	—	0.4 „
H_3BO_3	—	0.4 „
$ZnSO_4.7H_2O$	—	0.2 „
$CuSO_4.5H_2O$	—	0.1 „
$(NH_4)_2MoO_4$	—	0.05 „
1 % Ferric citrate	—	5 cc

C) Sucrose: 1) 80 gm 2) 120 gm 3) 180 gm

D) Growth factors:	VAN OVERBEEK (1942)	extra
1) *Ascorbic acid	20 mg	—
*Glycine	3 „	—
Thiamin	0.15 „	—
Nicotinic acid	1 „	—
Pyridoxin	0.2 „	—
*Adenine	0.2 „	—
Ca pantothenate	0.2 „	—
Inositol	—	0.5 mg
p-Aminobenzoic acid	—	0.5 „
2) Riboflavin	—	0.1 mg
Folic acid	—	0.01 „
Biotin	—	0.0004 „

E) Amino acids: 1 gram in accordance with the composition of edestin (Tristram, 1949).

Alanine	4.31 %	Lysine	2.4 %
Valine	5.7 %	Aspartic acid	12.0 %
Leucine	4.7 %	Glutamic acid	20.7 %
Iso-leucine	7.5 %	Amide NH_3	2.15 %
Proline	4.25 %	Serine	6.3 %
Phenylalanine	5.45 %	Threonine	3.85 %
Half cystine	0.93 %	Tyrosine	4.34 %
Cysteine	0.50 %	Tryptophane	1.48 %
Arginine	16.7 %	Methionine	2.4 %
Histidine	2.9 %		

MEDIUM	I:	combination of A, B 1, C 1, D 1.
„	II:	„ „ A, B 1, C 1 or C 2.
„	III:	„ „ A, B 1, C 2, E.
„	IV:	„ „ A, B 2, C 2 or C 3, (D 1 + 2), E.

unsterile parts. This was achieved by tearing the walls down from the ribs by means of a pair of coverglass tweezers after KÜHNE. The remainder of the silicle, including the ovules, was then placed in a sterilized watch glass; then the ovules were pulled off from the placenta with sterilized needles under a dissecting microscope. Another watch glass with 5 ml sterile medium, covered by a larger watch glass, used to be ready for the receipt of the ovules. The excision of the embryos followed by cutting a gap in the top of the ovule and exerting a slight pressure on its base near the micropyle. To prevent infection during these manipulations, the operators head, except the eyes, was covered by a sterile cloth, as is usual in surgery.

About 20 embryos could be excised within ten minutes. By means of a braking pipette we then brought them to a second, and from this to a third covered watch glass with medium in order to clean them from adhering rests of endosperm and from eventual contaminations.

These operations having been repeated for a number of silicles, the culture was started. Though one person may be able to perform this part of the technique, sterility was served best by a rapid sequence of manipulations as achieved by a team of two persons. One of them made drops of about 10 mm^3 on the underside of the cover glass that had been clipped between coverglass tweezers after CORNET; the other person introduced the embryos into the drops with the braking pipette. Then the coverglass was laid over the cell, the brim of which had been lined with vaselin. Also a bottom drop of nutrient solution was inserted in the moist chamber in order to prevent evaporation and change of concentration of the hanging culture drop.

When using the large culture cells, we could omit the repetition of several manipulations, which meant a lot of time saved.

§ 5. *General culture conditions*

After sealing the culture cells, they were always incubated in the same dark room with a constant temperature of 25° C. Measuring was done in the same room, in orange light, as in use in experiments on growth (Orange filter; SCHOTT OG 2).

In some cases we used other temperatures or light during the cultivation. This will be mentioned in the sections in question.

§ 6. *Growth measurements*

Cultivated embryos are always straight. Their lengths were determined with an ocular micrometer. The measuring can be warranted correct to half a scale unit (the usual unit was 12.8 μ; small or very big embryos were measured with units being 8.8 μ and 24.3 μ resp.).

In most cases the growth is expressed as the procentual increase of the initial length (length of first measurement). By means of a t test (FISHER, 1946) we checked, whether the differences in growth between the various series were significant. Each experiment included a control series. In the tables the standard error is marked: $\pm$.

B. Preliminary experiments

The experiments, that will be reported under this heading, are short preambulary tests as to a number of general milieu requirements. They were carried out at the start of our investigation.

It soon became clear, that the nutrient solution we used (van Overbeek c.s. 1942) did not meet the needs of experiments of longer duration. The administration of amino acids proved to be a considerable improvement (section 12).

The composition of the numbered media can be found in table III.

§ 7. *The growth of embryos, varying in initial length*

As it is quite impossible to gather a sufficient number of exactly equal embryos for an experiment, we first had to find out whether embryos, varying in initial length, would yield the same relative growth.

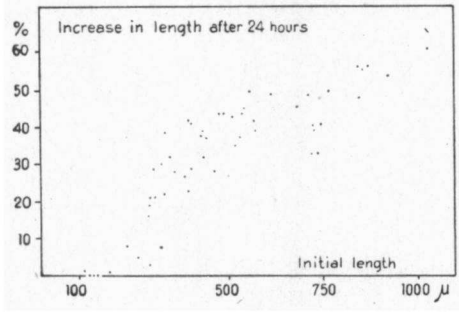

Fig. 9. Explanation see text.

To this end we started the culture of 60 embryos from 100—900 μ. Conditions: medium I; pH 6.6; darkness; temp. 25° C. Figure 9 shows the growth in 24 hours in dependence on the initial length, as a number of points, making a diagram. On the whole, there certainly appears to be a correlation between the growth rate and the initial length; on closer examination however the differences in the growth of embryos, initially between 500 μ and 750 μ seem to be caused by chance. The data on these embryos are given in table IV. From these a correlation coefficient r could be calculated. The result was: $r = -0.304$, with $0.3 > P > 0.2$, which proved that this variability is merely incidental. Therefore, we further took care not to surpass the limits of 500 μ and 750 μ.

Another fact, that can be learned from table IV is the strong decrease in growth percentage in the course of some days. The possibility that a change in the medium did cause this decrease, had to be examined. For that purpose we made two experiments.

In the first experiment 6 out of 13 cultivated embryos got their medium replaced by a fresh drop after 48 hours. As is shown in figure 10 the decrease in the growth in this case is essentially the same as that of the control embryos, the medium of which had not been renewed.

In the second experiment freshly excised embryos were inoculated

TABLE IV

The growth of embryos from the group of 500—750 μ during 4 days in medium I, pH 6.6; dark; temp. 25° C.

Embryo-number	Initial size in μ	Procentual increase in length				
		1st day	2nd day	3rd day	4th day	total 4 days
1	510	43	25	3	3	89
2	514	35	21	9	4	84
3	528	37	26	7	5	93
4	537	44	18	6	3	85
5	537	44	20	5	5	90
6	546	50	19	—	–	—
7	564	39	21	11	7	99
8	564	42	21	5	3	86
9	610	49	22	—	–	97
10	683	46	20	8	4	97
11	701	49	15	7	5	92
12	719	33	27	6	6	90
13	719	40	22	5	4	87
14	719	39	21	8	4	90
15	728	33	16	11	4	77
16	742	41	24	8	8	105
Mean:		41.5	21.1	7	4.6	90.7
Standard error		1.35				1.7
Standard deviation: . . .		5.4				6.3

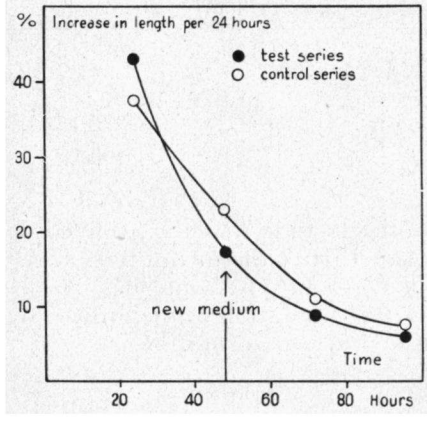

Fig. 10. Explanation see text.

into the drops, in which other embryos (series 1) had already been cultivated for 4 days. These embryos grew only 8 % during the last day; yet the new embryos (series 2), started growing in the same drops as if the medium had been fresh (cf. table V).

The results of these experiments justify the conclusion, that the growth of cultivated embryos was limited, if not yet on the first day, at least during the next days, by the shortage of one or more nutritional factors, lacking in the medium, and gradually getting exhausted in the store of the embryo.

TABLE V

The growth of two series of embryos; series 2 has grown in drops, in which the embryos of series 1 had been cultivated for 4 days, the growth rate of which had decreased to 8 %. Medium I with 120 gm sucrose / liter; pH 6.6; darkness; temp. 25° C.

Series	Number of embryos	Mean value of initial length	% Growth after 24 hours
1	14	628 μ	43.5
2	10	595 μ	44.7

§ 8. *Influence of the pH*

In a series of experiments the influence of the pH was also tested. The nutrient used was medium I; the pH was: 4, 5.4, 6.6, and 7.5. Its value was determined immediately after inoculation on the remainder of the medium with a quinhydrone electrode.

Table VI shows the results for embryos with an initial length between 500 μ and 750 μ. From the data of the first day it is clear that from pH 6 down the embryos do not thrive very well; a value below

TABLE VI

The growth of embryos from the group of 500—750 μ at different pH. Medium I, darkness, temp. 25° C.

pH	Number of embryos	Mean value of initial length	% Growth after 24 hours	% Growth after 96 hours
4*	16	590 μ	23 $\pm$ 2.7	98
5.4	15	597 μ	35.3 $\pm$ 1.7	93
6.6	16	620 μ	41.5 $\pm$ 1.35	91 $\pm$ 1.7
7.5	11	570 μ	43 $\pm$ 1.3	90

* 20 % of the embryos died.

pH 5 appears to be even unfavourable. However, when considering the total growth in four days, we must conclude that the growth rate decreased in all cases independent of the pH of the medium from the first day on, but so as to yield a total growth of about 90—100 %. This again is in favour of our view on the limitation of the growth by nutritional factors, mentioned in the previous section.

§ 9. *Influence of the temperature*

A great number of embryos was cultivated for the temperature test. They were distributed over five incubators with temperatures of resp. 21°, 25°, 30°, 35° and 40° C. The nutrient was medium I; pH 5.8. The results show the optimum curve of figure 11.

Again the possibility that the growth rate is mastered by general —non nutritional—milieu factors during the first 24 hours, is confirmed. In previous sections we stated, that this is not the case when considering the total growth in 96 hrs. The only way to increase this total must be the addition of nutritional factors unknown as yet. So we may have found a criterion for the identification of these factors.

§ 10. *Influence of light*

Freshly excised embryos are green when over 250 μ, and not yet mature. Cultivation without light causes the green colour to disappear; the embryos become white within the course of some days.

The main difficulty of cultivation in light was the necessity of maintaining the same constant temperature for the lighted and for

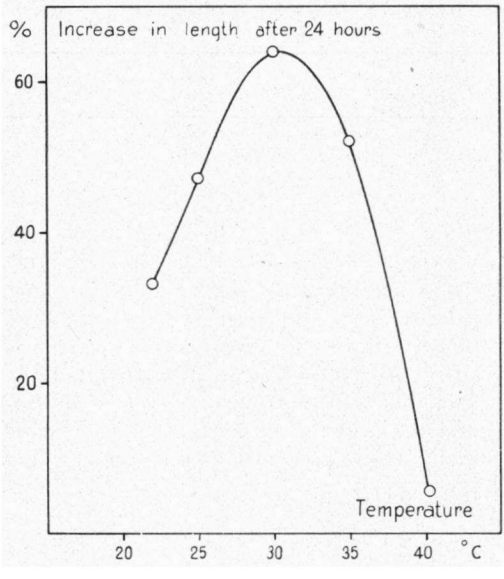

Fig. 11. Explanation see text.

the control (dark) series. So we put the control series in a lightproof zinc box on top of which the light-series was placed. Over this a lamp was installed, the infra red of which being mostly absorbed by a water filter. The whole set was placed in a room with constant temperature.

In our first experiment we used a high-pressure-mercury lamp (Philips Philora HP 75 W) of a light-intensity of $\pm$ 500 Lux near the culture cells. Table VII shows the results. Though light does not seem to have any influence, we continued the cultivation in the dark in all experiments to follow.

At the end of our investigations the experiment was repeated. We then knew that the growth is far better in a medium with glutamine

TABLE VII

The growth of embryos in light and darkness. Medium I; pH 6.7; temp. 25° C.

	Number of embryos	Mean value of initial length	% Growth after 48 hours
light. . .	17	504 μ	55 $\pm$ 2.4
dark. . .	18	491 μ	54.3 $\pm$ 2.9

added to it (Chapter III C). In this case there appeared to be a slight inhibiting action of light, but only after 4 days its significance could be shown (cf. table VIII). Tropistic phenomena were not noticed.

The light source was a Philips T.L. tube; the intensity near the culture cells was ± 450 Lux.

TABLE VIII

The growth of embryos in light and darkness. Medium IV with 120 gr. sucrose and 760 mgr. glutamin per liter. pH 6.1; temp. 25° C.

	Number of embryos	Mean value of init. length	% Growth 24 hours	% Growth 48 hours	% Growth 96 hours
light. . .	11	446 μ	82.9 ± 1.3	165 ± 3.4	297 ± 8
dark. . .	15	428 μ	87.5 ± 1.8	175 ± 2.75	341 ± 5.6

§ 11. *The influence of various sources of carbon*

Among our first preambulary experiments was a test as to the most favourable source of carbon. In a number of one-day-cultures we tried several substances, mainly sugars, viz.: sucrose, maltose,dextrose, dextrose + fructose, raffinose, lactose, mannitol and glycerol. Isotonic concentrations were used in order to get comparable results. We feared that errors might be caused by heat sterilization of the media; so we did not sterilize all of them, and moreover we added some unsterilized control series. Sterilization proved to have no effect (cf. table IX).

TABLE IX

The growth of embryos in medium II, with 8 % sucrose, and aequimolar substitutes; pH 6.6 or 6.7; darkness; temp. 25° C.

C-source	Sterilised				Not sterilised		
	gr./L	Number of embr.	Mean value of init. length	% Growth 24 hrs.	Number of embr.	Mean value of init. length	% Growth 24 hrs.
sucrose	80	12	466 μ	48	9	566 μ	48 ± 1.6
maltose	80				8	564 μ	40 ± 2.5
dextrose	45.8	10	460 μ	30			
dextrose	45.8				8	571 μ	29 ± 1.4
fructose	44.2						
raffinose	139				12	550 μ	17
lactose	80	9	449 μ	6	9	550 μ	7
mannitol	42.6	6	480 μ	11.5			
glycerol	21.5	10	550 μ				

Sucrose turned out to be the most favourable carbon source for growth, though it is not certain that maltose does not match it; the *t* test gave for the difference: P = 2 %.

On the other hand it is clear that dextrose is less suitable. No better results were obtained by mixing equal parts of isotonic solutions of dextrose and fructose. DORMER and STREET (1949), who cultivated

tomato roots, met with the same phenomena and demonstrated, that they could not be accounted for by possible impurity of the sugars.

Later, after glutamic acid had turned out to be a rather good source of nitrogen (Chapter III C), we made a closer examination of the difference between sucrose and dextrose by means of concentration series of both sugars (table X). The higher concentrations of both sugars did not yield any difference in this more suitable medium, but in lower concentrations sucrose still appeared to be a better carbon source.

TABLE X

The growth of embryos in media with different sugar-concentrations, in the presence of glutamic acid. Medium II, with 1.54 gr/l glutamic acid; pH 5.8; darkness; temp. 25° C

C-source	Number of embryos in each series	% growth after 24 hours				
		0.1 mol	0.2 mol	0.3 mol	0.4 mol	0.5 mol
sucrose	16	60 ± 1.33	71 ± 2.0	64 ± 1.27	67 ± 1.96	
dextrose	18		46 ± 1.41	64 ± 1.15	64 ± 0.97	57 ± 1.85

§ 12. *The limiting nutritional factors*

When the embryos are mature their cells are crowded with aleuron grains that give evidence of an intensive protein synthesis during the development. The materials for this synthesis are quite probably obtained as amino acids from the ovular sap, in which we could show their presence (cf. p. 172.).

In all preambulary experiments the medium contained nitrate as the sole nitrogen source; so the most plausible inference was that amino acids would be the growth limiting factor.

Our first check of this hypothesis was the administration of casein hydrolysate, added to medium II in a series of concentrations. As we explained in section 9, the total growth in four days should be the criterion. The results confirm our view (cf. table XI). A series with

TABLE XI

The growth of embryos (500—750 μ) on medium II with different concentrations of hydrolysate of casein; pH 6.7; darkness; temp. 25° C.

Added to 100 ml medium		Number of embr.	% Growth after 48 hrs.	% Growth after 96 hrs.
Control.		15	51.8 ± 1.1	71.8 ± 1.8
Casein	100 mg	15	52.7 ± 2.3	
	0.089 ml	15	79.6 ± 4.7	
	0.196 ml	14	92.3 ± 3.3	
± 20 % solution of hydrolysate of casein	0.382 ml	14	109.4 ± 4.4	174.4 ± 5
	0.734 ml	8	116.0 ± 3.9	
	1.344 ml	13	99.5 ± 5.6	145.5 ± 7.9

non-hydrolysated casein did not prevail over the blank control series.

Sterilization of the media containing casein hydrolysate was done by SEITZ filtration, as autoclaving of such media proved inadequate; precipitates appeared and an inhibiting effect resulted.

A more profound analysis of various sources of nitrogen is given in the following pages.

C. The analysis of the source of nitrogen

§ 13. *Administration of the complete mixture of amino acids*

After detecting the favourable effect of casein hydrolysate, we obviously had to continue our research by testing the effect of pure amino acids, a complete set of them in the first place. As to the mutual proportion of the amino acids in the mixture, it seemed the best thing to imitate the composition of globulins, present in the mature seeds. Of most plant globulins, however, the composition is not exactly known; so we had to content ourselves with the data about edestin, this being the only well-known plant globulin. In table III its composition is given. A striking feature of it is the high percentage of glutamic acid, aspartic acid and arginine. There are indications that many plant globulins show the same characteristics; on the other hand it is known that the globulins of the various plant families differ to a certain degree (Lugg, 1949).

The amino acids tyrosine, cysteine and cystine are hard to solve. Therefore they weer solved apart from the others in a little drop of concentrated hydrochloric acid. Sterilization of the media from now on was always done by filtration through glass filters (Jena, G5 auf 3).

Figure 12 gives the growth of embryos, "torpedoes", initially between 500 μ and 750 μ long, in dependence on the concentration of the amino acids. The measure of the latter is expressed in mg per 100 ml; the composition of the mixture in all cases is that of edestin. The graph shows, that the amino acids limit the growth rate in the lower concentrations. The concentration required for unlimited growth appears to increase with the duration of the experiment. A culture period of four days requires about 50 mg per 100 ml.

The embryos show a similar dependence on amino acids, increasing with increasing culture time, as stated for the unknown nutritional factors in the experiments without amino acids.

Henceforth media always got either 106 mg amino acids (complying with the composition of edestin, cf. table III) per 100 ml or other nitrogen containing substances. In the latter case we took care to maintain nitrogen aequivalence (106 mg amino acids contain 14.63 mg N).

Our first experiment in the spring of 1951 served to find out whether nitrogen, supplied as an organic ammonium-compound viz. ammonium lactate, could match the effect of amino acids. The growth proved to be abnormally high, both in the control and in the experimental series, probably because of better storage in the embryos early in the season, but the difference between the two series left no doubt as to the issue of the experiment (cf. table XII). Ammonium lactate can by no means replace the amino acids.

TABLE XII

Ammonium lactate and the complete mixture of amino acids (edestin) as N-source. Medium IV (12 % sucr.); pH 5.75; darkness; temp. 25° C.

N-source	Number of embryos	Mean value of initial length	% Growth after 96 hours
Amino acids	15	abt. 600 μ	289 $\pm$ 10.5
NH_4-lactate	14	abt. 600 μ	174 $\pm$ 2.8

TABLE XIII

Glutamic acid and the complete mixture of amino acids (edestin) as N-source. Medium IV (12 % sucrose); pH 6.7; darkness; temp. 25° C.

N-source	Number of embryos	Mean value of initial length	% Growth after 96 hours
Amino acids.	26	abt. 600 μ	209 $\pm$ 3.3
Glutamic acid	36	abt. 600 μ	185 $\pm$ 4.6

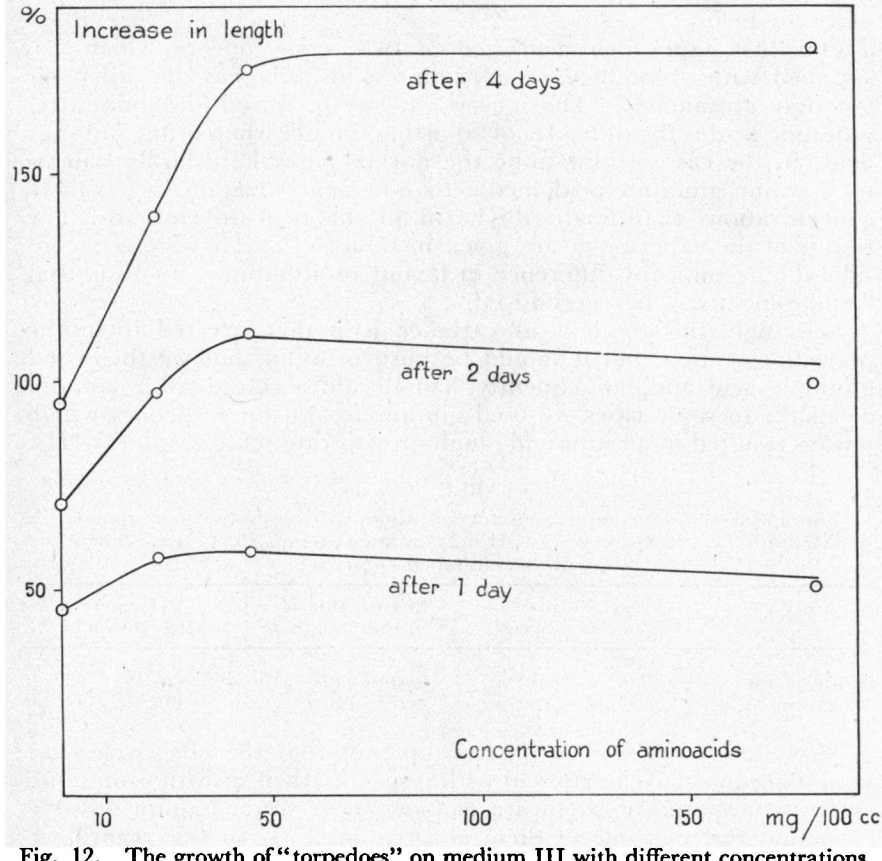

Fig. 12. The growth of "torpedoes" on medium III with different concentrations of amino acids (composition according to edestin); pH resp. 7.2, 6.94, 6.94, 6.94; darkness; temperature 25° C.

The next problem we wanted to solve was the possibility of replacing the complete set of amino acids by glutamic acid alone, which is supposed to take a key position and to play an important part in transaminations. The results of these experiments were hardly conclusive; a significant, though slight difference is shown in the case of table XIII, in which the glutamic acid series falls behind the amino acid series. As these results were not remarkable, we decided to continue the analysis with young heart-shaped embryos, in the hope that these would show a more pronounced heterotrophy in this respect.

§ 14. *The influence of various single amino acids in the culture of heart-shaped embryos*

For the cultivation of heart-shaped embryos the same conditions held as for that of torpedoes, except the sugar concentration that has to be increased up to 18 per cent (0,526 mol). After some experimentation we switched over to this concentration because some phenomena had indicated 12 per cent to be hypotonic in the case of heart-shaped embryos before.

Our first experiment consisted of two series, one of which was supplied with a complete set of amino acids, whereas the other one got only glutamine *. The reason why we preferred glutamine over glutamic acid is the difficulty of adjusting the pH when using glutamic acid; in the case of glutamine the normal phosphate buffer can be used, while glutamic acid media require neutralization by alkali in concentrations that might do harm to the delicate embryos. The results of the experiment are given in table XIV. They show a considerable, significant difference in favour of glutamine, an issue that could repeatedly be reproduced.

Seemingly this result is at variance with that referred to in the preceding section, but it should be born in mind that we then used glutamic acid and, consequently, had to add a considerable amount of alkali. In some cases we used glutamine also for torpedoes which always resulted in an unusually high growth rate (cf. e.g. table VIII).

TABLE XIV

Glutamine and the complete mixture of amino acids (edestin) as N-source. Medium IV (18 % sucrose); pH 6.2; darkness; temp. 25° C. Experiment with heart-shaped embryos

N-source	Number of embryos	Mean value of initial length	% Growth after 96 hours
Amino acids.	35	abt. 140 μ	225 $\pm$ 5.5
Glutamine.	42	abt. 140 μ	295 $\pm$ 7

After these experiments it was apparent that the effectiveness of various amino acids is different with respect to their growth promoting activity in the embryo culture of *Capsella*. It seemed quite possible even that one or some of them act as inhibitors. In this regard we

* We thank Dr F. van Walraven, scientific agent for the Netherlands of Hoffmann-La Roche & Co, Basel, for his kind help in procuring us glutamine.

especially had suspicion of tyrosine, because of its molecular structure, which holds the possibility of "auxin" activity, according to the theoretical considerations of VELDSTRA (1949). Indole-3-acetic acid had then already turned out to inhibit growth (cf. chapter III D). Our surmise was checked by cultivating embryos in a medium that contained, apart from glutamine, 10 mg tyrosine per 100 ml. In this medium growth was much lower indeed (cf. table XV). If trypto-

TABLE XV

Glutamine, glutamine + tyrosine and asparagine as N-source. Medium IV (18 % sucrose); pH 6.2; darkness; temp. 25° C.

N-source	Number of embryos	Mean value of initial length	% Growth after 96 hours
Glutamine.	25	abt. 170 μ	315 $\pm$ 11
Glutamine + tyrosine.	13	abt. 170 μ	167 $\pm$ 10.5
Asparagine	28	abt. 170 μ	58 $\pm$ 3.5

phane or phenylalanine, which are somewhat similar in structural features, were administered instead of tyrosine no significant difference with the control series (glutamine alone) could be stated.

Another point of interest was the question whether the beneficial influence of glutamine is exceptional for this very amino acid, a view, endorsed by recognized biochemical arguments. For a highly important key position as a link between carbohydrate- and protein-metabolism is ascribed to glutamic acid. A similar function, however, is attributed to aspartic acid as well and to some degree also to alanine (BRAUNSTEIN, 1947).

In conformity with this view alanine proved to cause a considerable growth, though its mean value was much lower than that of the glutamine series (table XVI). The homologous compound glycine, however, could by no means satisfy as a source of nitrogen: one third

TABLE XVI

Glutamine, complete amino acid mixture and alanine as N-source. Medium IV (18 % sucrose); pH 6.2; darkness; temp. 25° C.

N-source	Number of embryos	Mean value of initial length	% Growth after 96 hours
Glutamine.	17	abt. 170 μ	298 $\pm$ 9.0
Amino acids.	27	abt. 170 μ	237 $\pm$ 5.95
Alanine.	15	abt. 170 μ	209 $\pm$ 3.0

of the embryos died in it within four days, while the rest did not grow more than 40 per cent in total.

When administering asparagine, remarkable results were obtained. The growth rate in a medium with this amino acid was surprisingly low (cf. table XV). Yet the embryos did not die, as they did in a glycine medium. This effect was obtained with crystalline asparagine

of different origin. So the unexpected phenomenon cannot be ascribed to impurities of the preparation. Apparently asparagine, unlike glutamine, cannot play a crucial part in the protein synthesis of *Capsella* embryos. A different pattern of metabolism came into action as became clear already after 24 hours of cultivation when comparing embryos in the glutamine and the asparagine series: even the smallest embryos (150 μ) of the asparagine series had formed much starch in their cells as early as that, whereas, in the glutamine series starch could scarcely be noticed. In the discussion (p. 195) we will go deeper into the matter.

D. The influence of ergones

§ 15. *Bios factors*

To check the influence of growth factors we carried out a number of experiments, each comprising a control series and some series in which several bios factors were added, the richest medium containing nine of the best known factors (cf. table III). In all cases the results were negative, in the early experiments made before the necessity of amino acids in the medium had been shown, as well as in the later ones, when an adequate source of nitrogen was present. Tables XVII and XVIII may suffice in illustrating the facts; the first comprises data about "torpedoes", the latter about heart-shaped embryos.

TABLE XVII

Bios-factors and purine-derivates added to Medium IV (12 % sucrose); pH 6.7; darkness; temp. 25° C.

Ergones	Number of embryos	Mean value of initial length	% Growth after 96 hours
——	26	abt. 600 μ	209 $\pm$ 3.3
Bios-factors	17	abt. 600 μ	208
Purine-derivates . . .	22	abt. 600 μ	212

These negative results are not at variance with the findings of other authors as will be shown in the discussion (p. 195).

Many experiments were performed with media, lacking growth factors; yet such cultures yielded viable embryos.

TABLE XVIII

Bios-factors and adenosine-3-phosporic acid to Medium IV (with glutamine as N-source and 18 % sucr.) pH 6.2; darkness; temp. 25° C.

Ergones	Number of embryos	Mean value of initial length	% Growth after 96 hours
——	21	175 μ	325 $\pm$ 7.1
Bios-factors	13	190 μ	316 $\pm$ 12.5
Adenosine-3-phosphoric acid . .	25	170 μ	326 $\pm$ 5.8

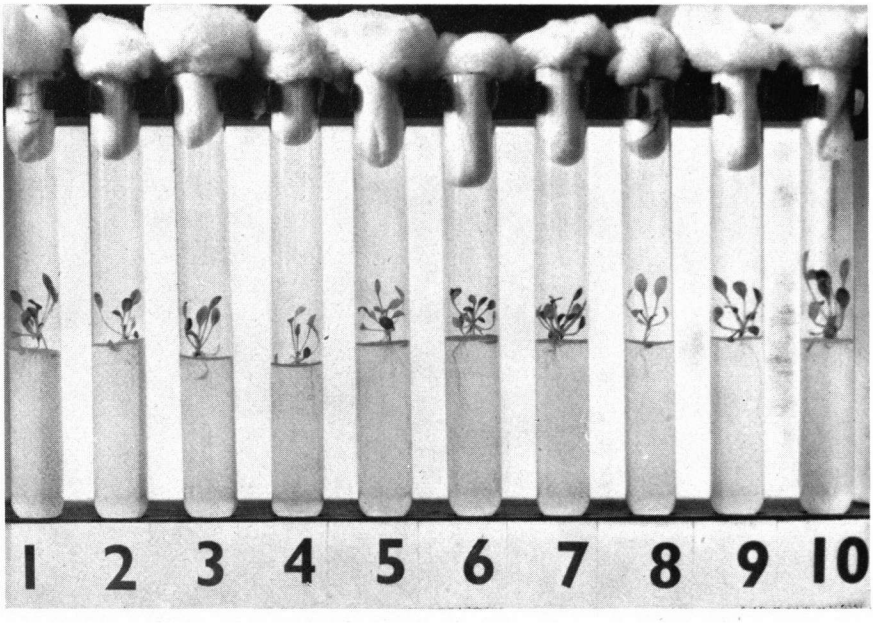

Fig. 13. Explanation see text.

Fig. 14. Explanation see text.

§ 16. *Purine derivatives*

The tables XVII and XVIII give also the results of some series of embryo cultures, the media of which contained purine derivatives. When preparing the medium, our nucleic acid, however, did not solve completely. The series of table XVII had nucleic acid and adenosine-3-phosphoric acid added, each of them in a concentration of 10 p.p.m.; that of table XVIII only adenosine-3-phosphoric acid in the same concentration. Also these experiments yielded negative results, which will be discussed below (p. 196).

§ 17. *Hetero-auxin*

In connection with the multifarious activities, accredited to indole-3-acetic acid, we decided to try this substance in our embryo cultures too. Two experiments were made, from which we could gather the data given in table XIX. In the last column the growth rates are expressed as percentages of the controls. An influence is obvious and becomes significant in the concentrations 10, 1 and 0.001 p.p.m. The

TABLE XIX

Two experiments on the influence of indole acetic acid on the procentual growth of "torpedoes" in 24 hours. Medium IV (12 % sucrose; exp. A with glutamine as N-source, exp. B with complete mixture of amino acids); pH 6.2; darkness; temp. 25° C.

Conc. I.A.A. p.p.m.	Number of embr.		% Growth		Difference of means and its s.e.	P in %	Growth in % of control
	A	B	A	B			
0	28	15	68.6 ± 1.23	57.9 ± 1.73			100
10	27		62.2 ± 1.2		4.2 ± 1.7	1—2	90.7
1		24		53.2 ± 1.3	4.7 ± 2.15	2—5	91.9
0.1	22		68.3 ± 1.95		0.3 ± 2.5		99.6
0.01		20		60.1 ± 0.86	2.2 ± 1.95		103.8
0.001	28		72.6 ± 1.4		4.0 ± 1.85	2—5	107
0.0001		12		62.6 ± 2.2	4.7 ± 2.8		108.1

effect is inhibitory in concentrations over 0.1 p.p.m. and promoting in the lower concentrations. The influence, however, is rather weak, too weak to justify any conclusion for a longer duration of the experiment, as the variation became too large after some days. In the discussion we will also consider these results (p. 196).

CHAPTER IV

THE POSTGERMINAL EMBRYO CULTURE

Actually a successful postgerminal culture is the best proof of the viability of the cultivated embryos. Therefore we also gave some

attention to this kind of culture, though our experiments did not cover the field systematically.

In the culture cells the embryos never spontaneously started germination; so "künstliche Frühgeburt" was out of the question (cf. page 160 and 162) and the growth we measured certainly was not cell-elongation but embryonic growth. The most likely cause of this is the high osmotic value of the used media, viz. isotonic with the embryo environment in the ovule.

The nutrient for the postgerminal culture consisted of a salt solution with 0.8 % agar and 2 % sucrose. The embryos, grown to maturity, became transferred with big braking pipettes to the tubes, containing the nutrient. Figure 13 shows a number of young *Capsella* "seedlings": they were 200 μ when excised; the duration of the pregerminal culture in darkness was one week, after which postgerminal culture was started (photograph four weeks later); from this stage the young plants can be set out in earth. Figure 14 shows some plants, grown from cultured embryos, which accomplished their life-cycle in vitro.

Some interesting data about the germination of cultivated embryos were collected by P. VAN OORDT, B.Sc.. In a series of tubes, containing resp. 0 %, 3 %, 6 % and 12 % sucrose mature embryos were inoculated. No germination followed on the 12 % nutrient; on 3 % and 6 % sucrose growth after germination was about equal but on 0 % much less.

For a closer examination of the germinating embryos, they were laid out on moist filterpaper. Germination turned out to make its first appearance as an elongation of the cells near the basal end of the hypocotyl; next the rootlet developed and root-hairs grew out from the elongating cells. Soon this centre of elongation spread in both directions, causing participation of the entire hypocotyl after some time; in the mean time the cotyledons became green in colour. The cells of the hypocotyl elongated during this process from 20 μ to 120 μ. Figure 15 gives some drawings by P. VAN OORDT of germination stages. It was noticed that, coinciding with cell-elongation, for the first time tropistic phenomena came through.

In our opinion these data are noteworthy because they focuss the attention on the essential difference that exists between pre- and postgerminal growth; accordingly the conditions of cultivation in both stages ought to differ, as we postulated when starting this investigation. A further evidence for this view is offered by microscopical observation of immature embryos; excised torpedoes exhibit a precocious germination after transfer to a medium with an inadequately low osmotic value. This result forms a striking conformity with that of ZIEBUR and BRINK (1951) on *Hordeum*.

DISCUSSION

1. There are two different criteria for judging in how far the embryo culture has succeeded. One of them lies in the success of

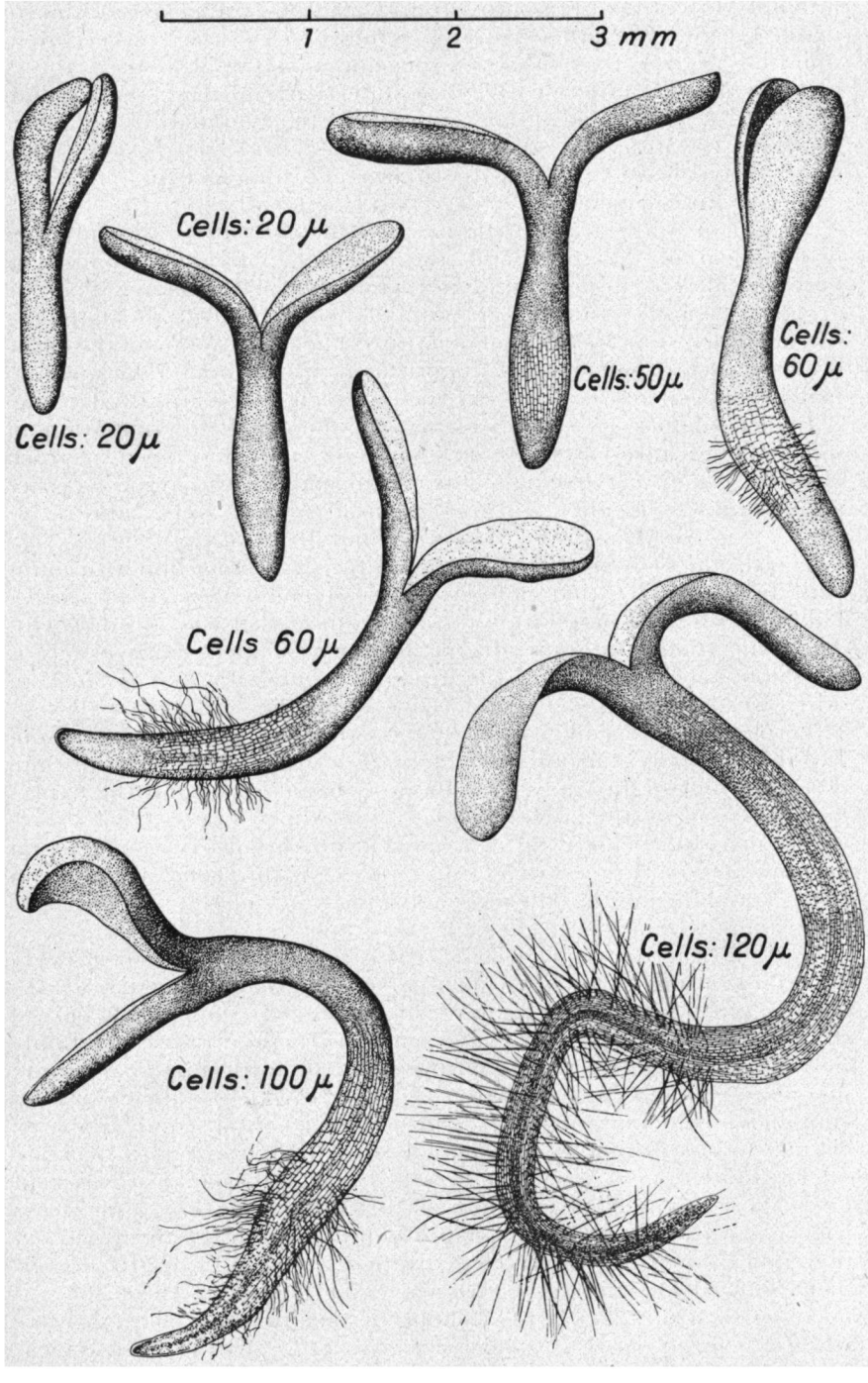

Fig. 15. Explanation see text.

attempts to cultivate embryos from a stage as young as possible to viable germs. The other criterion is how far one has succeeded in imitating *in vitro* the embryonic development in the ovule.

As to the first criterion we must state that embryos, smaller than abt. 100 μ, could not yet be cultivated; the globular stages never survived a transfer to *in vitro* conditions. The heart-shaped 100 μ stage proved to be very delicate: a large percentage of the inoculated embryos always died.

So we got stuck at about the moment the cotyledon-differentiation first appeared. At this point the original polyradiate symmetry becomes altered into a biradiate symmetry. This change may be accounted for by assuming an induction of it from gradients in the surroundings, that are biradial of nature. The cross-section of the ovule is elliptic, which offers some support for this hypothesis. The medium being homogeneous *in vitro*, here such gradients are naturally lacking.

In accordance with this we met an abnormal differentiation in some, though rare cases in young embryos, that developed a crown of more, e.g. 6, cotyledons at the apical end. In this respect earlier photographs of cultivated *Datura*-embryos are very illustrative (fig. 2—5, VAN OVERBEEK, CONKLIN and BLAKESLEE, 1942).

Yet the lack of gradients in the culture-medium cannot account entirely for the fact, that globular embryos could not be grown *in vitro*. Undoubtedly the composition of the medium itself was still deficient. The same holds to some degree for heart-shaped embryos, as is taught by a comparison of the growth *in vitro* with that *in ovulo*, in other words, by a judgement according to the second criterion.

Torpedo-shaped embryos, however, can stand such a comparison. In table VIII we find for these embryos a growth-rate of more than 80 % during the first day; this by no means lags behind the expectations, based on the growth *in ovulo*, as shown in figure 4. Conformity in metabolism may be concluded from the observation that fat accumulates *in vitro* as well as *in ovulo*. The presence of starch in both cases indicates that the interacting mechanism of fat-carbohydrate conversion is intact.

In table XX the data of a heart-shaped embryo, measured every 48 hours during 6 days, are compared with the expected growth: even in this favourable example growth lagged considerably behind that in the ovule. For such young embryos excision in itself certainly means a violent interference and the synthetic medium a change into new chemical and physico-chemical properties of the milieu. Another complication in imitating the embryo-milieu is, that quite probably changes occur during the development in the ovule, e.g. a decrease in the osmotic value. In the case of table XX the embryo was kept for 6 days in the same medium drop, containing 18 % sucrose, a concentration that is sure to be supra-optimal from the third day on, the embryo then having developed into a torpedo. A control experiment showed for embryos with an average initial length of 260 μ a mean growth of 42.5 % in 24 hours in media with 18 % sucrose whereas a mean of 63.5 % was reached in 12 % sucrose. So it is clear

that the culture of small embryos possibly would be markedly improved by a repeated transfer to media that change gradually.

In our cultures embryos did not show any cell-elongation; they kept growing as embryos do. Our statement in chapter IV may substantiate that the osmotic value of the milieu takes a major part in determining the type of growth that occurs.

TABLE XX

The growth *in vitro* of a young heart-shaped embryo during 6 days compared with the expected growth *in ovulo*. Medium IV with glutamine as N-source and 18 % sucrose; pH 6.2; darkness; temp. 25° C.

	Time in hours			
	0	48	96	144
Length *in vitro* in μ . . .	104 →	260 →	488 →	739
Procentual increase . . .		150	88	52
Length *in ovulo* in μ . . .		490 →	950 →	1360
Procentual increase . . .		364	265	179
Difference μ		230	462	521
Difference %		214	177	127

2. Our statement that glutamine surpasses the complete mixture of amino acids, provided nitrogen aequivalence has been guarded, is seemingly at variance with the paper of SANDERS and BURKHOLDER (1948) on the need of amino acids of *Datura* embryos (cf. page 164). Unfortunately the authors did not test glutamine, so that we cannot certify the reality of this difference.

Our results suggest that some amino acids, e.g. tyrosine and glycine, as administered to the embryo in our culture medium, are not suitable for *Capsella* embryos. Surely several amino acids in the complete mixture depreciate its potential nutritional value. In our experiments glutamine as the only source of nitrogen gave the best results. Yet the possibility exists that, as compared to glutamine alone, a still better growth might be secured by adding some other amino acids to a medium as the main nitrogen-source. The deficiency of our knowledge about compounds containing NH_2-groups, that certainly have a function in plant-tissues, is obvious when considering the review by STEWARD and THOMPSON (1950): in paper chromatograms of alcohol-soluble plant substances still 20 unidentified spots occurred that were stained by ninhydrin.

According to our experience, however, we sustain that protein synthesis, even in the youngest cultivated embryos, can proceed unlimitedly from glutamine as the only nitrogen-source. In this connection it is rather amazing to state, that the homologous compound asparagine appears to be of little use: very low growth values resulted when using media containing asparagine as the sole source of nitrogen (cf. table XV).

Apparently glutamine is unchallenged in taking a key-position in the nitrogen-metabolism. The prevalence of one amide over the other

is a well-known phenomenon with seedlings. Whether asparagine or glutamine plays the predominant role seems to vary with the species. *Lupinus* has become a textbook example, in which the etiolated seedlings accumulate asparagine. In *Pisum* this very amide could be found in the pods during ripening and quite likely figures in a similar way in protein synthesis too (after a review by ROBINSON, 1929). STREET (1949) mentions in a review glutamine as the prevailing amide in the seedlings of *Brassicaceae* (*Cruciferae*), which agrees with our observations on the pregerminal phase.

Why in seedlings either of the two amides predominates, is still an unsolved problem. SCHWAB (1936) raised the hypothesis that the presence of a specific amidase would discriminate in this respect; after this view the plants could be divided in two groups: asparaginase plants and glutaminase plants. Apart from this generalization, which has been criticized already by STREET (1949), we move against this view our experience with *Capsella* embryos. The vivid starch formation, noticed with asparagine as a nitrogen-source, indicates a "short-circuit" with the carbohydrate metabolism, presumably via the KREBS-cycle. In this case an explanation of the prevalence of glutamine should rather contain a consideration of a specificity of the trans-amination systems that interact with the keto-acids of the KREBS-cycle.

3. As mentioned above the growth of young embryos, cultivated in a medium containing salts, sucrose and glutamine, lags behind the growth *in ovulo*. Yet no improvement could be acquired by the addition of growth factors, viz. a large number of bios factors and some purine-derivatives, all of them substances that are generally considered to be important factors in metabolism. So we agree with the negative results of SANDERS (1950), who also tried a number of these factors on *Datura* embryos. In the experiments of RAPPAPORT, SATINA and BLAKESLEE (1950) nucleic acid even proved inhibitory.

These results seem to be at variance with the issue of the *Pisum* research, where a great number of the factors in question has been found to be growth-promoting in the postgerminal culture. The embryos, however, used in these experiments, were highly differentiated, decotylated germs; removing of the cotyledons may quite well eliminate an important nutritional system, thus causing a heterotrophy on bios factors.

In the pregerminal embryo culture the investigators met with a number of obstacles that were due to a deficiency of the medium. Attempting to surmount these difficulties, they took refuge in using natural substrata, such as coconut-milk. According to STEWARD and THOMPSON (1950) in this sap a "protein stimulating substance" should be present besides the nitrogen containing nutritional factors; this argument is based on the investigation of CAPLIN and STEWARD (1948) on the culture of carrot root phloëm, but the facts mentioned by these authors do not justify this hypothesis. VAN OVERBEEK, SIU and HAAGEN SMIT (1944) cannot persuade us either of the ergone character of their "embryo factor" in coconut-milk. The mere statement of a

growth promoting activity in a dilution 1 : 19000 (5 mg/100 ml) is no sound argument, because it does not inform us on any optimal concentration. In reality their embryo factor could be an amino acid as well; we proved clearly that amino acids act favourably in a concentration of 20 mg/100 ml, even though we did not use glutamine in that experiment (cf. fig. 12).

At the "Cold Spring Harbor Symposion" (1942) VAN OVERBEEK suggested "auxin" to have an important regulatory function in embryonic development. SANDERS (1950) could not notice any influence of indole-3-acetic acid on embryo growth. Our experiments, however, yielded a real, though small influence. The results of a concentration series ranging from 0.001 to 10 p.p.m. show an effect somewhat similar to that on root growth, viz. stimulating in very low concentrations and inhibiting in higher ones. These effects, however, are far too small to implicate an important direct regulatory influence of hetero-auxin in embryogenesis.

SUMMARY

1. The distinction between pregerminal and postgerminal embryo culture has been defined and emphasized. The literature on the former has been surveyed from a physiological point of view.

2. Chapter II gives an account of the development of the embryo of *Capsella in ovulo*. The embryogenesis was divided in stages. The formula of the sigmoid growth curve could be determined by means of a graphical treatment of the data. Chemical and physico-chemical properties of the embryo and the endosperm were studied; the pH of the latter is 6.0 and the osmotic value is isotonic with 3/8 mol mannitol (8.4 atm). The osmotic value refers to ovules with "torpedoes."

3. Chapter III reports methods of and results with pregerminal embryo cultures.

a Culture cells were used for the cultivation of embryos, that were suspended in drops between two glasses so as to enable the observation of 25 embryos per vial. The growth was measured by a microscope with an ocular-micrometer.

b. In preliminary experiments on torpedoes (0.5 mm) the influence of some general conditions (pH, temperature and light) was examined.

c. Sucrose proved to be a superior source of carbon.

d. Casein-hydrolysate favoured the growth strongly.

e. A concentration series of a complete amino acid mixture verified that amino acids are limiting growth.

f. Ammonium lactate could not substitute amino acids as a food factor.

g. In cultures of heart-shaped embryos glutamine gave better growth than a nitrogen aequivalent, complete amino acid mixture. In

this respect glutamine proved to be exceptional and outstanding among the amino acids tried.

h. In contrast with glutamine, asparagine gave extremely low growth values, but in the embryos an abnormally rapid and strong starch production was observed.

i. Experiments on the influence of bios factors and purine derivatives gave negative results. Indole-3-acetic acid had a real, though small influence: it stimulated growth in a concentration of 0.001 p.p.m. and inhibited it in concentrations up from 1 p.p.m..

4. Chapter IV demonstrated the viability of cultivated embryos by means of postgerminal cultures. Germination appeared, when the osmotic value of the medium was lowered.

5. In the discussion the results of the cultures are confronted with the embryogenesis *in ovulo* and with the data of the literature.

I should like to express my appreciation to Prof. Dr V. J. KONINGSBERGER, Department of Botany of the State University, Utrecht, who suggested the problem, for his interest and his kind help in preparing this paper for the press. I should also like to thank Mrs. M. A. RIJVEN–SIMONS for her constant technical assistance and for translating the manuscript.

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