CHEMICAL STUDIES ON THE DEVELOPMENT OF PLANT EMBRYOS

Thesis by

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PREFACE

Although considerable advances have been made in plant embryogeny from the morphological standpoint, the knowledge of the physiological and the chemical factors involved in the development of the embryo is meager. The present work is a study of the conditions and the chemical substances, which are necessary for the development of plant embryos.

During the course of this investigation, the author has been given much help and encouragement. He has received valuable advice and many personal favors from Professor A.J. Haagen-Smit. Many of the ideas in this work have originated from Professor J. van Overbeek, who also has given much of his time and energy to the project. Helpful suggestions have been tendered by Professor F. Went, Professor J. Bonner, and Dr. Harriet Bonner. Messers. S. Gottlieb, W.D. Bonner Jr., P.R. Saunders, Dr. J. Kirchner and Dr. H.D. Michener have been very generous with their help during various phases of this project. Mrs. Gertrude Wilson assisted in the culturing of the thousands of embryos, which was necessary to arrive at the conclusions drawn in this thesis. To all of the above persons the author wishes to express his sincere gratitude and appreciation. The author also wishes to express his indebtedness to the American Cyanamid Co. and to the American Philosophical Society for grants received during the course of this investigation.

INTRODUCTION

Investigations on the culture of plant embryos *in vitro* have been stimulated by the study of the nutrition of plant embryos, the question of lethal factors and incompatibility in plant breeding, the germination and the development of otherwise abortive seeds, and artificial parthenogenesis.

Of the many investigators who studied the nutrition of plant embryos over a decade ago (2, 13, 14, 16, 21, 22, 25, 36) relatively few undertook the culturing of immature embryos. The earliest of these was Hannig (23) in 1904. He excised immature embryos, which were only 1.2 mm. long, from the ovules of *Raphanus* and *Cochlearia* and grew them to maturity in a mixture of glucose, asparagine, leucine, glycocoll, tyrosine, mineral salts, and peptone digest. As a result of improvements in cultural techniques, smaller embryos were grown *in vitro* by later workers (26, 29, 42).

This culturing of immature embryos *in vitro* became a very useful technique for the propagation of otherwise abortive embryos. When *Linum austriacum* was crossed with *L. perenne*, for example, viable seeds were not obtained. However, Laibach (26) excised the embryos, which were about a fortnight old and 1.5 mm. long, and placed them on a cotton wadding watered by a 10 to 15 per cent sucrose solution in a test tube. After a week they were transferred to a moist blotting paper. Germination occurred in a few days and the embryos subsequently grew into normal mature plants, which were inter-fertile and capable of being pollinated by either

parent. Thousands of immature hybrid embryos of cherry, apricot, peach, and other horticultural plants have been cultured similarly *in vitro* and grown to maturity (26, 38, 40, 41, 42). As was the case with the earlier investigators Tukey (39) found that the "sterility" of some varieties of sweet cherry (*Prunus avium* L.) was due to nutritional and cultural conditions. He also suggested (40) that the abortion of the embryos might have been correlated with the earliness with which some varieties ripened their fruit. "Abundance" cherry, which ripened in early August, produced nearly 100 per cent viable seeds. "Black Tartar" cherry, which ripened in early July, gave only 70 to 85 per cent viable seeds, while the "Early Purple Guigne" cherry, which ripened in early June, produced only abortive seeds. Although Tukey was not able to grow cherry embryos which were in the early stages of development, he was able to culture those which were in the period of rapid growth prior to their collapse in the seed. From the standpoint of the plant breeder, then, the realization that the early-ripening varieties produced abortive seeds might have argued against the use of an early-ripening variety as a female parent in a cross. But through the culture of excised embryos *in vitro* it was possible to obtain offspring from crosses involving two parents with early-ripening characteristics. The newly-bred variety, if satisfactory, could be perpetuated by the usual vegetative methods of propagation.

All of the immature embryos which were cultured *in vitro* by the above mentioned workers were relatively large.

The failure of these workers to grow small embryos *in vitro* made prevalent the idea of a limit in the size of excised embryos which could be cultured *in vitro*. Dietrich (20), for example, asserted that embryos less than one third of their full size were incapable of development outside of the seed. LaRue, who was able to culture immature embryos of *Chrysanthemum Leucanthemum* and *Bryophyllum crenatum*, which were only 0.5 mm. in length, suggested (29) that "....it does appear to the writer that at a length of about 0.5 mm. we may have reached a new lower threshold which it will be difficult to pass."

LaRue's statement went unchallenged until van Overbeek, Conklin and Blakeslee (46, 47), who were interested in artificial parthenogenesis in plants, began their investigations on the culture of small *Datura* embryos. These workers divided the problem of artificial parthenogenesis into 2 steps: a) the inducement of the egg cell to divide and b) the development of the resulting proembryo into a viable plant. In their attempts to grow small immature *Datura* embryos and proembryos they discovered the following facts: a) Excised mature embryos could be grown in dim light on a medium of 0.8 to 1 per cent agar, 1 per cent dextrose, and Tukey mineral solution. Root, hypocotyl, and cotyledons developed, which resulted in normal seedlings. b) Larger immature embryos at the "torpedo" stage formed roots and hypocotyl but no cotyledons in the above medium. But they could be grown into normal plants with the addition of glycine, thiamine,

ascorbic acid, nicotinic acid, vitamin B_6, adenine, succinic acid, and pantothenic acid to the above medium. c) Embryos smaller than 0.5 mm. in length were not capable of growth even in the modified medium. With the addition of non-autoclaved coconut milk, however, embryos as small as 0.15 mm. in length could be grown to maturity.

It was at this point that the present investigation began.

EXPERIMENTAL SECTION

Chapter 1

Technique for culture of Datura embryos in vitro

The method devised by van Overbeek et al (47) was used in this investigation. Capsules were picked 12 to 15 days after pollination from Datura plants grown in greenhouses and in the field. They were brought to the dissecting room where the embryos were isolated and grown under aseptic conditions. The capsules were first soaked in 70 per cent ethyl alcohol for about one minute. They were then held with sterile forceps and about 200 cc. of sterile water poured over each capsule, in order to wash off the alcohol. After the washing process, the capsules were placed each in a sterile Petri dish and the carpels cut with the aid of a sterile scalpel and a sterile pair of forceps. The carpels were then discarded. The ovules were removed from the placental tissues and mixed in a sterile Petri dish. This made possible a random sampling of the ovules, which was necessary for the quantitative assay of the embryo factor present in different media. As a rule, a single capsule, which contained about 300 ovules, was used for each test.

About 5 to 10 ovules were placed and held between 2 microscope slides, which had been dipped previously in 70 per cent ethyl alcohol and flamed. In order to avoid the use of hot slides, 2 pairs were needed. While 1 pair was being employed to hold the ovules, the other pair, which had

been dipped previously in alcohol and flamed, was being cooled. The ovules between the glass slides were cut longitudinally by drawing a sterile thin razor blade through them. It was found that although the use of hot razor blades had to be avoided, greater cutting facility was obtained with a warm blade than a cold one. When small embryos had to be isolated, the ovules were cut into approximately equal parts. However, when embryos over 0.5 mm. in length had to be isolated, the ovules were cut into 2 unequal parts in order to avoid injury to the embryo.

The halves were placed on the slides with the cut surface facing upwards. These were then examined under a preparation microscope. The back of the microscope was fitted with a celluloid shield to protect the exposed embryos from contamination by the operator's breath. If the cut had been made properly an embryo could be seen in one corner of the embryo sac of each of the cut ovules. The embryo was removed with the aid of dissecting needles, which had been sterilized by dipping in alcohol and flaming. Here again, 2 pairs of needles were used alternately, as in the case of the microscope slides. The embryos were transferred directly to sterile shell vials (retempered, short-style type, 3.6 cm. long x 1 cm. diameter), containing the culture medium.

The culture medium (hereafter referred to as 1 per cent dextrose medium), which was used at the beginning of this investigation, was made up according to table 1. As a result of new findings during the course of this work, this culture medium was subjected to modifications, which

will be described in the appropriate subsequent parts of this thesis.

Table 1. Composition of 1 per cent dextrose culture medium.

Weight in grams	Chemical		Source
0.680	potassium chloride	Tukey's salt mixture (42)	Baker's C. P.
0.170	calcium sulfate		Baker's C. P.
0.170	magnesium sulfate		Baker's C. P.
0.170	calcium phosphate		Baker's C. P.
0.170	ferric phosphate		Baker's C. P.
0.136	potassium nitrate		Merck reagent
8.0	bacto-agar		Difco
10.0	bacto-dextrose		Difco
0.003	glycine		Eastman Kodak
0.00015	thiamine hydrochloride		Merck U. S. P.
0.020	ascorbic acid		Merck U. S. P.
0.001	nicotinic acid		Merck U. S. P.
0.0002	vitamin B_6 hydrochloride		Merck
0.0002	adenine		Eastman Kodak
0.025	succinic acid		Eastman Kodak
0.0005	calcium d-p antothenate		Merck
1 liter	distilled water		

3/4 cc. of this medium, the pH of which was 5.5, was placed in each culture vial, which was fitted with a cotton plug and autoclaved at 15 pounds pressure for 20 minutes. While the agar was still liquid, the test solution, which

had been sterilized previously by means of a Seitz bacteriological filter, was introduced into the vials. Pasteur pipettes, delivering 27 drops per cc., were used. The agar medium was allowed to solidify on a slanting rack, after which one embryo was introduced into each vial. Unless otherwise stated, 10 embryos were used for each test solution. For greater uniformity of growth, the embryos were all inserted about 1 mm. below the surface of the agar.

The embryos were cultured in the dark at constant temperatures. During the early part of this investigation, the embryos were grown at 25±1° C. This was changed later to 32±0.5° C. Measurements of the length of the embryos were made after different lengths of time by means of an ocular micrometer. But since the measurements were made through the curved walls of the vials, the figures given in this thesis are larger than the actual dimensions of the embryos. The average relative growth in the length of the embryos (final/initial length) and the percentage of the embryos which finally developed (hereafter referred to as "per cent viability") were taken as indices of the amount of embryo factor in the medium.

The above isolation and culturing of the embryos were carried out in a room which was kept closed and free of wind currents. It was found that if the described manipulations were performed with a reasonable speed contamination was negligible. No special precautionary measures, such as antiseptic sprays and face masks, were necessary. Illumination

was furnished by an 85-watt fluorescent lamp, hung about 2 feet above the dissection table. Incandescent lamps were not used because the heat produced by them dried out the exposed embryos rather rapidly.

Chapter 2

Effect of coconut milk on growth of Datura embryos in vitro

a) Growth of embryos in 1 per cent dextrose medium.

Embryos varying from 0.1 to 1.7 mm. in length were isolated from Datura capsules of different ages and cultured in the 1 per cent dextrose medium. Measurements of the length of the embryos were taken after different number of days of culture at 25 ± 1° C. The growth of a representative group of these embryos is given in table 2 and plotted in figure 1.

From the data it can be seen that the 1 per cent dextrose medium was satisfactory for embryos which were over 1 mm. in length. Smaller embryos did not respond as well. About 10 per cent of the embryos between 0.5 and 1.0 mm. in length and rarely embryos less than 0.5 mm. in length were capable of development in this medium. It is also evident that growth could be detected after only 2 days of culture. Embryos which failed to show any signs of growth within 2 days did not develop any further. In fact, a slight shrinkage of the embryo was observed in most of the failures.

b) Growth of embryos in 1 per cent dextrose medium containing coconut milk.

Heart-shaped embryos smaller than 1 mm. in length were excised and cultured in the 1 per cent dextrose medium with and without the addition of coconut milk (liquid in the nut of Cocos nucifera). Measurements of the length of the em-

bryos were taken after 0, 2, and 6 days of growth at 25± 1° C. The growth data for the individual embryos are shown in table 3 and figure 2.

Table 2. Growth of Datura embryos in 1 per cent dextrose medium.

Embryo no.	Length of embryo in mm.		
	0 days	2 days	6 days
1	1.2	1.2	1.1
2	0.4	0.35	0.35
3	0.25	0.2	0.15
4	0.75	0.6	0.6
5	0.1	0.15	0.15
6	0.9	0.8	0.8
7	1.7	2.9	5.0
8	1.4	1.5	5.0
9	1.6	1.9	5.0
10	1.1	1.3	4.0
11	1.0	1.25	5.0
12	1.7	2.8	7.0
13	1.0	1.0	1.0
14	1.2	1.1	1.2
15	0.55	0.5	0.5
16	0.6	0.55	0.55
17	0.7	0.7	0.6
18	0.9	0.9	0.8
19	0.35	0.3	0.3
20	0.5	0.95 (3 days)	1.8 (7 days)

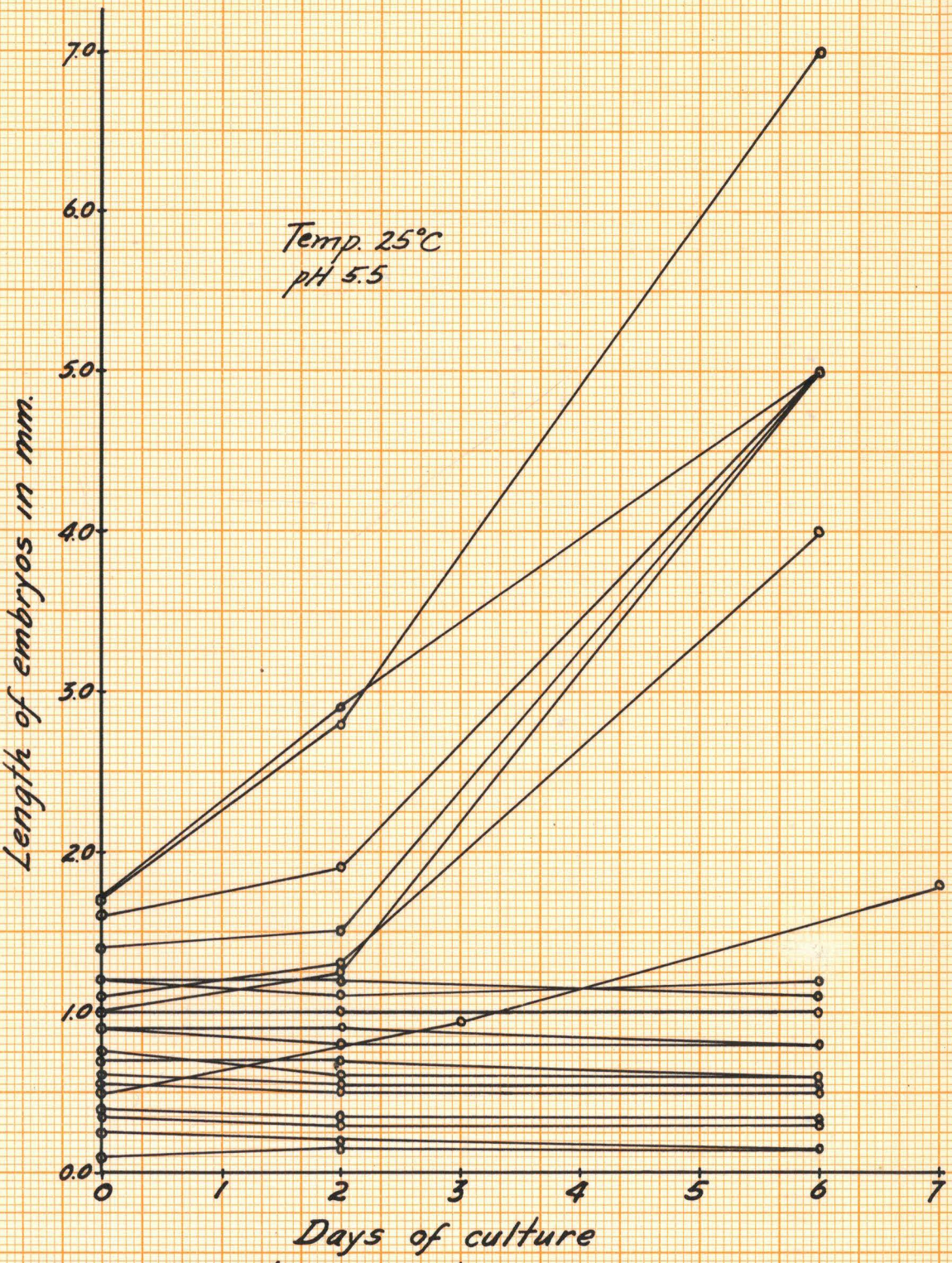

Fig. 1. Growth of *Datura* embryos in one per cent dextrose medium.

Table 3. Effect of coconut milk on growth of *Datura* embryos at 25° C.

Medium	Embryo No.	Length of embryo in mm.		
		0 days	2 days	6 days
No coconut milk added	1	0.95	1.0	1.0
	2	0.25	0.2	0.2
	3	0.4	0.35	0.35
	4	0.25	0.2	0.15
	5	0.4	0.35	0.3
	6	0.5	0.6	1.5
	7	0.1	0.15	0.15
	8	0.15	0.2	0.2
	9	0.45	0.45	0.35
	10	0.9	0.8	0.8
	Av.	0.44	0.43	0.50
5 drops coconut milk per vial	1	0.25	1.0	5.0
	2	0.6	1.4	3.3
	3	0.6	1.9	8.0
	4	0.35	1.55	6.5
	5	0.25	0.25	0.25
	6	0.35	0.55	2.5
	7	0.6	1.15	3.0
	8	0.75	1.8	6.5
	9	0.6	1.7	4.0
	10	0.6	2.0	5.0
	Av.	0.50	1.33	4.4

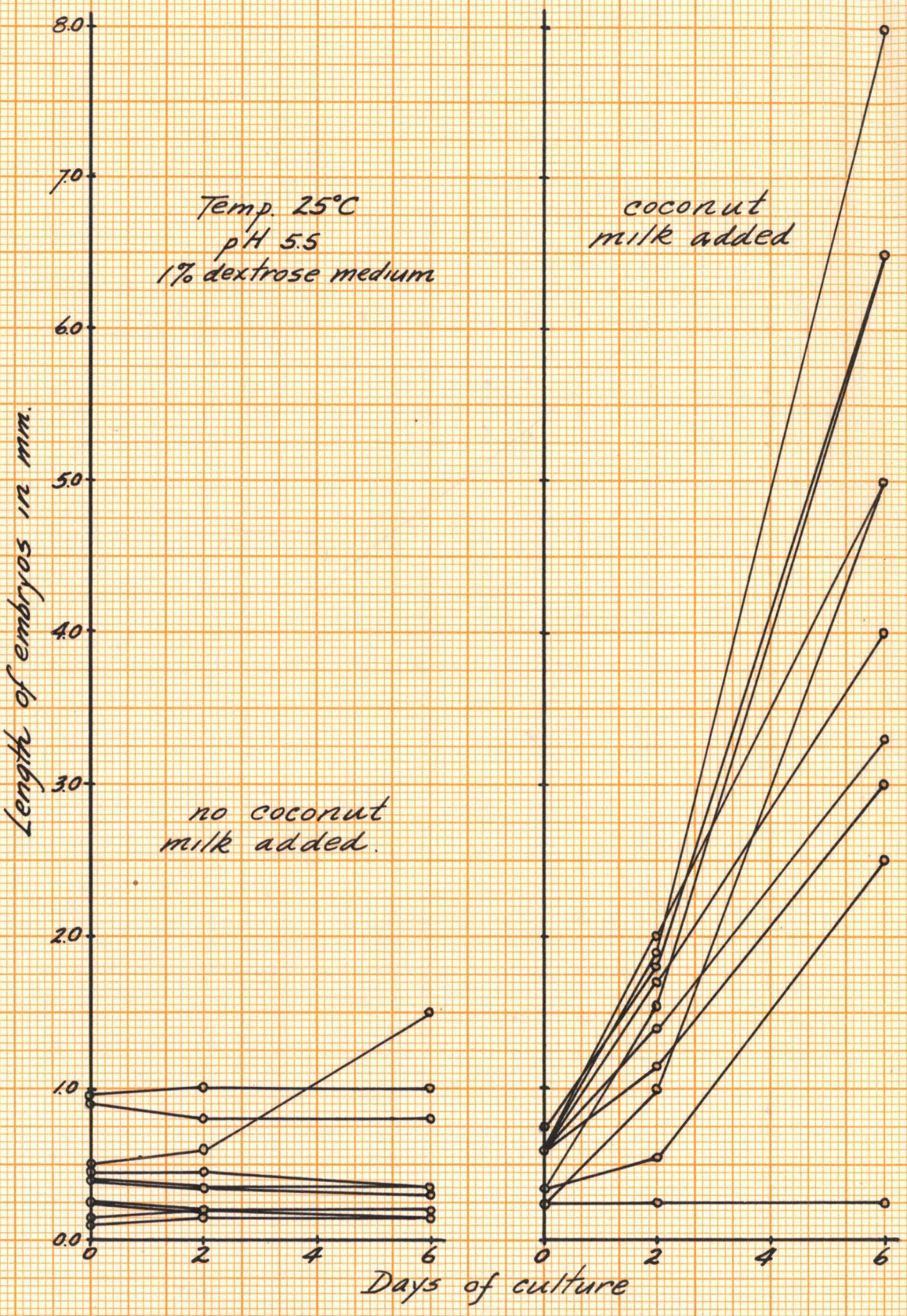

Fig. 2. Effect of coconut milk on growth of *Datura* embryos *in vitro*.

The necessity of coconut milk for the growth of small immature embryos is obvious from figure 2. Without the addition of coconut milk only 1 out of 10 embryos grew. However, the growth of this one embryo was abnormal and did not give rise to a normal differentiated seedling. When coconut milk was added to the dextrose medium, 9 out of the 10 embryos developed into normal viable seedlings.

c) Effects of different concentrations of coconut milk on the growth of embryos.

Heart-shaped embryos, 0.3 to 0.6 mm. in length, were isolated and grown in the 1 per cent dextrose medium containing different concentrations of coconut milk. Coconut milk was concentrated in vacuo at 50 to 55° C. to one fifth of its original volume. Stock solutions were prepared by diluting this concentrate. One to 5 drops of the respective stock solutions were added to each of the culture vials to give the desired concentrations. The embryos were cultured at 25±1° C. and measurements were made after 0, 2, 4, and 7 days, respectively. The growth data of these embryos are presented in table 4 and figure 3.

There were no significant differences in the percentages of viability in the embryos grown in media containing the equivalent of 3 to 15 drops of coconut milk per vial. Optimum growth of the embryos, however, took place in media containing 3 to 7 drops of coconut milk per vial. With increasing concentrations the growth of the embryos decreased rapidly, so that at a concentration equivalent to 20 drops of original coconut milk per vial ~~no~~ little growth was obtained.

Table 4. Growth of *Datura* embryos in 1 per cent dextrose medium containing different concentrations of coconut milk.

Coconut milk per vial		Per cent viability	Growth measurements						
			Av. length in mm.				Av. relative growth		
no. of drops	mg. dry wt.		0 days	2 days	4 days	7 days	2 days	4 days	7 days
0	0	0	0.37	0.34	0.33	0.33	0.9	0.9	0.9
1	5	70	0.47	0.64	1.43	2.15	1.4	3.0	4.6
3	15	90	0.38	0.69	1.72	2.58	1.8	4.5	6.8
5	25	100	0.42	0.94	2.10	3.59	2.2	5.0	8.5
7.5	37.5	100	0.44	1.21	2.10	3.24	2.8	4.8	7.4
10	50	100	0.48	0.98	1.66	2.42	2.0	3.5	5.0
12.5	62.5	100	0.33	0.58	0.97	1.63	1.8	2.9	4.9
15	75	100	0.48	0.80	1.08	1.51	1.7	2.3	3.1
20	100	40	0.35	0.44	0.52	0.64	1.3	1.5	1.8
25	125	20	0.43	0.50	0.56	0.53	1.2	1.3	1.2

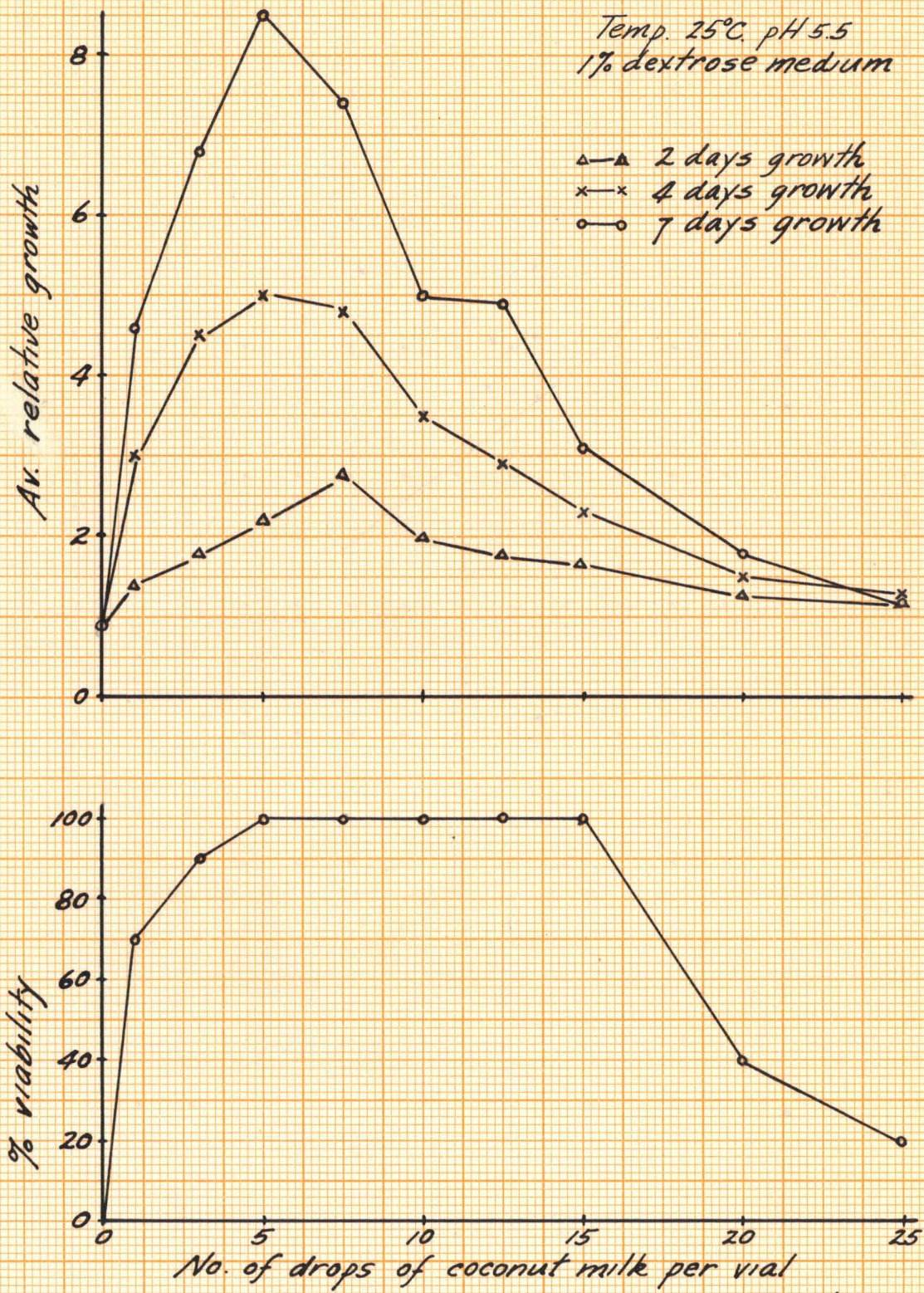

Fig. 3. Effect of coconut milk concentration on embryo growth.

d) Effect of coconut milk on the growth of Datura embryos provided with or without supplementary nutrients.

Embryos between 0.2 and 0.6 mm. in length were grown in the following media: a) 1 per cent dextrose medium, as given in table 1, b) 1 per cent dextrose medium, as given in table 1, plus 5 drops of coconut milk per vial, and c) 0.8 per cent agar, plus 1 per cent dextrose, plus 5 drops of coconut milk per vial. Measurements of the length of the embryos were taken after 0, 3, and 7 days of culture at 25±1° C. and are given in the table below.

Table 5. Growth of Datura embryos in coconut milk with and without supplementary nutrients.

Medium	Per cent viability	Growth measurements of embryos				
		Length in mm.			relative growth	
		0 days	3 days	7 days	3 days	7 days
a	0	0.38	0.35	---	0.9	---
b	100	0.41	1.74	3.37	4.2	8.2
c	100	0.29	1.24	1.89	4.3	6.5

Medium "c", which lacked the inorganic salts and the organic supplements except dextrose, did not support as vigorous a growth of the embryos as did medium "b", which contained all of the ingredients of the 1 per cent dextrose medium. The beneficial effect of the supplement, however, was not observed during the first 3 days of growth but became prominent only after about a week. The percentage viability was the same in both cases.

Chapter 3

Variability in growth of embryos

Before any quantitative estimation of the embryo factor* in a test solution could be obtained from the assay method described in the previous pages the degree of variability in the growth of the embryos had to be known.

Personal variations among different workers were tested in the following way. Ovules from a single capsule were mixed together in a sterile Petri dish. Ten ovules were taken randomly from the dish and transferred to vials containing 1 per cent dextrose medium by each of three persons. The embryos were subsequently cultured side by side at 25±1° C. However, all measurements of the lengths of the embryos were made by one and the same person. The results are recorded in table 6.

Because of the unsuitability of the 1 per cent dextrose medium for the growth of small embryos, those which showed an initial development did not continue their growth after reaching a size of about 1.5 to 2 times their original length. However, 80 per cent of the embryos isolated by person A showed the initial growth, as compared to 10 to 20 per cent in the case of persons B. and C. Examination of the culture vials after the tests showed that considerable quantities of the embryo sac contents were transferred

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* The term "embryo factor", as used in this thesis, includes all the unknown chemical substances, which are necessary for the growth of small Datura embryos in vitro.

along with the embryos by person A but not in the case of persons B and C. The presence of these substances in the 1 per cent dextrose medium might have stimulated the development of the embryos, which ordinarily would not have grown in the 1 per cent dextrose medium.

Table 6. Growth of embryos isolated by different persons.

Person	Embryo no.	Embryo length in mm.		% viability	Av. rel. growth (failures not counted)
		0 days	2 days		
A	1	0.3	0.8		
	2	0.6	1.05		
	3	0.35	dead		
	4	0.4	0.65		
	5	0.6	0.7		
	6	0.5	0.9	80	1.7
	7	0.65	1.0		
	8	0.25	dead		
	9	0.25	dead		
	10	0.35	0.5		
	Av.	0.42	0.80		
B	1	0.35	dead		
	2	0.3	dead		
	3	0.25	dead		
	4	0.40	dead		
	5	0.50	0.65		
	6	0.4	dead	20	1.5
	7	0.3	0.55		
	8	0.25	dead		
	9	0.5	dead		
	10	0.3	dead		
	Av.	0.36	0.60		
C	1	0.4	0.8		
	2	0.25	dead		
	3	0.7	dead		
	4	0.5	dead		
	5	0.4	0.6		
	6	0.45	dead	30	1.6
	7	0.25	dead		
	8	0.3	dead		
	9	0.5	dead		
	10	0.45	0.6		
	Av.	0.42	0.67		

However, preliminary experiments by van Overbeek (unpublished data) did not show any increase in the growth of embryos, when the endosperm was transferred together with the embryos from the ovules to the culture vials. Nevertheless, in order to avoid personal variations, all subsequent tests were performed each by one and the same person.

The following test was carried out to determine the effect of embryo size as a cause of variability. Ten embryos, which were about 0.5 and 1 mm. in length, respectively, were cultured in the 1 per cent dextrose medium containing different amounts of embryo factor. Measurements of the growth of the embryos were taken after 2 days of culture at 25±1° C. The results are shown in table 7 and plotted in figure 4.

Table 7. Growth of embryos of different sizes.

Relative conc. of embryo factor	Dry wt. of embryo factor in mg.	Initial embryo length in mm.	Per cent viability	Increase in length in mm.	Av. relative growth (failures not counted)
0	0	0.56 1.12	0 0	-0.01 -0.06	--- ---
1	0.59	0.50 1.18	30 100	0.20 0.46	1.4 1.4
2	1.18	0.50 1.10	40 100	0.26 0.68	1.52 1.62
6	3.54	0.47 1.12	70 100	0.43 1.02	1.92 1.90
12	7.08	0.51	100	0.49	1.96

From the data presented it can be seen that although

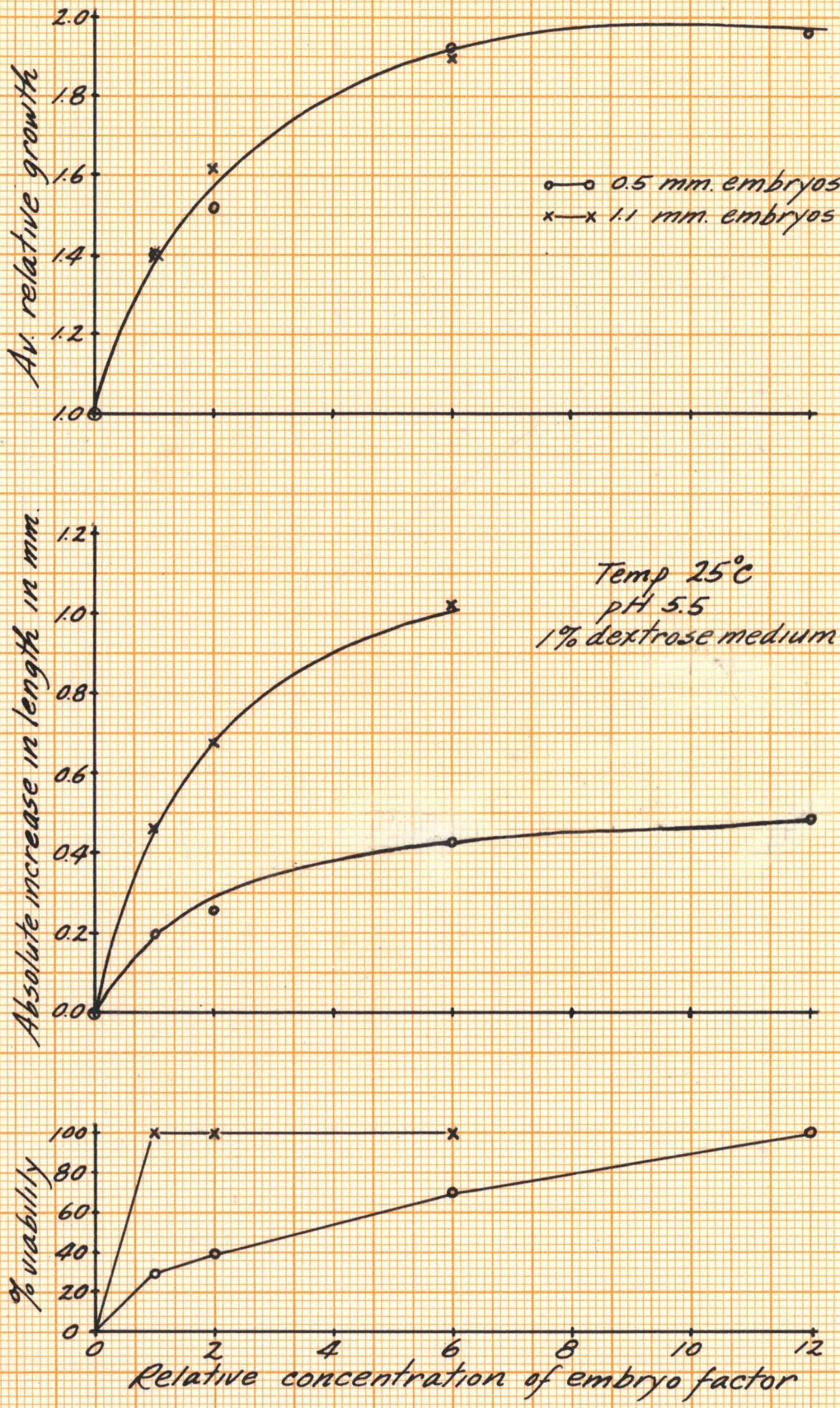

Fig. 4. Growth of embryos of different sizes.

the percentage viability and the absolute increase in embryo length were different for embryos of different sizes, the relative growths of the viable embryos were the same in both cases. From this point of view, then, the relative increase (final/initial length) in the length of the viable embryos would serve as a better index of the amount of embryo factor than the other 2 criteria. This index will be used in the subsequent parts of this thesis.

Two factors, viz., pH of the media and the temperature in which the embryos were cultured, were found to be of great importance in influencing the growth of the embryos. These will be treated in detail in Chapter 4.

With the control of these various factors a satisfactory bio-assay for the embryo factor was obtained. The variation in the tests was relatively small and the results could be duplicated to a satisfactory degree. This was shown by the following series of experiments, which were conducted over a period of several months.

In the first series, 2 sets of 10 embryos each, set A and set B, were cultured in a 2 per cent sucrose medium (2 per cent sucrose in place of dextrose in the medium given in table 1). These paired sets constituted the duplicate controls of larger tests. The medium was buffered with 0.01 molar monosodium-dipotassium phosphate at approximately pH 6.8. The embryos were cultured at 32±0.5° C. and measurements of the growth of the embryos were made after about 2 days. The average relative growths of the paired sets and their differences are listed in table 8.

Table 8. Variability in the growth of embryos in 2 per cent sucrose medium.

Pair no.	% viability			Av. relative growth*		
	Set A	Set B	Diff.	Set A	Set B	Diff.
1	50	50	0	2.9	3.0	0.1
2	60	70	10	2.0	2.2	0.2
3	80	60	20	2.3	2.3	0.0
4	90	80	10	2.7	2.4	0.3
5	80	70	10	2.6	2.8	0.2
6	70	80	10	2.8	2.7	0.1
7	80	70	10	3.1	3.4	0.3
8	90	80	10	2.6	2.4	0.2
9	50	70	20	2.0	2.3	0.3
10	60	60	0	3.1	2.6	0.5
11	70	70	0	2.3	2.4	0.1
12	60	40	20	3.3	2.9	0.4
13	80	100	20	2.8	2.4	0.4
14	60	50	10	2.2	2.4	0.2
15	50	50	0	2.2	2.4	0.2
16	40	40	0	2.7	2.8	0.1
17	80	40	40	2.4	2.6	0.2
18	30	70	40	2.0	2.2	0.2
19	60	90	30	2.5	2.6	0.1
20	70	80	10	2.3	2.3	0.0
Av.	65		13	2.6		0.20

* Hereafter, the average relative growth of only the viable embryos will be presented in the tables. Dead embryos will not be included in the calculations.

The average difference between the relative growths of the paired sets was 0.20, which was only 7.7 per cent of the average relative growth of the 40 sets. However, the relative growth values among the individual sets differed considerably, ranging from 2.0 to 3.4. This variation might have been due in part to the fact that the pH and the time of measurement were not identical in all cases. Furthermore, the response of embryos from different capsules, which had been picked from plants grown under uncontrolled conditions in greenhouses, might have varied with the capsules used. This variability can be seen from table 9, which presents data taken over a period of 2 months.

Table 9. Variability in the growth of embryos from different capsules.

Capsule no.	Av. relative increase in length after 2 days		
	2% sucrose medium	2% sucrose medium + 3 drops coconut milk per vial	Difference
1	2.5	4.5	2.0
2	2.2	4.6	2.4
3	2.8	5.5	2.7
4	2.3	3.7	1.4
5	2.6	4.9	2.3
6	2.5	5.2	2.7
7	2.6	4.2	1.6
8	2.6	4.6	2.0
9	2.5	4.0	1.5
10	2.5	4.4	1.9
Average			2.0

The figures in the last column of table 9 give a measure of the amount of embryo factor activity in coconut milk when tested on a 2 per cent sucrose medium. The values for the same sample of coconut milk, as tested on different days, ranged from 1.4 to 2.7. Because of this variability in the response of embryos from different capsules, a standard active solution, usually 3 drops of coconut milk per vial of culture medium, was included in each test. Also included in each test were duplicate controls of 10 embryos each. In this way, a satisfactory quantitative value for the amounts of embryo factor in a given test solution could be obtained.

An idea as to the quantitative variations which might be expected from a typical embryo factor bio-assay could be obtained from the figures in table 10. In this test a 2 per cent sucrose medium, which was buffered at pH 6.7 with 0.01 molar monosodium-dipotassium phosphate, was used and the embryos were cultured for 3 days at 32±0.5° C.

Table 10. Growth of embryos supplied with different concentrations of embryo factor.

Set	Relative conc. of embryo factor	No. of embryos	Per cent viability	Av. initial length in mm.	Av. relative growth ±std.error	Coefficient of variability
A	0	36	55	0.45	4.0±0.24	6.0%
B	1	10	70	0.36	4.4±0.25	5.7%
C	2	10	70	0.44	4.9±0.40	8.2%
D	3	10	60	0.47	5.2±0.35	6.7%
E	5	10	60	0.50	5.1±0.31	6.1%

The relatively low degree of variation in the bio-assay, which was performed under controlled conditions, is shown by the coefficients of variability, which ranged from 5.7 to 8.2 per cent. Other tests showed similar magnitudes of variability. With a knowledge of these statistics for a pair of uncorrelated means one could calculate the standard error of the difference of the means, using the formula:

$$S.E._{diff.} = \sqrt{(S.E._1)^2 + (S.E._2)^2}$$

The absolute difference which was necessary for significance between the means could then be obtained, using the equation:

$$\text{Difference} = \text{"t" value} \times S.E._{diff.}$$

According to Fisher's "t" table, the "t" value for $p = 0.05$ is 2.09 and for $p = 0.01$ is 2.54 for 19 degrees of freedom. Through the use of these equations the differences necessary for significance between the average relative growth of the embryos cultured in the presence and in the absence of embryo factor were computed and listed in table 11.

Table 11. Differences necessary for significance between the average relative growths of pairs of means with different standard errors.

$S.E._1$	$S.E._2$	Differences necessary for significance	
		$p = 0.05$	$p = 0.01$
0.2	0.2	0.6	0.7
0.2	0.3	0.7	0.9
0.2	0.4	0.9	1.1

From the statistical data presented in table 11, it seems that 0.9 may be considered a significant difference between the relative growths of 2 sets of embryos cultured for 3 days in different media. The threshold difference for significance between 2 means will necessarily depend upon the length of time of culture. The means with their respective standard errors for Sets A and D of table 10 have been calculated for growth after 1, 2, 3, and 4 days and are given in table 12. Also included in the table are the differences necessary for significance between set A and set D after different lengths of time of culture. The means for set A and set D with the respective differences necessary for significance between the two at the $p = 0.05$ level are plotted in figure 5. From the graphs one can readily observe the significant differences in growth between the embryos supplied with embryo factor and those not supplied with embryo factor. The difference can be noted as early as after 2 days of culture. This criterion of significance, as expressed in the last 2 rows of table 12, will be followed throughout the subsequent parts of this thesis.

Table 12. Variation in embryo growth after different number of days of culture.

Set		Av. relative growth after 1 day			2 days			3 days			4 days		
		Av.	S.E.	Coef. var.	Av.	S.E.	Coef. var.	Av.	S.E.	Coef. var.	Av.	S.E.	Coef. var.
A: Control		1.6	0.07	4.4	2.7	0.09	3.3	4.0	0.24	6.0	4.8	0.25	5.2
D: Embryo factor		1.6	0.08	5.0	3.8	0.18	5.1	5.2	0.35	6.7	6.2	0.42	6.8
Diff. necess. for signif. at	P=0.05	0.22			0.38			0.52			0.70		
	P=0.01	0.29			0.51			0.70			0.95		

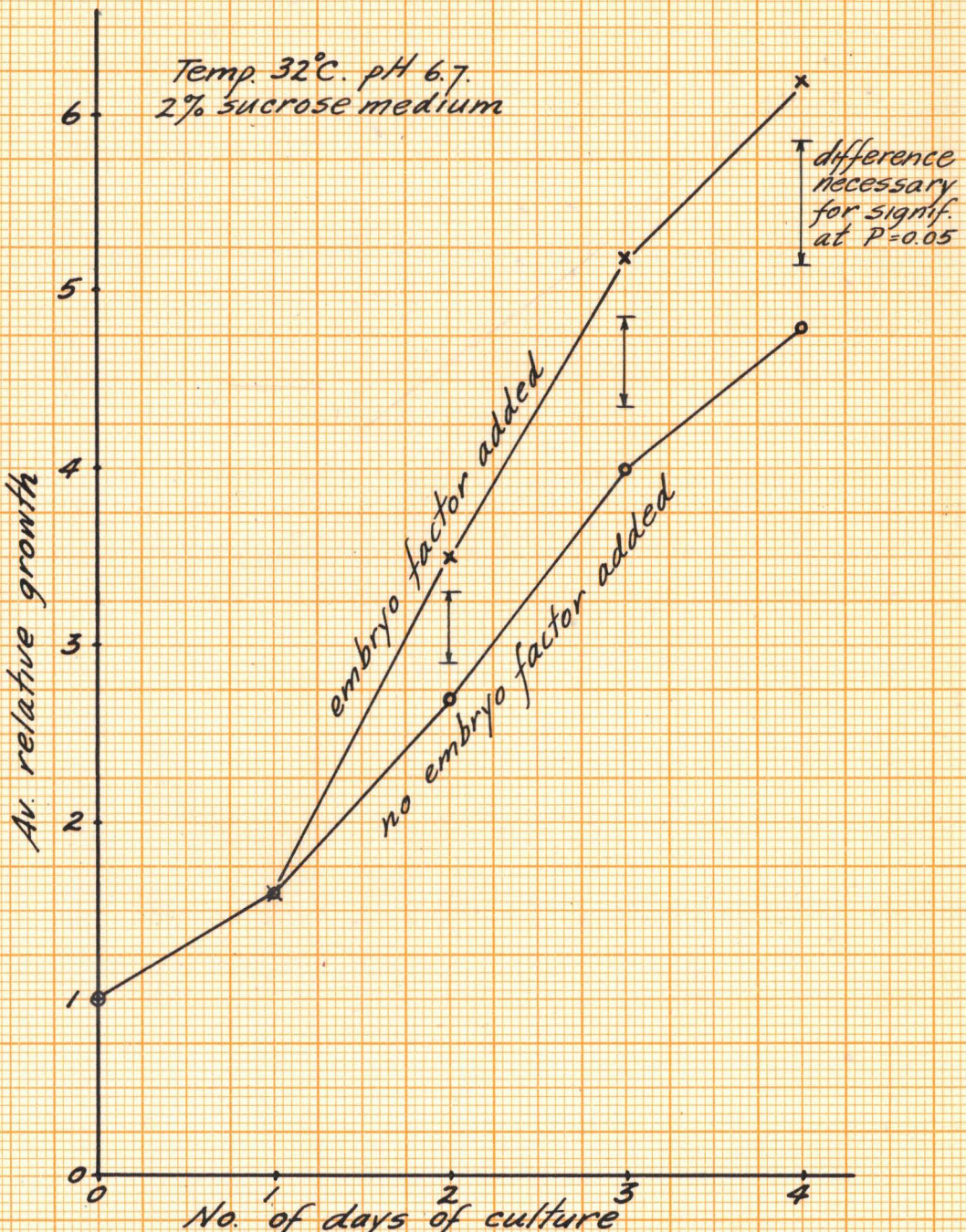

Fig. 5. Effect of embryo factor on embryo growth.

Chapter 4

Environmental factors influencing growth of Datura embryos

a) Temperature.

Embryos, which were 0.25 to 0.65 mm. in length, were grown in a 2 per cent dextrose medium (2 per cent dextrose in place of 1 per cent dextrose in the medium given in table 1) with and without the addition of embryo factor. The growth status of the embryos grown at different temperatures for different lengths of time are shown in table 13 and plotted in figure 6.

Table 13. Growth of embryos at different temperatures.

Temp.	Relative conc. of embryo factor	Per cent viability	Av. relative growth			
			23 hr.	42 hr.	91 hr.	117 hr.
17±1°C	0	40	1.2	1.4	1.9	2.7
	1	60	1.3	1.4	1.8	2.6
	2	60	1.2	1.4	2.1	3.1
25±1°C	0	70	1.3	1.8	2.8	3.9
	1	80	1.4	1.6	3.5	4.8
	2	70	1.4	2.1	4.0	5.3
32±0.5°C	0	60	1.6	2.3	4.4	7.5
	1	90	2.0	3.5	7.6	12.5
	2	90	2.0	3.0	6.3	10.2
36±1°C	0	40	1.6	2.3	4.2	7.3
	1	70	1.8	2.7	5.7	9.7
	2	70	1.6	2.6	5.1	9.2

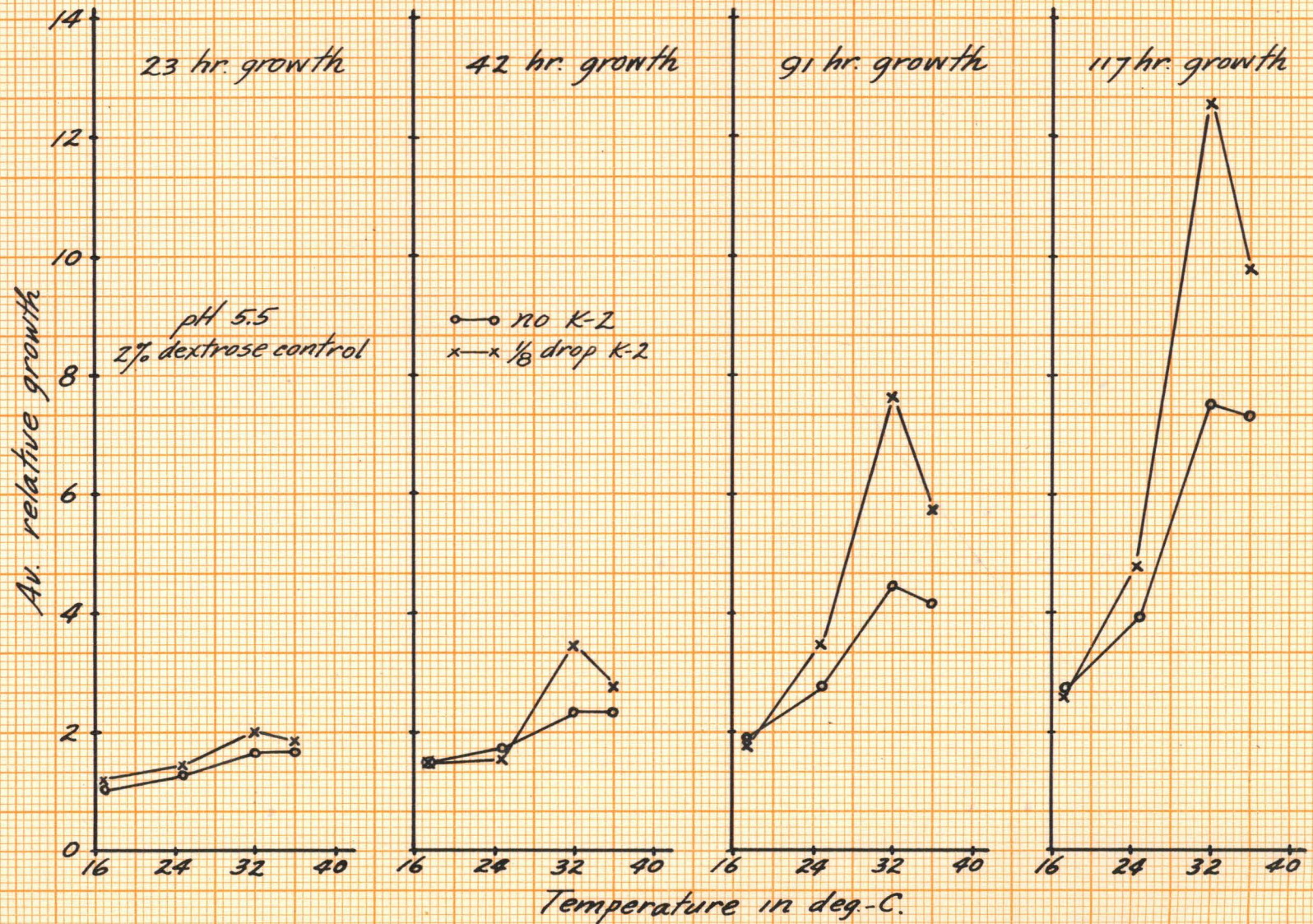

Fig. 6. Effect of temperature on growth of excised embryos.

The data show that the embryo growth increased rapidly with increasing temperatures up to 32° C., where an optimum was reached. At 17° C. the growth was equally poor in both the presence and the absence of embryo factor. A stimulation in the growth of the embryos by the addition of embryo factor was marked at the temperatures favorable to optimal embryo growth. It must be pointed out, however, that the embryos used in this test developed in the dextrose medium alone. This is an exception to the usual failure of embryos to grow in the dextrose control medium and will be treated in more detail in the Discussion section of this thesis.

b) pH of medium.

Preliminary tests on the effect of the pH of the medium on the growth of Datura embryos in vitro were conducted. During the early experiments the following buffers were used in 0.01 molar concentrations:

1) sodium citrate buffer for pH 3 to 4, and
2) monobasic-dibasic potassium phosphate buffer for pH 5 to 8.

In the cultures buffered with the above mixtures only a few of the embryos developed. The growth was very unsatisfactory even in the presence of embryo factor. It was found later, as shown by the figures of table 14, that a 0.01 molar dipotassium-monosodium phosphate buffer did not affect the growth of embryos adversely.

Table 14. Effect of K_2HPO_4-NaH_2PO_4 buffer on the growth of Datura embryos.

Medium	pH	No. of embryos	Per cent viability	Av. relative growth after 2 days
2% sucrose				
+ buffer	5.4	15	60	2.4
- buffer	5.2	15	40	2.4
2% sucrose + 3 drops of coconut milk per vial				
+ buffer	5.7	15	67	4.8
- buffer	5.5	15	60	4.3

Through the use of this buffer the following series of tests on the effect of pH on embryo growth was conducted.

Embryos about 0.2 to 0.6 mm. in length were grown in a 2 per cent sucrose medium which was buffered at pH's ranging from 5 to 9. The data were compiled from experiments extending over a period of several months. The pH values given in the table represent the pH's of the media at the time of the final measurements, which varied between 36 to 48 hours of culture at 32±0.5° C. The percentage viability and the average relative growth of the embryos, which developed at the various pH's, are presented in table 15 and figure 7.

Table 15. Effect of pH on the growth of embryos in 2 per cent sucrose medium.

pH	Test no.	Av. initial length of embryos in mm.	Per cent viability	Av. relative growth
5.0	421123	0.31	0	---
	421207	0.41	40	1.9
	421208	0.31	0	---
	421208	0.34	0	---
5.1	421123	0.38	0	---
	421207	0.44	30	2.4
5.2	421103	0.37	0	---
5.3	421119	0.33	40	1.5
	421123	0.39	20	1.6
	421130	0.42	20	2.2
5.6	421116	0.30	40	1.9
5.7	421223	0.28	60	2.0
5.8	421119	0.36	100	1.9
5.9	421203	0.39	60	2.2
	421203	0.35	70	2.2
6.0	430112	0.24	60	3.3
6.1	421130	0.37	60	2.6
6.4	421207	0.40	70	2.3
6.5	421116	0.28	60	2.4
6.6	421212	0.40	80	2.2
	421212	0.40	80	2.4
	421212	0.34	100	2.7
	421217	0.33	90	2.5

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Table 15. (Continued from previous page).

pH	Test no.	Av. initial length of embryos in mm.	Per cent via-bility	Av. relative growth
6.6	421217	0.33	70	2.7
6.7	421126	0.35	60	2.2
	421210	0.35	90	2.3
	421210	0.35	50	3.0
	421210	0.44	60	2.4
	421210	0.43	70	2.6
	430104	0.47	70	2.4
6.8	421130	0.38	80	2.6
	421203	0.34	40	2.8
	421203	0.33	80	3.2
	421210	0.37	50	2.9
	421231	0.32	90	3.4
	421231	0.38	90	3.1
6.9	421207	0.31	50	3.0
	430106	0.54	60	2.0
	430119	0.51	70	2.3
	430119	0.26	50	2.8
7.0	421215	0.34	60	2.6
	430111	0.46	50	2.3
	430113	0.36	90	2.8
	430118	0.43	50	2.4
7.2	421130	0.34	60	2.5
7.3	421210	0.38	60	2.4
8.3	421215	0.32	20	2.2
8.9	421110	0.27	0	---

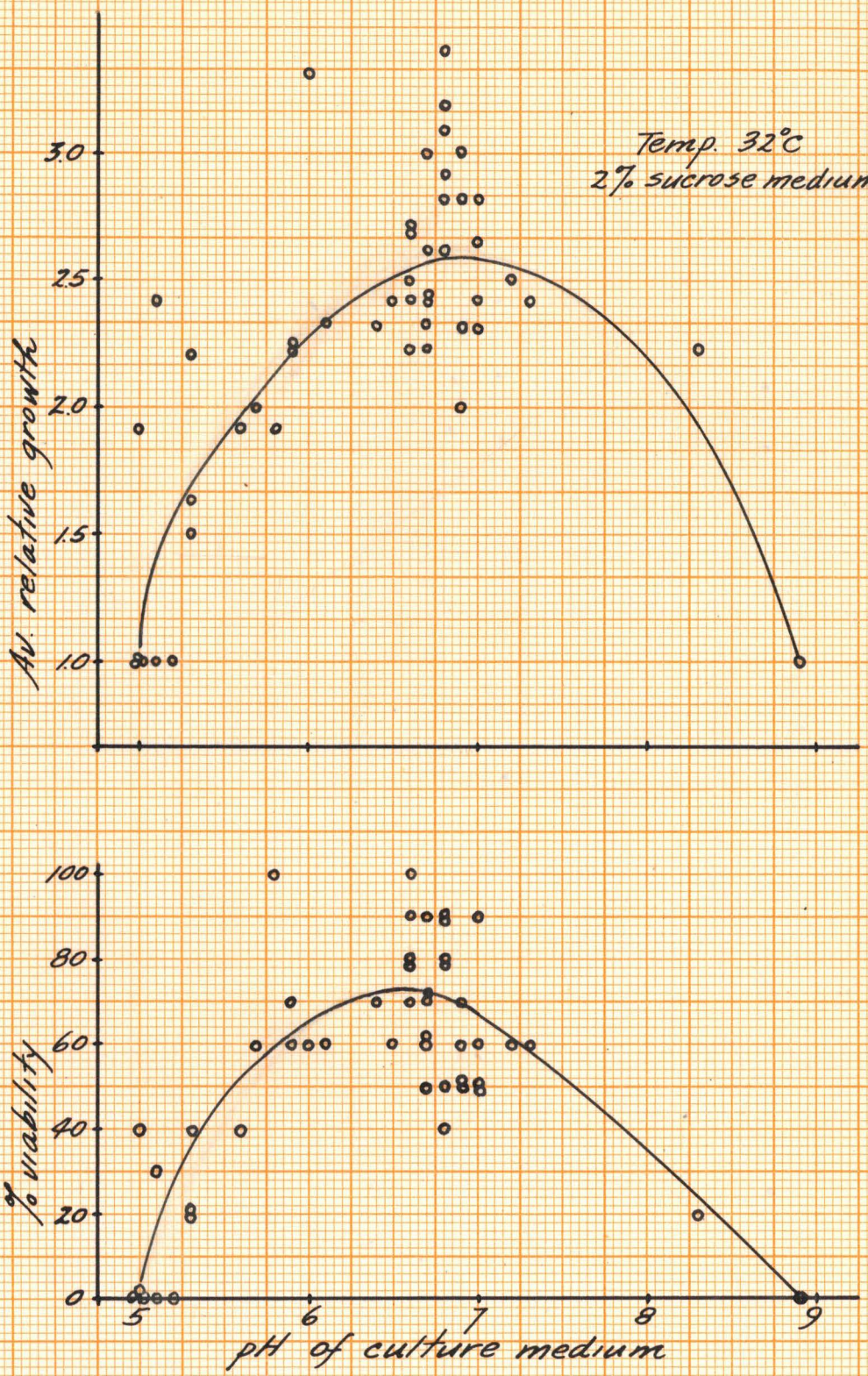

Fig. 7. Effect of pH on embryo growth.

From the data, it is apparent that the highest percentage viability and the fastest rate of growth of Datura embryos during the first 2 days of culture in vitro in the 2 per cent sucrose medium occurred between pH 6 to 7. Whether or not the same optimum applied to larger embryos and whether it applied to embryos grown in the presence of embryo factor was determined by the following test. Embryos about 0.35 and 1.0 mm. in length, respectively, were grown in a 2 per cent sucrose medium, which was buffered at 2 pH's, with and without the addition of embryo factor. The final measurements were made after 2 days and are given in table 16.

Table 16. Growth of embryos of different sizes at 2 pH's.

pH	Average length of embryos in mm.	Relative conc. of embryo factor	Per cent viability	Av. relative growth
5.6	0.35	0	30	2.6
	0.36	1	40	3.2
	0.39	5	50	4.1
6.5	0.36	0	50	3.1
	0.36	1	50	3.5
	0.35	5	70	4.6
5.7	1.2	0	70	1.9
	1.1	1	70	2.0
	1.0	5	70	2.6
6.7	0.92	0	100	2.8
	1.1	1	100	2.9
	0.93	5	40	3.4

The data clearly show that the larger, as well as the smaller, embryos responded to pH differences in the same way. Both groups of embryos grew better at pH 6.5 than 5.6. Furthermore, the same pH response existed in the presence and in the absence of embryo factor.

All of the previous tests on the effect of pH on embryo growth were terminated after 2 days. The question as to the effect of the pH of the medium on the continued growth of the embryos was investigated in the following experiment. Embryos, which were 0.25 to 0.5 mm. in length, were grown in the 2 per cent sucrose medium with and without the addition of 3 drops of coconut milk per vial. The medium was buffered at different pH's. Each set consisted of 15 embryos, which were cultured at 32±0.5° C. Measurements of the length of the embryos were made on 5 consecutive days after the isolation of the embryos and the embryos were weighed after 11 days. The data are given in table 17 and figure 8.

Table 17. Growth of embryos at different pH's.

Medium	pH	Per cent viability	Av. relative growth					Av. wet wt. in mg. after 11 days
			1 da.	2 da.	3 da.	4 da.	5 da.	
2% sucrose + 0 drops of coconut milk per vial	5.1	33	1.4	2.0	3.0	3.7	4.3	2.0
	6.9	73	2.1	2.9	3.6	4.1	5.5	0.7
	7.5	67	2.0	2.5	3.4	4.4	5.2	0.5
2% sucrose + 3 drops of coconut milk per vial	5.5	73	2.0	3.8	6.8	9.8	16.1	18.0
	6.9	81	2.7	5.0	7.1	8.3	9.1	1.7
	7.5	94	2.7	4.7	7.5	8.4	9.8	2.0

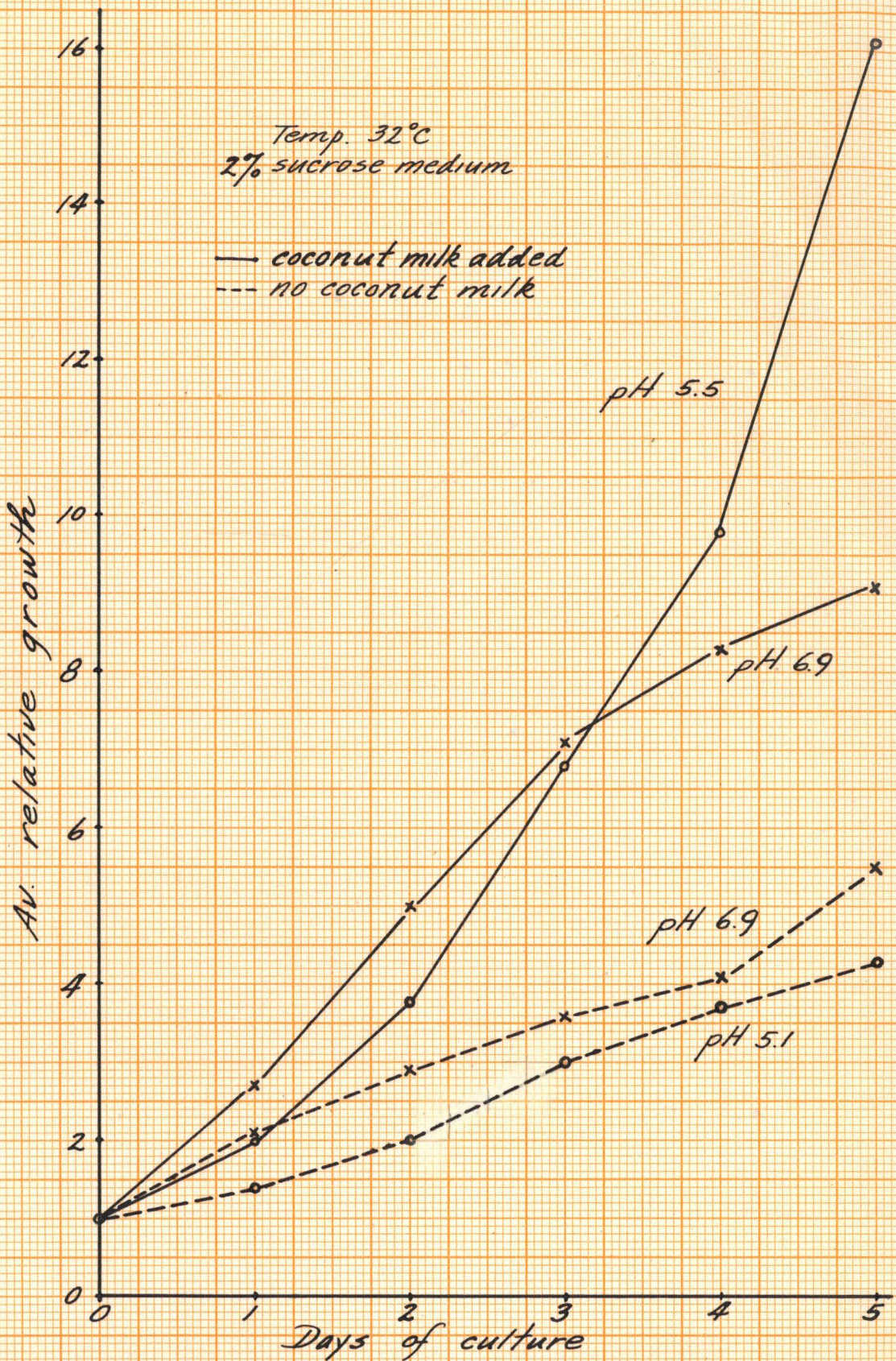

Fig. 8. Effect of pH on embryo growth.

As in the case of the previous tests, the optimum pH during the first 2 days of growth was around pH 6.9. However, after 4 days of growth the pH optimum changed and the embryos which originally grew slowly at pH 5.5 finally outgrew the embryos cultured at the more alkaline pH, despite the better start of the latter. The poor growth of the embryos at pH 5.1 was expected from the results of earlier experiments shown in figure 7.

The possibility that the decreased growth at the higher pH was due to the difference in the ratio between the sodium and the potassium ions, which were introduced with the buffer, was eliminated by the results of the following experiment. Embryos 0.2 to 0.4 mm. in length were cultured in 2 per cent sucrose media, which were buffered at pH 5 and 7 with the solutions listed in table 18 in 0.01 molar concentrations. It is to be noted that 2 buffers were used for each pH; one had a high sodium:potassium ratio, while the other had a low sodium:potassium ratio. 3 drops of coconut milk was added to each vial and the embryos were cultured at 32±0.5° C. The results are shown in table 18 and figure 9.

It can be concluded, therefore, that the pH of the medium and not the ionic ratio between sodium and potassium was the important factor in influencing the growth of the embryos. The rapid initial rate of growth at pH 6.8 was followed by a slowing down, while the slow initial growth at pH 5.5 was followed by a subsequent rapid rate of growth.

This was observed whether the medium was buffered with a solution with a low or one with a high sodium:potassium ratio.

Table 18. Effect of different buffer solutions on the growth of Datura embryos.

Buffer	Composition	pH	No. of embryos	Per cent viability	Av. relative growth			
					2 da.	3 da.	5 da.	7 da.
A	3cc.MNa_2HPO_4 50cc.MKH_2PO_4	5.5	13	32	2.5	4.0	8.8	15.2
B	10cc.MK_2HPO_4 3cc.MNaH_2PO_4	6.9	13	77	4.4	6.2	7.6	9.0
C	10cc.MNa_2HPO_4 0.6cc.MK_2HPO_4	5.7	13	62	2.3	3.7	7.4	13.5
D	50cc.MNa_2HPO_4 12cc.MKH_2PO_4	6.8	15	60	4.4	5.8	7.2	8.1

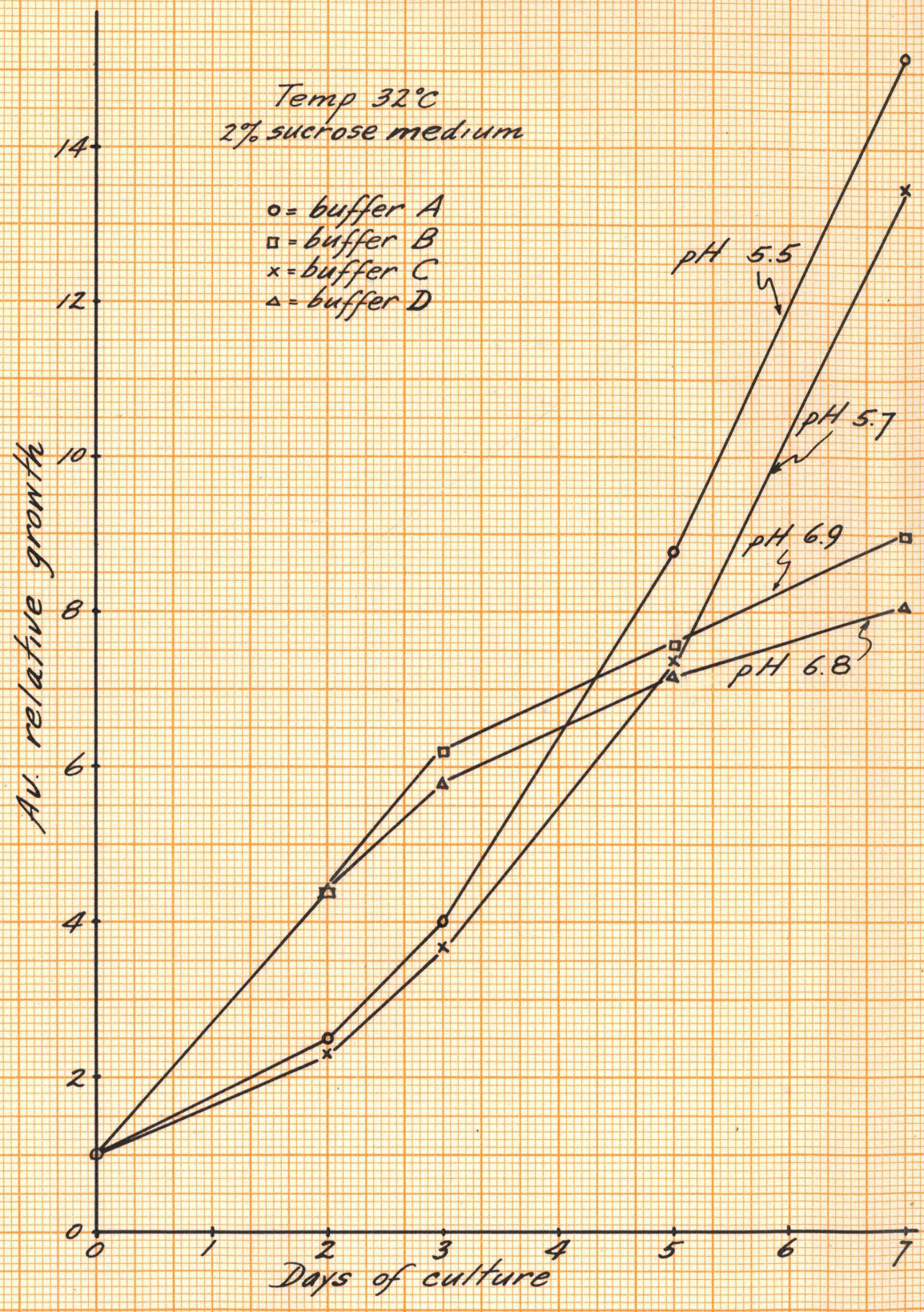

Fig. 9. Effect of different buffer solutions on embryo growth.

Chapter 5

Effect of chemical substances on growth of Datura embryos

During the course of this investigation the effect of different substances on the growth of embryos was tested. Of these, only sucrose and auxin were found to be beneficial, as will be discussed in chapter 7. Other substances, which are listed in table 19, showed very little or no effects in eliciting favorable growth responses in the embryos cultured *in vitro*.

Table 19. Substances inactive in promoting the growth of *Datura* embryos *in vitro*.

Substance*	Medium	Conc.per culture vial
KCl - C.P.	2% dextrose	0.001M to 0.03M
K_2SO_4 - C.P.	2% dextrose	0.001M to 0.03M
Hoagland's minor elements	2% sucrose	0.1 to 100x original
Mannitol	2% sucrose	0.01 to 0.1%
d-Galactose	0% dextrose	2.0%
Maltose	0% dextrose	2.0%
d-Lactose	0% dextrose	2.0%
d-Mannose	0% dextrose	2.0%
Mellibiose	0% dextrose	2.0%
Xylose	0% dextrose	2.0%
d-Arabinose	0% dextrose	2.0%
Lyxose	0% dextrose	2.0%
Dextrose + l-rhamnose + amygdalin	2% sucrose	0.27% of each

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Table 19. (Continued from previous page).

Substance	Medium	Conc. per culture vial
d-Mannose + d-arabinose + arbutin	2% sucrose	0.27% of each
Maltose + l-sorbose + salicin	2% sucrose	0.27% of each
d-Glucosamine + d-galactose + esculine	2% sucrose	0.27% of each
d-Lactose + d-fructose + phloridzin	2% sucrose	0.27% of each
d-Arabinose + α-methylglucoside + α-methylmannoside	2% sucrose	0.27% of each
Phytic acid	2% dextrose	0.0001 to 0.05M
Mixture of: dl-methionine, glutamic acid, l-aspartic acid, l-(-)-leucine, d-valine, dl-serine, l-(-)-proline, dl-lysine, l-histidine, cystine, d-ornithine, β-alanine, dl-cysteine, dl-isoleucine, dl-norleucine, dl-phenylalanine, l-(-)-hydroxyproline	2% sucrose	0.00003 to 0.25% of each
Xanthine	2% sucrose	0.005 to 0.05 mg.
Hypoxanthine	2% sucrose	0.001 to 0.01 mg.

* The author is indebted to Dr. Carl Niemann for the sugars and glycosides, which were used in this series of experiments.

Chapter 6

Embryo factor in natural extracts

A casual investigation was conducted on the promoting effects of various natural extracts on the growth of Datura embryos in vitro. The active extracts are listed in table 20. They were obtained in the following way:

1) Coconut meat extract: 30 g. of fresh coconut meat was ground in a Waring blendor and shaken mechanically with 400 cc. of 50 per cent ethyl alcohol for 2 days. The alcohol extract was evaporated to dryness at 50 to 60° C. in vacuo and the residue (1.0 g.) was taken up in 125 cc. of water. One drop of the filtrate was placed in each culture vial for the test.

2) Datura ovule extract: 16 g. of fresh Datura ovules from 9 capsules about 14 days after pollination was macerated in a mortar with 100 cc. of water and left overnight in the refrigerator. The filtrate was concentrated at 50 to 60° C. in vacuo to 1/4 of its original volume. One drop of this concentrate was placed in each culture vial.

3) Datura ovule water diffusate: 8.8 g. of fresh Datura ovules about 14 days after pollination was placed in a flask and covered with 56 cc. of water and kept in the refrigerator for about 4 days. 306 cc. of 95 cc. of ethyl alcohol was added to the diffusate and the mixture filtered. The filtrate was evaporated to dryness at 50 to 60° C. in vacuo and the residue taken up in 22 cc. of water. 5 drops of this solution was added to each vial for the test.

4) Datura ovule alcohol diffusate: 37 g. of fresh Datura ovules was placed in a flask and covered with 125 cc. of 80 per cent ethyl alcohol and kept at room temperatures for 2 days. The filtrate was evaporated to dryness at 50 to 60° C in vacuo and the residue taken up in 37 cc. of water. The solution was shaken 3 times with 100 cc. portions of purified diethyl ether. The water fraction was evaporated to dryness at 50 to 60° C in vacuo and the residue taken up in 37 cc. of water. One drop of this solution was added to each culture vial for the test.

5) Wheat germ extract: 30 g. of wheat germ (Horton and Converse Co, of Los Angeles) was soaked in 100 cc. of distilled water for 4 days. 550 cc. of 95 per cent ethyl alcohol was added to the filtrate, resulting in the formation of a precipitate. The precipitate was dried in vacuo and the dried residue taken up in 40 cc. of water. 5 drops of this solution was added to each culture vial.

6) Almond meal: 20 g. of powdered almond meal (S.B. Penick and Co. of New York City) was soaked in 200 cc. of distilled water and left in the refrigerator for 2 days. One drop of the filtrate was added to each test vial.

7) Yeast extract: Difco yeast extract powder was suspended in water. An aliquot of the filtrate was added to a 2 per cent sucrose culture medium at pH 6.8 to give a final concentration of yeast extract in the medium of 0.03 per cent. The vials were then autoclaved and used for the test.

All of the above extracts showed activity in promot-

ing the growth of Datura embryos in vitro. Little or no embryo growth-promoting activity was found in honey, dormant apple leaf and flower buds, potato sprouts, bean sprouts, potatoes, lemon pulp, corn meal, lemon peel, urine, and casein hydrolysate.

Table 20. Embryo growth stimulation by natural extracts.

Source	Method of extraction	Testing medium	Days of culture	Av. initial length of embryo in mm.		Per cent viability		Av. relative growth	
				Control	Extract	Control	Extract	Control	Extract
Coconut meat	water extract	2% sucrose	3	0.26	0.26	70	70	2.3	4.0
Datura ovules	water extract	1% dextrose	5	0.47	0.50	10	80	1.5	4.0
Datura ovules	water diffusate	1% dextrose	2	0.34	0.43	0	90	---	1.7
Datura ovules	alcohol diffusate	2% sucrose	2	0.35	0.29	70	50	3.4	5.3
Wheat germ	water extract	1% dextrose	2	0.34	0.43	0	70	---	1.9
Almond meal	water extract	2% sucrose	2	0.44	0.38	80	50	2.4	3.4
Yeast extract	water extract	2% sucrose	2	0.33	0.28	65	60	3.0	4.0

Chapter 7

Chemical studies on embryo factor in coconut milk

a) Preliminary fractionation of coconut milk.

The medium used in the testing for embryo factor activity in the fractions obtained during these preliminary fractionations was the 1 per cent dextrose medium, given in table 1. The embryos were measured after approximately 2 days of culture at 25°±1° C. As shown by the data in table 21, the embryo factor, as tested on 1 per cent dextrose medium, was insoluble in ether from both acid and alkaline extractions, butyl alcohol, acetone, ethyl acetate, benzene, carbon tetrachloride, and pyridine, but was soluble in water and 80 per cent ethyl alcohol. It was not adsorbed by Norit A, fullers earth, Lloyd's reagent (Eli Lilly and Co.), 60-100 mesh florisil (Floridin Co., Warren, Pa.), 40 to 60 mesh decalso (Air Conditioning Co., Los Angeles), and alumina (Mefford activated alumina).

Table 21. Fractionation of coconut milk, as tested on 1 per cent dextrose medium.

Chemical treatment	Fractions	% viability	Av. relative growth
	Original soln.	90	2.3
Acid ether extraction	Ether	0	---
Basic ether extraction	Ether	0	---
BuOH extraction	Alcohol	0	---

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Table 21. (Continued from previous page).

Chemical treatment	Fractions	% viability	Av. relative growth
Acetone extraction	Acetone	0	---
EtOAc extraction	Acetate	0	---
Benzene extraction	Benzene	0	---
CCl_4 extraction	CCl_4	0	---
Pyridine extraction	Pyridine	0	---
80% EtOH extraction	Alcohol	80	2.2
	Original soln.	90	2.3
Norit A	Filtrate	100	2.0
Fuller's earth	Filtrate	90	2.0
Lloyd's reagent	Filtrate	80	2.4
	Original soln.	80	1.7
Decalso chromatogram	Filtrate	90	2.0
Florisil chromatogram	Filtrate	90	1.9
Alumina chromatogram	Filtrate	60	1.8

b) Sucrose.

In view of the correlation in fractionation behavior between the embryo factor and sucrose, experiments on the effect of sucrose on the growth of embryos naturally suggested themselves. Indeed, the stimulating effect of sucrose on the growth of embryos was demonstrated through the following simple experiments.

In the first experiment embryos between 0.35 and 0.45 mm. in length were cultured in a 2 per cent and an 8 per cent dextrose and sucrose media, respectively. The sup-

plementary vitamins, agar, and inorganic salts were added in each case. Final measurements of the growth of the embryos were made after 2 days with the following results:

Table 22. Growth of embryos in dextrose and sucrose media.

Medium	Per cent viability	Av. relative growth
2% dextrose	20	1.3
2% sucrose	60	1.6
8% dextrose	0	---
8% sucrose	90	1.9

The second experiment which demonstrated the growth-promoting effects of sucrose consisted of the growing of embryos, which were 0.3 to 0.5 mm. in length, in a 2 per cent dextrose and a 2 per cent sucrose media, respectively, each containing 4 drops of coconut milk per culture vial. The growth of the embryos was measured after 1, 2, and 4 days of culture.

Table 23. Effect of sucrose on the growth of embryos.

Medium	Per cent via-bility	Av. relative growth		
		1 day	2 days	4 days
2% dextrose + coconut milk	50	1.4	2.3	4.3
2% sucrose + coconut milk	80	1.8	3.4	7.8

From the above data it is obvious that the growth of the embryos was stimulated by the presence of sucrose.

Embryos cultured in sucrose medium gave nearly twice as good growth and nearly twice as high a percentage viability as those cultured in dextrose medium. The same response was seen both in the presence and the absence of coconut milk.

To determine the response of embryos to different sucrose concentrations and different pH's the following experiment was conducted. Embryos, which were between 0.2 and 0.45 mm. long, were cultured in media containing 0.9, 1.8, 2.5, 3.5, 3.9, and 7.7 per cent sucrose, respectively, in the place of dextrose in the 1 per cent dextrose medium given in table 1. The pH was adjusted to an initial value of 5.2 and 7.0, respectively, with sulfuric acid and potassium hydroxide. At the end of the experiment the pH's of the media were measured with a Beckmann pH meter and the values are given in table 24, together with the growth of the embryos after 2 days.

According to the figures given in the table, the optimum concentration of sucrose included a wide range between 1 and 5 per cent at pH 6.4. No growth at pH 5.2 was obtained with any concentration of sucrose.

Although the embryos grew very well in the presence of sucrose during the first 2 days of culture it was found that other substances besides sucrose were needed in the basic medium for the continued growth of the embryos. Unlike the embryos supplied with coconut milk those cultured in the 2 per cent sucrose medium alone soon ceased growth, as illustrated in table 25, and figure 10. In this experiment

embryos about 0.35 mm. in length were cultured in media containing different concentrations of sucrose with and without the addition of an active solution (stock D).

In this respect, the graph in figure 11 is also pertinent. Here, the average fresh weight of the embryos cultured for 11 days at 32°±0.5° C in a 2 per cent sucrose medium at pH 6.8 is plotted against the relative concentration of embryo factor added. It is to be noted that the weight was a linear function of the embryo factor concentration. Furthermore, the weight in the absence of embryo factor was very low even at the optimum concentration of sucrose.

Table 24. Effect of different concentrations of sucrose on the growth of embryos at pH 5.2 and 6.4.

Per cent sucrose	pH	Per cent viability	Av. relative growth
0.0	ca. 5.2	0	---
	6.4	0	---
0.9	ca. 5.2	0	---
	6.5	80	1.9
1.8	ca. 5.2	10	1.5
	6.4	80	2.3
2.5	ca. 5.2	0	---
	6.4	80	2.3
3.5	ca. 5.2	0	---
	6.3	70	2.4
5.9	ca. 5.2	10	1.5
	6.4	80	2.3
7.7	ca. 5.2	0	---
	6.4	10	2.2

Table 25. Growth of embryos in sucrose medium in the presence and the absence of stock D.

Medium	Per cent viability	Av. relative growth	
		2 days	4 days
0.0% sucrose, no stock D	0	---	---
0.8% sucrose, no stock D	40	1.5	3.0
2.4% sucrose, no stock D	40	1.5	2.8
4.8% sucrose, no stock D	100	1.9	3.0
0.0% sucrose, stock D	0	---	---
0.8% sucrose, stock D	70	2.5	6.0
2.4% sucrose, stock D	90	3.0	8.2
4.8% sucrose, stock D	100	2.7	7.1

During the course of these investigations, it was found that the response of the excised embryos to sucrose could be decreased to zero by keeping the capsules off the plant for several days before the isolation and the culturing of the embryos. A typical investigation is presented below.

A capsule was picked from a plant in the field and the ovules were removed and placed in a sterile Petri dish in a moist chamber at 23° C. Ovules were taken randomly from the dish after different lengths of time and the embryos were isolated and cultured in a 5 per cent sucrose medium with and without the addition of an active solution (K-2). The growth of the embryos, which were initially from 0.3 to 0.6 mm. in length, is shown in table 26.

From the figures in table 26 it can be seen that the embryos which were isolated from the fresh capsule showed

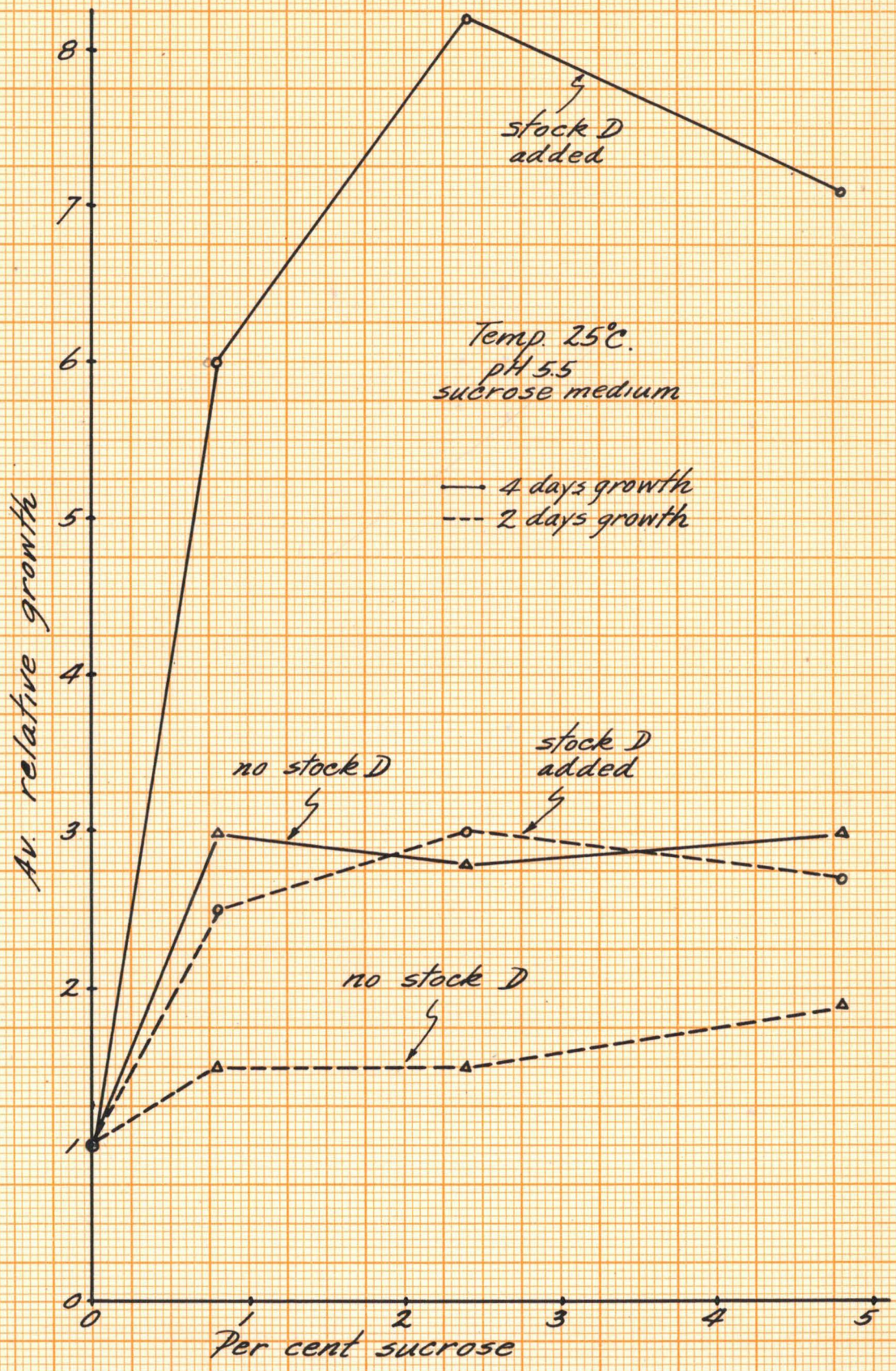

Fig. 10. Effect of sucrose on embryo growth.

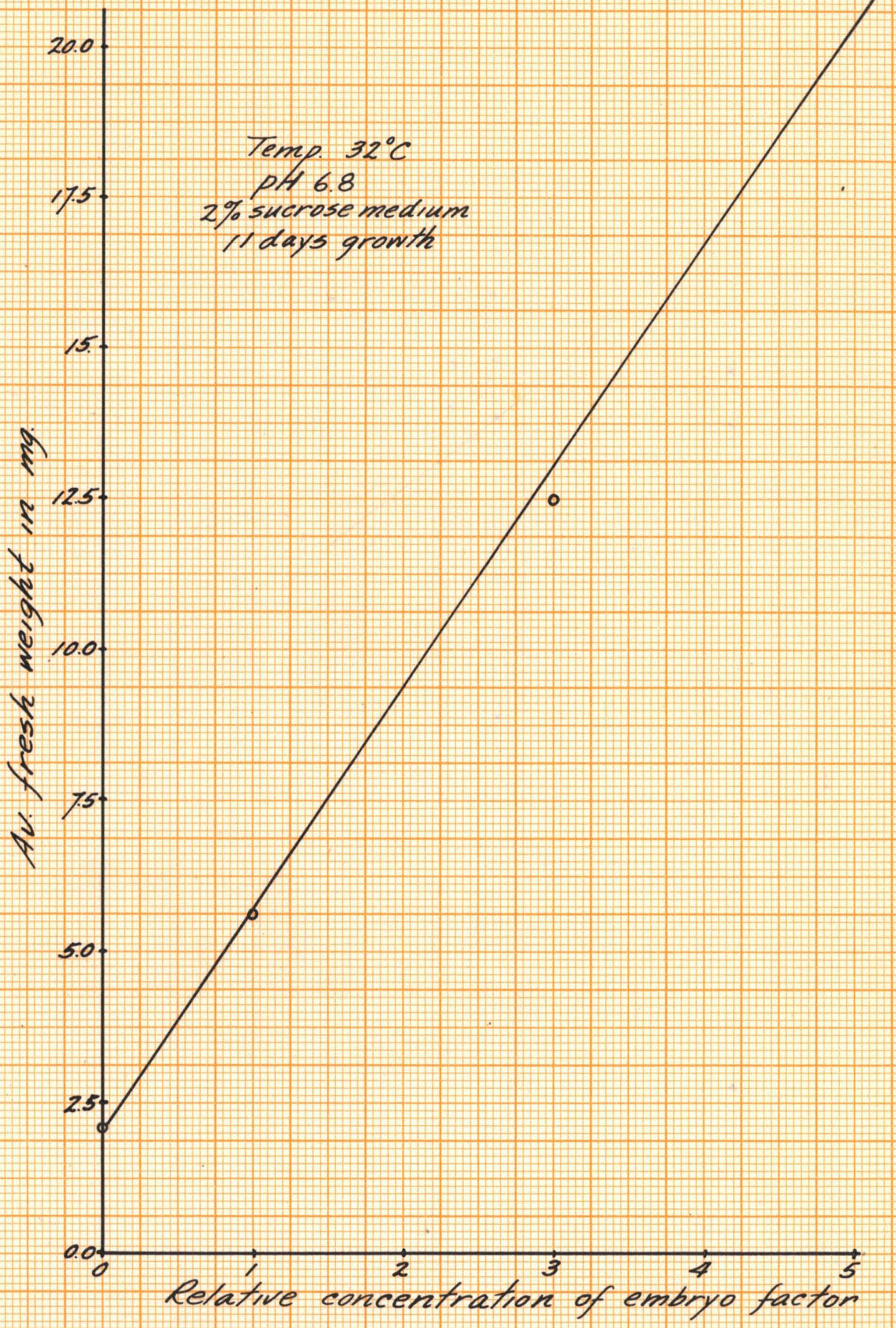

Fig. 11. Effect of embryo factor concentration on embryo growth.

considerable growth in the sucrose medium even in the absence of K-2 during the first 4 days of culture. After aging for a single day the growth of the embryos in the sucrose medium without K-2 dropped to a very low rate. No growth was obtained when the embryos were aged for 4 days or longer and grown in the 5 per cent sucrose medium without the addition of K-2. Similar embryos, however, grew very nicely in the sucrose medium when K-2 was added.

Table 26. Growth of aged embryos in 5% sucrose medium.

Length of aging	Medium	Per cent viability	Av. relative growth		
			2 days	3 days	4 days
0 day	no K-2	60	2.1	---	3.4
	K-2	50	2.4	---	4.3
1 day	no K-2	40	---	1.3	---
	K-2	80	---	3.9	---
4 days	no K-2	0	---	---	---
	K-2	70	1.9	---	---
6 days	no K-2	0	---	---	---
	K-2	70	2.0	---	---

c) Growth inhibitor .

One of the chief difficulties encountered in the use of the bio-assay described in the previous pages is the sensitivity of Datura embryos in vitro to growth inhibitors. Often, a solution which did not promote the growth of the embryos did so after certain chemical treatments, whereby the growth inhibiting substances had been removed. The

presence of a growth inhibitor was indicated from the experiments on the effect of sucrose on the growth of Datura embryos in vitro. Sucrose (Chemical Co., C.P. grade) was recrystallized from a 50 per cent ethyl alcohol solution. The original and the recrystallized sucrose were added, respectively, in place of the dextrose in the medium given in table 1. Embryos, which were 0.25 to 0.55 mm. in length, were used and the final measurements were made after 2 days of growth at 32 ± 0.5° C. The results of the test are presented in table 27.

Table 27. Growth of embryos in recrystallized sucrose.

Conc. of sucrose	Per cent viability	Av. relative growth
0.00% sucrose	0	---
1.0% C.P. sucrose	0	---
2.3% C.P. sucrose	40	1.3
4.4% C.P. sucrose	70	2.0
9.6% C.P. sucrose	50	1.3
1.0% recrys. sucrose	10	1.8
2.3% recrys. sucrose	40	1.4
4.4% recrys. sucrose	80	1.8
9.6% recrys. sucrose	90	2.0

The figures for the non-recrystallized sucrose showed a sharp drop in the relative growth of the embryos cultured in the highest sucrose concentration of 9.6 per cent. A decrease in the percentage of viable embryos paralleled this

decrease in relative growth. An identical drop was not obtained in the embryos, which were grown in media containing recrystallized sucrose. This, of course, could be explained on the basis of the removal of a growth inhibitor from the sucrose through the recrystallization process. Inasmuch as no inhibitory effect was observed in the low sucrose concentrations, the C.P. sucrose was used for the culture media of subsequent bio-assays.

A similar inhibitory effect was also found in previously active extracts, which had been autoclaved or heated at 60° C for a few hours. Data for a typical experiment of this nature are shown in table 28 and figure 12. An active extract of Datura ovules was added to a 2 per cent sucrose medium. Half of the culture vials were autoclaved and the other half were not autoclaved after the addition of the extract. Embryos, which were 0.25 to 0.6 mm. in length, were cultured at 32 ± 1° C and their growth was measured after 2 and 3 days, respectively.

The growth of the embryos in the non-autoclaved ovule extract increased with increasing concentrations up to 1 drop per vial, at which point the growth remained more or less constant. However, in the case of the autoclaved ovule extract, the growth of the embryos increased up to a concentration of about 0.5 to 1.0 drop per vial, after which a sharp decrease in growth occurred. Furthermore, the embryos in the higher concentrations of the autoclaved extract appeared brown and transparent, despite their growth.

Table 28. Growth of embryos in autoclaved and non-autoclaved ovule extract.

Drops per vial		% viability	Av. relative growth	
			2 days	3 days
Controls (2% sucrose)	A	60	2.2	3.5
	B	50	2.4	3.4
Not autoclaved				
	0.25*	70	2.7	4.4
	0.5	60	2.7	4.7
	1.0	80	3.0	5.1
	1.5	90	3.2	5.0
	2.0	60	3.1	5.0
Autoclaved				
	0.25	40	2.0	3.6
	0.5	70	2.6	4.6
	1.0	50	2.8	4.2
	1.5	80	2.3	3.8
	2.0	50	1.5	1.8

The embryo growth in the vials containing 2 drops of autoclaved extract was far less than even that of the controls.

Some of the inhibitory substances could be removed by extraction with ethyl alcohol and butyl alcohol and by precipitation with lead acetate. Supporting data for this statement are included in table 29. In the first experiment 100 cc. of a coconut milk concentrate (c), which was obtained by concentrating coconut milk at 55°-60° C <u>in vacuo</u>, was

* "0.25 drop" per vial means: The extract was diluted 4 times and 1 drop of the diluted solution was added to each culture vial.

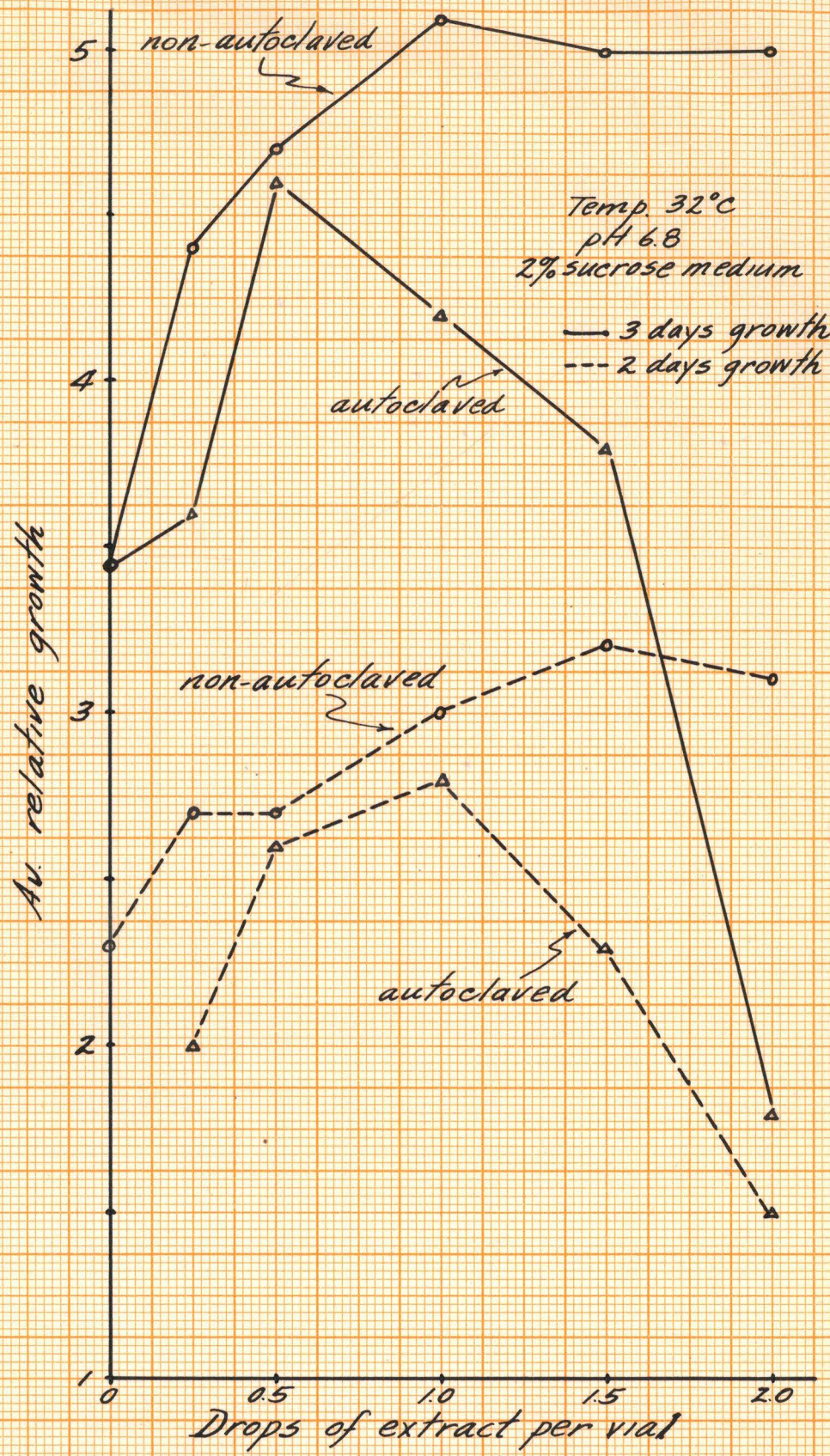

Fig. 12. Embryo growth in Datura ovule extracts.

shaken with 350 cc. of 95 per cent ethyl alcohol and the supernatant liquid decanted from the pasty residue. Both fractions were evaporated to dryness in vacuo at 55 to 60° C. The dried residues, which were approximately equal in weight, were taken up in equal volumes of water for the test. Embryos, which were 0.5 to 0.8 mm. in length, were cultured in a 2 per cent sucrose medium at 25±1° C.

In the second experiment 15 cc. of coconut milk concentrate (B) was shaken 2 times with 100 cc. portions of butyl alcohol. Two per cent of the dry weight was found in the alcohol fraction. Both alcohol and water fractions were evaporated to dryness in vacuo at 55 to 60° C and the residues were taken up in equal volumes of water. Embryos 0.25 to 0.5 mm. in length were cultured on a 1 per cent dextrose medium for this test.

In both of the above fractionations the residues, after being separated from the solvent, gave a much higher growth than the original extracts. In the first experiment the addition of the alcohol fraction to the control medium stopped the growth of the embryos completely, which gave considerable growth in the sucrose medium alone. The assumption that inhibitory substances were present in the alcohol fraction is, therefore, logical. In the second experiment the water fraction remaining after the extraction with butyl alcohol promoted a much higher rate of growth than the original concentrate prior to the butyl alcohol extraction.

Table 29. Removal of growth inhibitor by ethyl and butyl alcohol.

Treatment	Fraction	Conc. per vial	% viability	Av. relative growth
Ethyl alcohol	Control (2% sucrose)	---	100	1.9
	Alcohol	1 drop	0	---
		3 drops	0	---
	Residue	1 drop	100	4.3
		3 drops	70	2.5
Butyl alcohol	Control (1% dextrose)	---	0	---
	Conc. B	1 drop	50	1.7
		2 drops	80	2.4
		4 drops	90	2.3
	Alcohol	1 drop	0	---
		2 drops	0	---
		4 drops	0	---
	Water	1 drop	90	2.6
		2 drops	100	4.2
		4 drops	100	4.9

Another simple way to remove part of this growth inhibitor was found to be the precipitation with lead acetate. The toxic principle was precipitated, while the embryo factor remained in solution. A typical purification scheme is given in fractionation I. The starting material in this fractionation was the residue, which resulted from the treatment of coconut milk concentrate with 95 per cent

ethyl alcohol. The fractionation included first a precipitation with lead acetate (Merck, reagent grade). The lead ions were then removed through a series of steps involving the addition of excess sulfuric acid and the extraction of the embryo factor with 80 per cent ethyl alcohol. Finally, the embryo factor was precipitated from the active alcohol solution by the addition of sulfuric acid.

Datura embryos, which were initially 0.15 to 0.4 mm. in length, were cultured at 32±1° C in a 2 per cent sucrose medium, to which different aliquots of the different fractions were added. The growth of the embryos after 2 days is given in table 30 and figure 13.

From the graphs in figure 13, it can be seen that the embryos which were cultured in media containing the lead precipitate fraction (A) were markedly suppressed in their growth. Fractions D and E, which were not precipitated by lead acetate, remained active. The possibility that lead was the toxic factor involved seemed unlikely because similar toxicity symptoms were not observed in fractions B, D, and E, which were subjected to the same treatments as fraction A. These data, therefore, strongly suggest that at least a part of the inhibitory substances could be precipitated by lead acetate.

Fractionation I. Scheme for lead precipitation of growth inhibitors.

10 cc. concentrate (C), dry wt. ca. 2.8 g.

↓ satd. $PbAc_2$ soln. till no further pption.

precipitate → filtrate (Cont. next page).

↓ 4 cc. 1:1 H_2SO_4; 10 cc. H_2O.

filtrate → precipitate (Discarded).

↓ 95% EtOH to make final conc. = 80%.

filtrate → precipitate (Discarded).

↓ KOH till nearly neutral.

filtrate → precipitate (Discarded).

↓ evapd. at 55-60° C in vacuo.

residue

↓ 20 cc. 80% EtOH.

filtrate → insol. residue (Discarded).

↓ evapd. at 55-60° C in vacuo.

residue, dry wt. ca. 0.4 g.

↓ 15 cc. H_2O, KOH till pH = 8.7.

test soln. A.

(Continued on next page).

Fractionation I. (Continued from previous page).

filtrate (from previous page).

↓ 95% EtOH to make final conc. = 80%.

filtrate → ppt. (F) (Cont. next page).

↓ 55-60° C in vacuo.

15 cc. soln.

↓ H_2SO_4 till no further pption.

filtrate → precipitate (Discarded).

↓ 55-60° C in vacuo.

residue

↓ 20 cc. 80% EtOH.

filtrate → residue (Discarded).

↓ 55-60° C in vacuo.

residue

↓ H_2O

15 cc. solution

↓ 2 drops H_2SO_4

filtrate → faint ppt. (Discarded).

↓ KOH til pH = 7, 55-60° C in vacuo.

residue

↓ 20 cc. 80% EtOH

clear soln.

↓ H_2SO_4 till no further pption.

precipitate (E) → filtrate (D)

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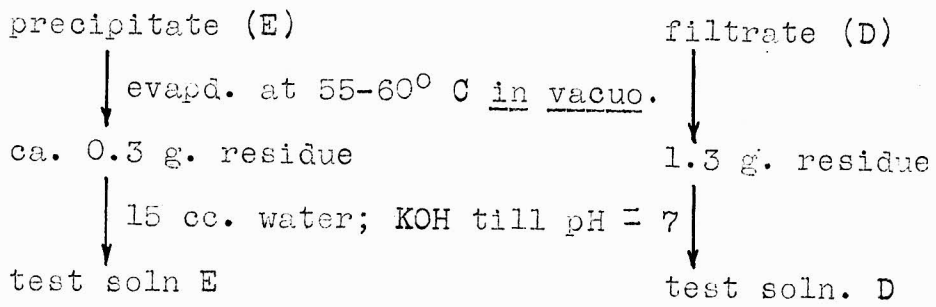
Fractionation I. (Continued from previous page).



precipitate (F) (Continued from previous page).

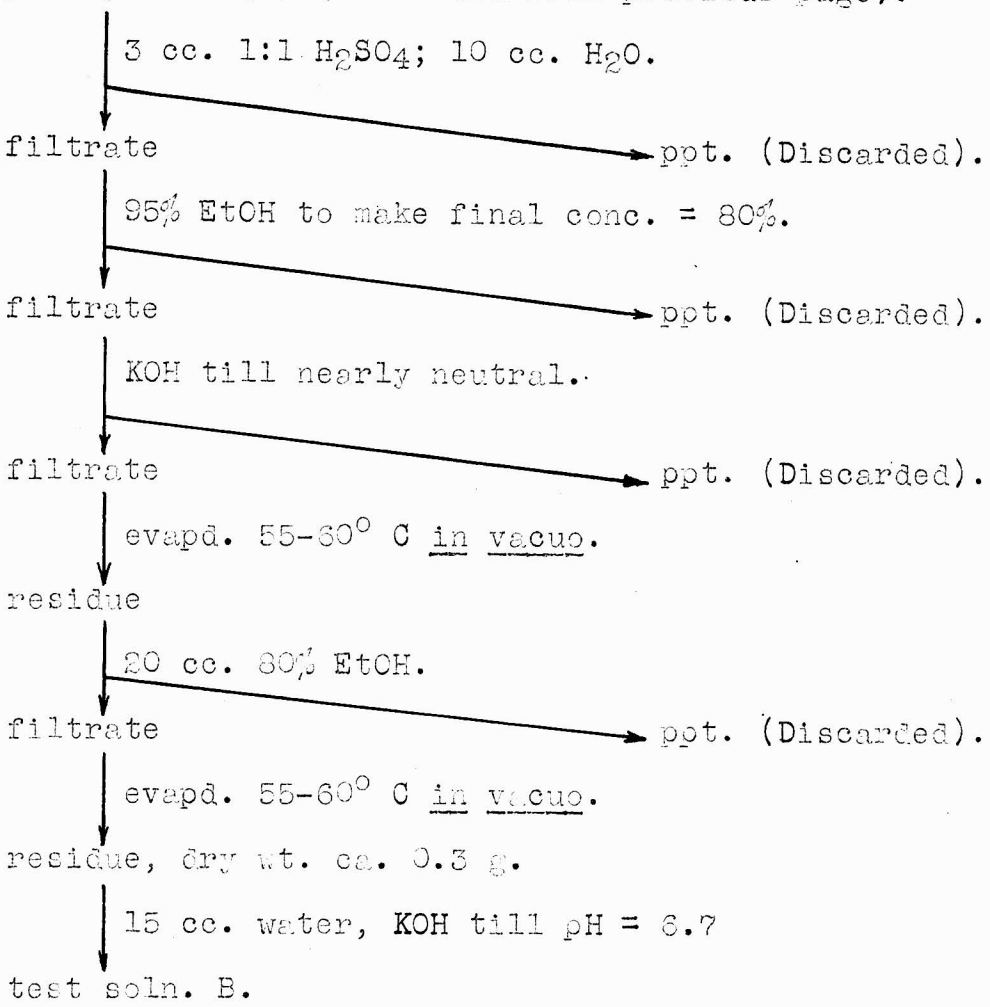


Table 30. Growth promoting activity of test solutions from fractionation I.

Test	Fraction	dry wt. in mg. per vial	Per cent via-bility	Av. relative growth
430112	Control (2% sucrose)	0.0	65	3.1
	C	0.7	80	4.0
		3.5	40	4.2
		7.0	90	3.5
	A	0.5	60	2.8
		1.0	80	2.7
		3.0	90	2.3
	B	0.35	60	3.3
		0.7	90	3.3
		2.1	60	3.3
430111	Control (2% sucrose)	0.0	90	2.3
	C	0.7	90	2.9
		3.5	90	3.0
		7.0	80	3.1
		21.	80	2.0
	D	0.3	90	2.5
		1.6	80	2.6
		3.2	90	2.8
		9.6	80	2.4
	E	0.07	90	2.8
		0.37	80	3.0
		0.74	90	2.7
		2.2	80	3.1

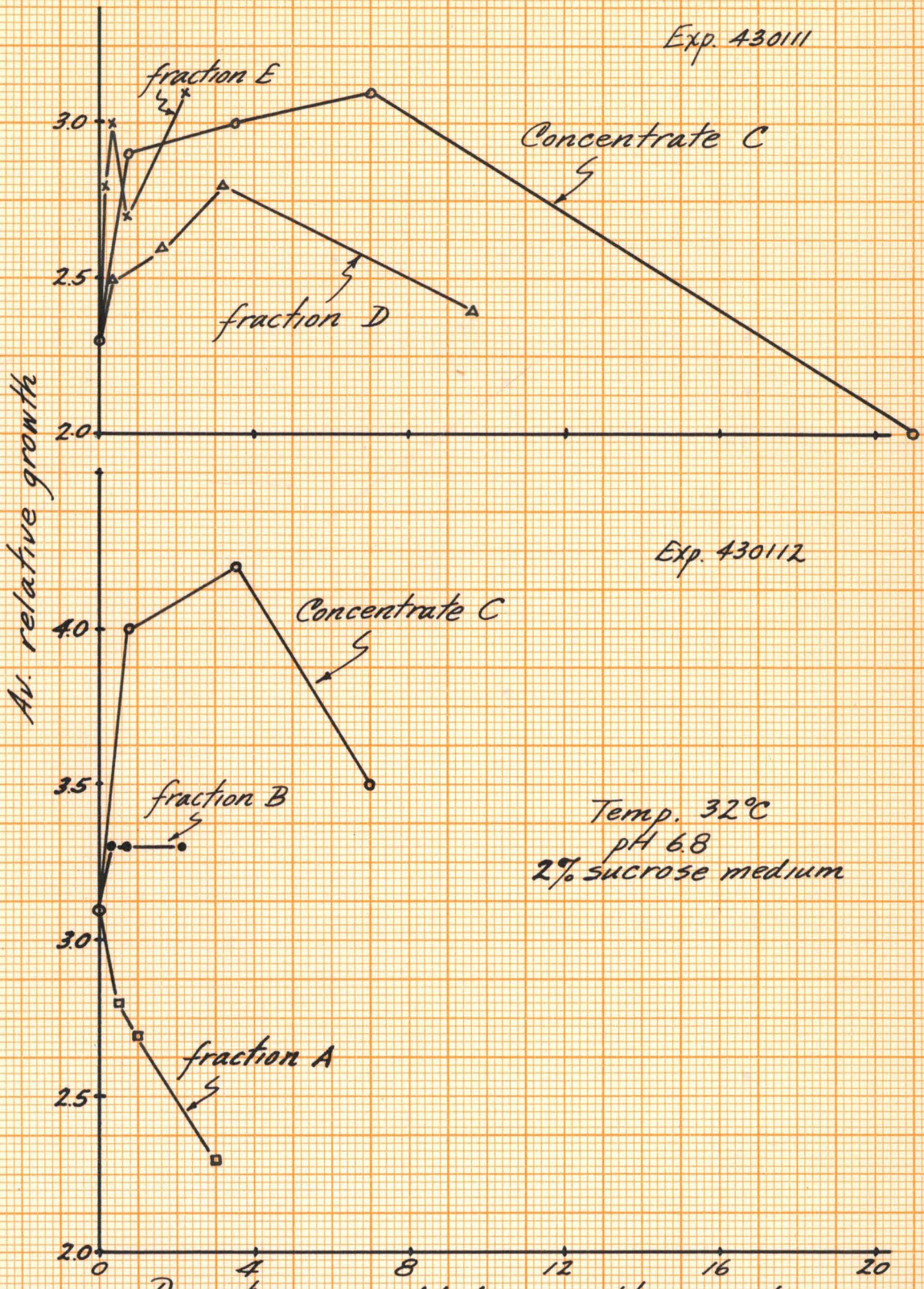


Fig. 13. Growth of embryos supplied with test solutions from fractionation I.

d) Root inhibitor.

A growth characteristic of Datura embryos cultured in media containing fresh coconut milk was the lack of root formation. The cotyledons and the hypocotyl enlarged but no roots appeared as long as the embryos were left in the same medium. But if the embryos, after reaching a size of several millimeters in length, were transferred to a 1 per cent dextrose medium, which did not contain any coconut milk, root formation occurred (47). Attempts to remove this root inhibitor were successful through the use of solvents, such as ethyl alcohol, butyl alcohol, and diethyl ether. A typical example of the methods used for the removal of the root inhibitor is the following:

14.7 liters of fresh coconut milk was concentrated at 55 to 60° C in vacuo for 8 hours to a volume of 3.45 liters. 846 cc. of 95 per cent ethyl alcohol was added slowly with vigorous stirring to 100 cc. of this concentrate (test solution B). The supernatant alcoholic fraction was separated from the pasty residue, which was dissolved in 25 cc. of water. The latter solution was shaken with 100 cc. of butyl alcohol and the 2 layers separated. The water layer was shaken with 100 cc. of diethyl ether and dialyzed with a cellophane tubing in distilled water. The dialyzate was then concentrated at 55 to 60° C in vacuo to a volume of 50 cc. (test solution E). 270 cc. of 95 per cent ethyl alcohol was added to this concentrate in small portions with vigorous stirring. A precipitate was formed upon the addition of the alcohol. This precipitate was dried in

vacuo at 55 to 60° C and taken up in 50 cc. of water (test solution F). Of all the different fractions tested in a 1 per cent dextrose medium with embryos, which were 0.35 to 0.6 mm. long, only fractions E and F were active. The data for fractions B, E, and F are shown in table 31.

Table 31. Removal of root inhibitor.

Test Solution	% viability	Av. relative growth		% rooting in 5 days
		2 days	5 days	
Control (1% dextrose)	0	---	---	0
B	0	---	---	0
E	100	2.3	6.9	30
F	100	2.2	5.9	80

Thus, it can be seen that the root inhibitor and also the growth inhibitor were removed by successive extractions with ethyl alcohol, butyl alcohol, diethyl ether, and again with ethyl alcohol. Although the original concentrate B gave no stimulation of growth in the embryos, the final fractions E and F gave excellent growth. Very good root formation was observed in the embryos which were supplied with fraction F.

Because of the similarity in the behavior of the root inhibitor in coconut milk and the auxins to solvent extractions, Dr. van Overbeek investigated the content of the latter in coconut milk. Coconut milk was subjected to different treatments, such as ether extraction, alkaline

hydrolysis, boiling, and autoclaving and the auxin content of these fractions determined by the standard Avena coleoptile test (48). Results of such a test are given in table 32.

Table 32. Liberation of auxin from coconut milk by different treatments.

Treatment	Avena deg. curvature (Comparable data)
Agar blocks soaked in coconut milk concentrate.	0.6
Concentrate extracted with ether; residue from ether fraction taken up in volume of water equal to that of the original concentrate.	21.5
Concentrate hydrolyzed at pH 10 for 24 hours at 4° C.	7.9
Hydrolysis followed by ether extraction; residue from ether fraction taken up in volume of water equal to that of the original concentrate.	28.5
Concentrate boiled for 5 minutes.	0
Concentrate autoclaved 3 times at 15 pounds pressure for 20 min.	8.7
Boiling followed by ether extraction; residue from ether fraction taken up in volume of water equal to that of original concentrate.	21.6

The data show that although untreated coconut milk concentrate showed only a very low Avena curvature the treated concentrates produced large curvatures. Therefore, a good supply of auxin or precursors was present in coconut milk.

Since the presence of auxin in coconut milk was established the effects of the addition of auxin with a somewhat purified extract were studied. Different concentrations of indole-3-acetic acid (Merck) were added to a 5 per cent sucrose medium with and without an active extract (K-2). K-2 was obtained from coconut milk concentrate after successive treatments with ethyl alcohol, diethyl ether, calcium oxide, and ethyl alcohol. Embryos, which were 0.2 to 0.4 mm. in length, were used. The results of the experiment are shown in table 33.

Table 33. Effect of indole-3-acetic acid on the growth of Datura embryos in vitro.

Medium	Conc. of IAA/liter	% viability	Av. relative growth		Condition of embryo
			2 days	8 days	
- K-2	0	70	2.1	3.9	transparent
	10	50	2.0	3.1	transparent
	100	60	1.9	3.3	transparent
	750	30	2.2	---	callus
	1000	50	1.7	---	callus
+ K-2	0	60	2.7	34±2.0	normal; no roots
	10	90	2.7	50±6.8	normal; 45% rooting
	100	70	2.7	28	callus-like
	750	60	2.1	6.9	callus
	1000	80	2.0	3.4	callus

A significant stimulation in both root and cotyledon and hypocotyl growth was found with the addition of 10 gammas

of indole-3-acetic acid per liter of medium in the presence of K-2 only. No beneficial effects were found in the absence of K-2. In concentrations above 100 gammas per liter callus formation resulted in both the absence and the presence of K-2. Although the results of this particular experiment were clear and convincing, the stimulating action of 10 gammas of indole-3-acetic acid on embryos supplied with K-2 could not be duplicated a month later. However, the changes undergone by K-2 could not be ascertained to explain this apparent discrepancy. ~~It may be that sufficient amounts of free auxin were formed in the K-2 on standing, so that added auxin was not required for optimal growth.~~ About 6 months later, however, a similar stimulation in embryo growth was obtained through the addition of 10 gammas of naphthalene acetic acid to a 2 per cent sucrose medium, containing a purified embryo factor preparation.

e) Ash.

Coconut meat, milk, and husk were ashed separately in platinum dishes in a muffle furnace and tested for their effects on the growth of Datura embryos in vitro. No beneficial effects were found in all cases. The media and the concentrations of the ash samples, which were tested, are given in the following table:

Table 34. Concentrations of coconut ash found to be inactive in promoting the growth of embryos.

Test no.	Ash of:	Test medium	Concentration
420902	Meat	2% dextrose	equiv. to 0.04 to 135 mg. of dry meat per culture vial.
420831	Husk	2% dextrose	equiv. to 0.04 to 125 mg. of husk per culture vial.
430326	Milk	2% sucrose	equiv. to 1 to 6 drops of milk per culture vial.

Among the many subsequent repititions of these experiments, occasionally a beneficial effect of the ash of the growth of the embryos was observed. In all cases, however, the effect was slight, if significant.

f) Fractionation of coconut milk for embryo factor.

Based on the facts known from the experiments described in the previous pages of this chapter a fractionation of coconut milk was performed. The scheme, which was followed, is diagrammed in fractionation II. Tests for embryo factor was conducted with embryos, which were about 0.25 to 0.6 mm. in length, cultured in 2 per cent sucrose medium. The medium was buffered at pH 6.5 to 7.0 with 0.01 molar dipotassium-monosodium phosphate. The final measurements were made after 2 days of culture at 32±0.5° C. Most of the active fractions were tested several times in different dilutions.

In order to calculate the amount of embryo factor

present after the various fractionation steps a quantitative unit was adopted. The unit was designated as the Datura unit and defined as follows: One Datura unit was the quantity of embryo factor which had to be added to a standard culture medium to give a relative growth difference in one Datura embryo of 0.5 over that of the control. The standard medium was the 2 per cent sucrose medium (2 per cent sucrose in place of 1 per cent dextrose in the medium given in table 1). The medium was buffered at pH 6.8 with 0.01 molar dipotassium-monosodium phosphate. 3/4 cc. of this medium was placed in each culture vial, which contained one embryo. The relative growth measured after 2 days of culture at 32±1° C was taken as the ratio: final length/ initial length. The initial length of the embryos used varied from 0.2 to 0.5 mm.

An example is presented to show the application of the above definition.

Embryo growth in the standard culture medium to which 0.002 g. of a test substance was added:

Av. initial length of 10 embryos = 0.40 mm.
Av. final length of 10 embryos = 1.20 mm.

Relative growth = 1.20/0.40 = 3.00

Embryo growth in the standard culture medium only:

Av. initial length of 20 embryos = 0.35 mm.
Av. final length of 20 embryos = 0.87 mm.

Relative growth = 0.87/0.35 = 2.49

Therefore, relative growth difference = 3.00 - 2.49

= 0.51

Hence, 0.002 g. of the test substance contained 1 Datura unit of embryo factor.

It must be realized, however, that the Datura units given in fractionation II are only rough approximations of the actual quantity of embryo factor present. In the first place, the embryos were sensitive to inhibitors. Furthermore, the pH of the culture medium was not always maintained at exactly 6.8 and the final measurements were not always made after exactly 48 hours.

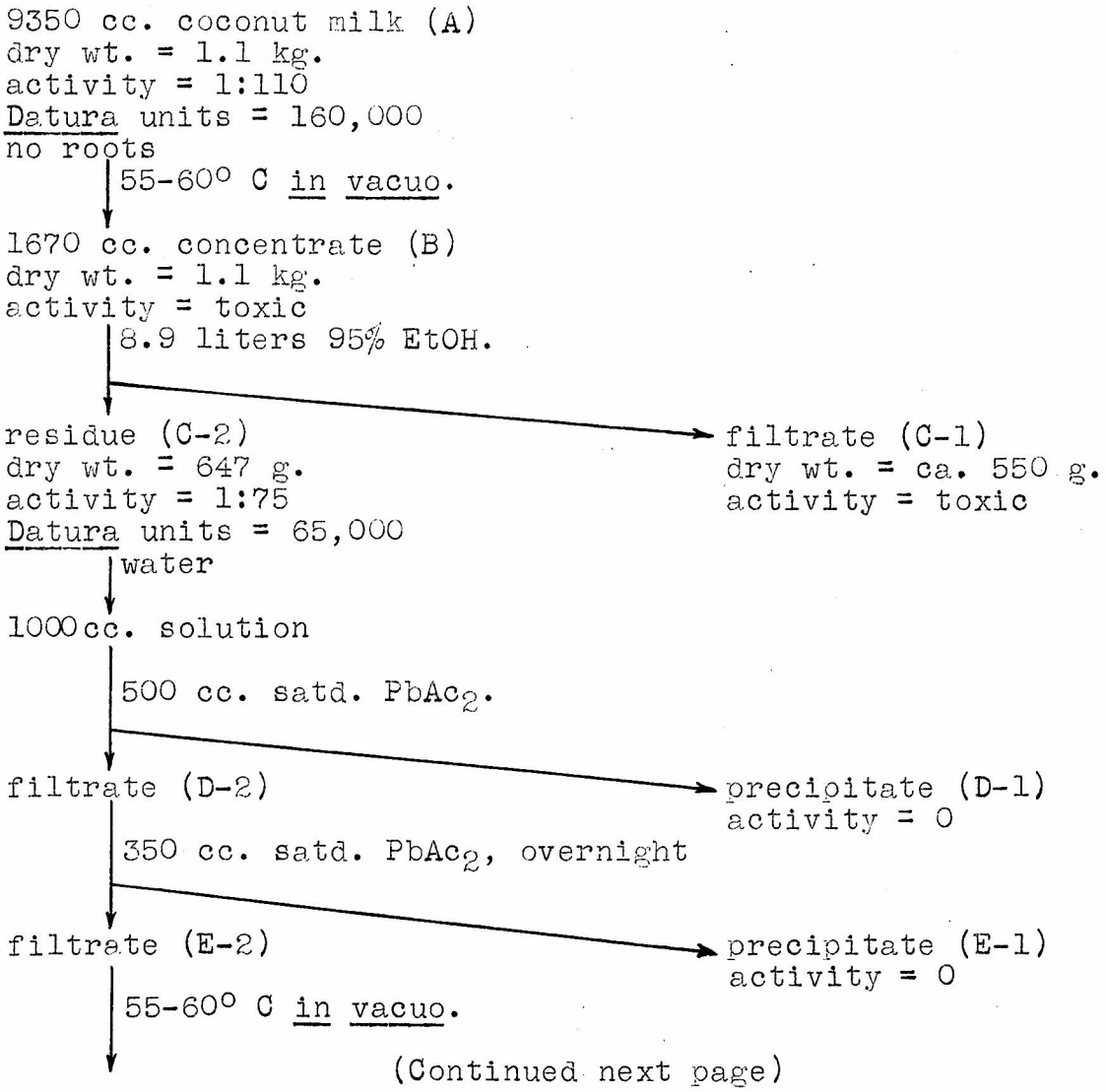
Milk from fresh coconuts (from L.A. Nut House of Los Angeles) was concentrated in vacuo at 55 to 60° C in a glass still. After this concentration procedure and standing for several months in the refrigerator the concentrate showed no activity in promoting the growth of embryos. The possibility of a liberation of toxic substances suggested the alcohol extraction step as described in part "c" of this chapter. After the alcohol treatment activity was again observed in the residue fraction (C-2); the toxic substances were taken up by the alcohol (C-1).

The residue fraction was dissolved in water and subjected to a lead precipitation process. To the resulting 80 per cent alcohol solution, after the removal of the lead ions, the embryo factor was precipitated with sulfuric acid. The resulting precipitate (I-2) was active in a dilution of 1:1200, which represented an enrichment of 11 times. The type of growth closely resembled that of embryos cultured in a medium containing coconut milk. No roots were formed. It was therefore subjected to several ethyl alcohol extractions in order to remove the root inhibitor. The

final residue (L-2) promoted the growth of embryos at a dilution of 1:19,000. This represented an enrichment of 170 times that of the original coconut milk. Furthermore, two thirds of the embryos cultured in media containing optimal concentrations of L-2 produced healthy roots.

Further fractionations were accompanied by a loss of activity so that it was not yet possible to obtain fractions with greater activity than L-2.

Fractionation II. Fractionation scheme of coconut milk.



(Continued next page)

filtrate (E-2)

↓ 55-60° C *in vacuo*.

1200 cc. solution

↓ 6N H_2SO_4 till no further pption. → precipitate (F-1) activity = 0

filtrate (F-2)

↓ 6N KOH till pH = 7

neutral soln.

↓ 55-60° C *in vacuo*.

residue

↓ 1500 cc. 80% EtOH. → lower syrup (G-1) activity = toxic

upper liquid layer (G-2)

↓ 55-60° C *in vacuo*

residue
dry wt. = 190 g.

↓ water added.

400 cc. soln.

↓ 1600 cc. 95% EtOH added.

clear solution

↓ 6N H_2SO_4 till pH = 6.0 → precipitate (H-1)
dry weight = 31.9 g.
activity = 1:1400
enrichment = 13x
Datura units = 59,000
no roots
filtrate (I-1)
activity = toxic

filtrate (H-2)

↓ 6N H_2SO_4 till pH = 3.5.

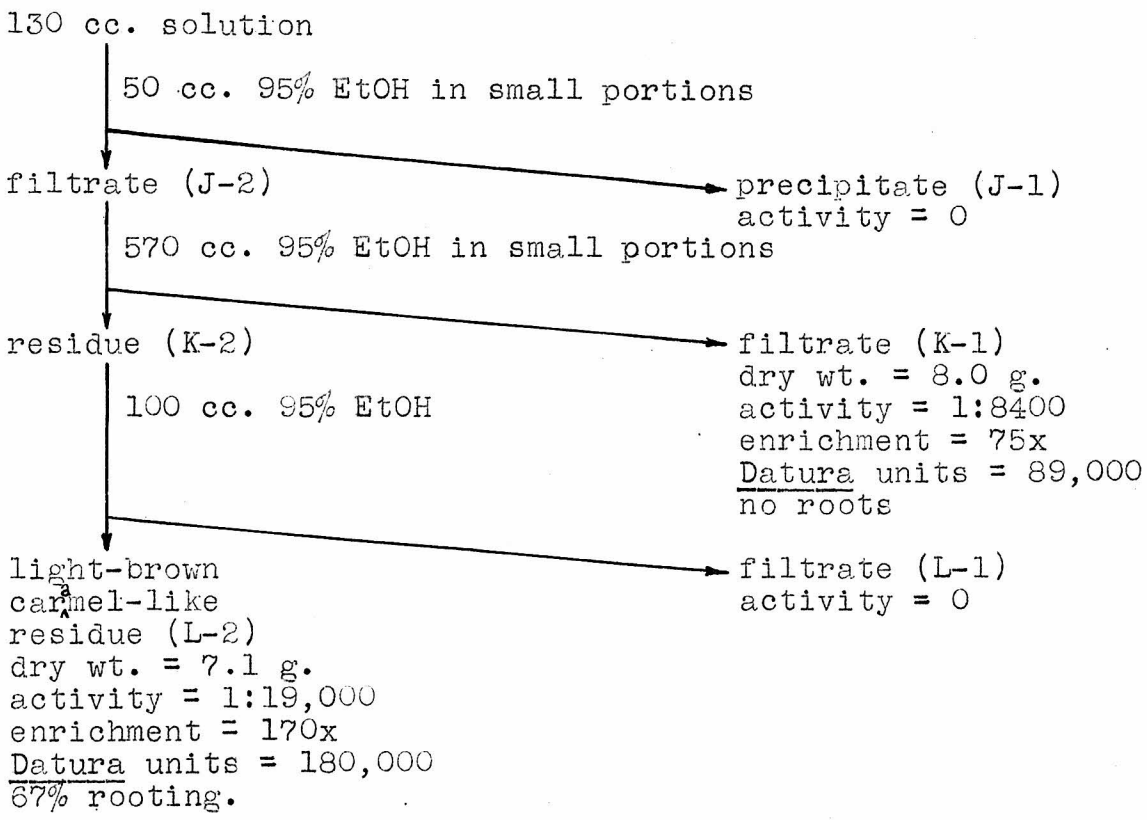
precipitate (I-2)
dry wt. = 38.4 g.
activity = 1:1200
enrichment = 11x
Datura units = 62,000
no roots

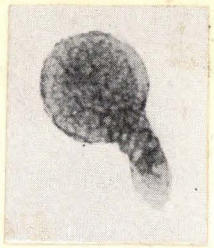
↓ water added.

130 cc. solution

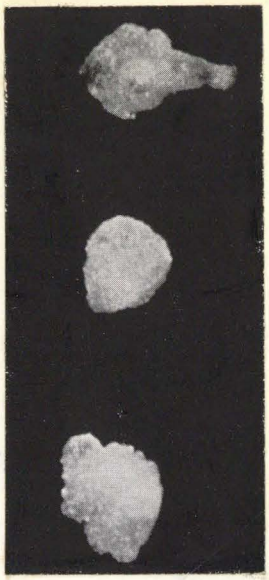
↓ 50 cc. 95% EtOH in small portions.

(Cont. next page)

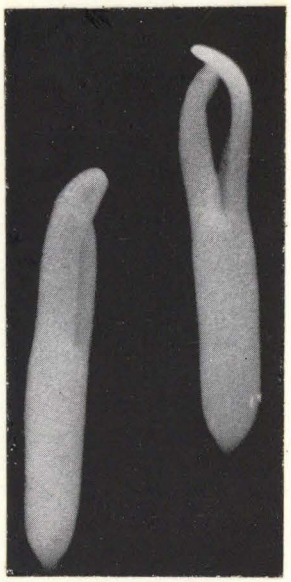

130 cc. solution
50 cc. 95% EtOH in small portions
filtrate (J-2)
precipitate (J-1)
activity = 0
570 cc. 95% EtOH in small portions
residue (K-2)
filtrate (K-1)
dry wt. = 8.0 g.
activity = 1:8400
enrichment = 75x
Datura units = 89,000
no roots
100 cc. 95% EtOH
light-brown
caramel-like
residue (L-2)
dry wt. = 7.1 g.
activity = 1:19,000
enrichment = 170x
Datura units = 180,000
67% rooting.
filtrate (L-1)
activity = 0



1) Proembryo of Datura stramonium, 0.1 mm. in diameter. These do not grow in media lacking embryo factor.



2) Callus-like bodies, 0.35 mm. in diameter, which developed from heart-shaped embryos, 0.35 mm. in length, after 11 days of culturing in media containing 1 per cent dextrose, inorganic salts, vitamin supplements, and autoclaved coconut milk.



3) Normally differentiated embryos, 4.7 mm. in length, which had developed from proembryos, 0.17 mm. in diameter, after 7 days of culturing in media containing inorganic salts, 9 per cent sucrose, vitamin supplements, and embryo factor.

4) Embryos, originally 0.4 mm. in length, cultured for 1 month in the dark. Upper embryo: 3 mm. in length, was grown in a medium containing 2 per cent sucrose, inorganic salts, and vitamin supplements. Lower embryos: 15 mm. long, was grown in the same medium, with the addition of fresh coconut milk. Note the absence of roots, as discussed in Chapter 7.

Plate I. Photographs showing different types of growth in Datura embryos cultured in vitro.

(Photographs 1, 2, and 3 presented through courtesy of Dr. J. van Overbeek)

DISCUSSION*

Ideas suggesting a limit in the size of the embryos which can be cultured *in vitro*, as proposed by LaRue (29) and Dietrich (20), were shown by van Overbeek *et al* (46, 47), and confirmed in this investigation, to be untenable. In view of van Overbeek's success (unpublished data) in the culturing *in vitro* of *Datura* proembryos as small as 0.06 mm. in diameter (approximately the 64 cell stage), the question is clearly one of nutrition and not one of a vitalistic threshold as suggested by earlier workers. The failure of these investigators to grow very small embryos was due merely to the lack of a proper culture medium.

The culture of mature embryos, which were shown to be autotrophic, did not meet with any great difficulty. They could be maintained on strictly inorganic media (42). In some cases the addition of sucrose or vitamin B_1 was necessary (27).

For large immature embryos of deciduous trees, Lammerts (27) found that sugars were required in the ~~inorganic~~ medium. Similar results were found by other workers (29, 42). With smaller immature embryos, LaRue (29) and White (51) found that the heat-stable factors in yeast or fibrin digest were necessary. The addition of organic supplements was also found necessary for the growth of immature

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* A considerable portion of the material in this section has been presented in a symposium (44) by Dr. J.van Overbeek, who discussed the results of the investigations on embryo culture carried on at the Institute and at the Carnegie Institution of Washington at Cold Spring Harbor, New York. For the sake of completeness, however, the subject is repeated in this section.

Datura embryos in vitro (47). The medium given in table 1 was found by van Overbeek et al (47) to be suitable for the culturing of medium-size immature Datura embryos in vitro. Whether of not all of the ingredients were necessary has not been established experimentally. The choice of these substances, however, was not without good reason.

Although some argument existed as to the beneficial effects of glycine for tissue cultures (33, 53, 54) it was included in the medium. The essentiality of thiamine and pyridoxine for the growth of roots was established more clearly through experiments on root cultures (1, 6, 9, 32) and mature pea embryos (7, 24). In similar experiments (11, 15), ascorbic acid was shown to give a vigorous increase in the growth of some varieties of pea embryos in vitro. No stimulation, however, was observed in those varieties which were capable of synthesizing ascorbic acid in appreciable quantities. Datura roots in vitro were shown (8) to be completely heterotrophic for thiamine, pyridoxine, and nicotinic acid. The response of isolated tomato roots to nicotinic acid in the presence of thiamine and pyridoxine varied with the different clones and strains. Some responded and some did not (9). With isolated pea roots, however, the absence of nicotinic acid and thiamine resulted in a decrease in growth with each weekly transfer. The inclusion of adenine in the culture medium of Datura embryos was based on the discovery of its leaf growth promoting effects (5). Succinic acid was suggested (37) to

be essential as one of the members of the 4-carbon-acid hydrogen-carrier cycle, which was presumed to be coupled with auxin-controlled respiration. Because of the universal occurrence in living things (56) of pantothenic acid and its stimulatory activity in some plants and plant tissues *in vitro* (10, 31, 57) it was also included in the medium.

The above medium proved satisfactory for the culture of immature *Datura* embryos over 0.5 to 1.0 mm. in length (table 2X). Smaller embryos were not capable of growth in this medium. Since the embryo is nourished by the endosperm in the seed it seemed logical to van Overbeek *et al* (47) that the addition of a natural endosperm may stimulate the growth of smaller *Datura* embryos *in vitro*. Indeed, it was found to be so when coconut milk was tried (table 3).

From the fact that extracts of coconut milk and meat, *Datura* ovules, wheat germ, almond meal, and yeast extract were found to be active in supporting the growth of very small immature *Datura* embryos *in vitro* (table 20), it seems that little specificity existed among the substances necessary for the growth of very small embryos from different species of plants. The same source which nourished the coconut or the almond or the wheat embryo was found capable of nourishing the *Datura* embryo. This non-specificity of nutritive factors for plant embryos was also suggested from the results of earlier investigators. Arnaudov (3) found that moss sporophytes could be removed from their own gametophytes and transplanted successfully into other

gametophytes either of the same or of different species. Similar types of transfer were accomplished in the higher plants. Brown and Morris (13) transferred embryos from one barley grain to another. If proper adhesion was obtained between the transplanted embryo and the new endosperm the grafted embryo would develop as readily as if it were dependent on its own endosperm. The contents of the foreign endosperm underwent all of the usual changes attending their absorption and, if molds were excluded, the resulting growth of the embryo was entirely normal. Partial development was obtained in the transplantation of barley embryos on wheat endosperm. In this case, however, the differences in the size and the shape of the embryos made proper contact between the transplanted embryo and the new endosperm difficult. Nevertheless, that there was a partial development is significant. Better success was obtained by Colla (16) who was able to transplant and grow wheat embryos on the endosperm of rye, barley and oats.

The relative growth curves of embryos cultured in medium containing embryo factor (figures 2, 8) resembled those of autocatalytic reactions. This suggested to van Overbeek (44) that all of the cells of the embryos were involved in the process of growth of the embryos in media containing the growth factors. He, therefore, concluded that the embryo factor promoted cell proliferation chiefly, as contrasted to the action of auxins, in promoting cell elongation in the Avena coleoptile. To further support

this contention he brought out the fact that the curves for the relative growth of small and of large embryos (figure 4) were the same, while the absolute increase in length was much larger in the case of the larger embryos. Furthermore, a cytological examination by Dr. Jack Schultz revealed actively dividing nuclei in the embryos supplied with embryo factor, while the nuclei in embryos not supplied with embryo factor quickly became pycnotic. Embryos deprived of embryo factor for a few days were not able to resume growth when supplied with embryo factor.

On the basis of the data in figure 4 it also seems likely that the concentration of embryo factor must reach a certain threshold value, before its effect can be manifested. Only 30 per cent of the embryos, which were 0.5 mm. in length, developed at the lowest embryo factor concentration. At the same level 100 per cent of the larger embryos developed. With increasing amounts of embryo factor the percentage of viable embryos increased in the smaller embryos and remained at 100 per cent for the larger ones. This, of course, is in harmony with the increasing autotrophic nature of increasingly mature embryos, as discussed above. As pointed out by van Overbeek (44) the formation of embryo factor in the parent plant and the supply to the embryo may vary with the conditions under which the plants were grown. The embryos whose growth was plotted in figure 4 were taken from plants which were grown during the winter months in a relatively poorly lighted greenhouse. In this

case, embryos as large as 1.2 mm. in length were not capable of growth in the absence of added embryo factor. On the other hand, occasionally embryos, as small as 0.5 mm. in length, which were taken from plants grown in the Spring in the field, were capable of growth in the 1 per cent dextrose medium alone (figure 6). This suggested a sufficiency of embryo factor in the embryo, as a result of increased production by the mother plant in the Spring.

A characteristic feature of Datura embryos cultured in media containing fresh coconut milk is the lack of root formation (plate I.). Root development was markedly suppressed even on mature embryos cultured in medium containing autoclaved coconut milk (47). The fact that the hypocotyl in the embryos cultured in coconut milk was not suppressed along with the root suggested to van Overbeek et al (47) the possibility that auxin was the agent concerned. The suspicion was strengthened by his determination of auxin in coconut milk (table 32) and by the removal of the root inhibitor from coconut milk through extractions with solvents, in which the auxins were soluble (table 31).

The state in which auxin is present in coconut milk can be settled only with further more conclusive experiments. When an agar block was soaked directly in fresh coconut milk, only a very slight Avena ~~coleoptile~~ curvature was detected (table 32). Some auxin was released by boiling or autoclaving. Considerable amounts of auxin were detected when the milk was extracted with ether and the

ether fraction tested. In all cases there was a three- to a four-fold increase in the Avena curvature. The fact that coconut milk after being forced through a collodion membrane and extracted with ether gave the same auxin curvatures as undialyzed coconut milk suggested that the auxin liberated upon ether extraction cannot be due to a liberation from a protein complex as postulated by Skoog and Thimann (34, 38, 55).

On the basis of preliminary experiments, van Overbeek et al (47) tentatively proposed the presence of at least 3 factors in coconut milk, which produced the type of growth characteristic of Datura embryos in a medium containing coconut milk: a) thermolabile factor causing both growth and differentiation of the Datura embryo, b) heat stable factor causing in some cases a callus-like growth but no differentiation, and c) heat stable root inhibitor. In the light of the results obtained in the present investigation, these tentative assumptions must be altered. The failure of autoclaved coconut milk to support organized embryo growth was probably not solely a result of the heat lability of the growth-promoting factor. This was suggested by the fact that autoclaved yeast extract was able to support normal embryonic growth (table 20). The more probable reason was the liberation of growth inhibitors. Inhibitors might have arisen from autoclaving (figure 12) or from a prolonged heating of coconut milk at about 60° C and standing (fractionation II). Indications of growth

inhibitors in untreated plant material were first found in the work of van Overbeek et al (47). They found that when halved ovules containing the embryos were placed in the nutrient medium, such embryos did not grow whereas similar embryos, when completely isolated, did grow. There was some suggestion that auxin was concerned in the above relationships. The experimental facts which led towards this possibility were the following: There was a similarity in behavior between the root inhibitor in coconut milk and auxin. Both were heat stable and extracted with the same solvents. Autoclaved coconut milk supported a callus type of growth (47), as contrasted to the normal differentiated type of growth supported by non-autoclaved coconut milk. It was found that autoclaving liberated auxin (table 32). Furthermore, it was found that high concentrations of auxin added to the medium containing a somewhat purified embryo factor preparation induced callus growth (table 33). The callus formation induced by high ~~auxin~~ concentrations of auxin might have been superimposed upon the growth stimulated by the heat stable substances, resulting in an undifferentiated growth. The results are in agreement with the findings of Skoog (44) that Nicotiana callus failed to differentiate into shoots and roots when supplied with a high auxin medium. Although there were some similarities in properties between the growth inhibitor discussed in chapter 7 and auxin it is not certain whether the two are identical. More experiments are needed before any con-

clusions can be drawn in this respect.

On one hand, therefore, a high auxin concentration induced callus formation. On the other hand, the figures in table 33 indicated that there was a stimulation in embryo growth upon the addition of 10 gammas of indole-3-acetic acid per liter of medium containing a somewhat purified embryo factor preparation. Rooting was also stimulated by this low auxin concentration. The implication of the necessity of a minimum amount of a substance or substances for root formation was supported further by the experiment illustrated in figure 11. Of the 4 sets of embryos in this experiment only those which were given the highest concentration of embryo factor formed any roots after 11 days. No roots developed in the others, despite their good hypocotyl and cotyledonary growth. Of course, much more experiments are needed before conclusive data can be presented. However, the work of Stephenson (35) is interesting in this respect. He obtained a marked stimulation in the growth of lettuce embryos with the addition of 0.1 mg. of indole-3-acetic acid or naphthalene acetic acid per liter of medium. With the addition of 1 to 10 mg. of auxin per liter cancerous and nodule-like types of growth were obtained. Stephenson's investigations, like those in this thesis, were conducted under sterile conditions.

Anyway, we see present in the endosperm of coconut a good source of auxin, which is apparently bound in some unknown way but which can be liberated by different methods.

As suggested by the results of this investigation, the auxin may be liberated in small amounts to stimulate normal embryonic development. Under abnormal or unfavorable conditions the liberation of unusually high amounts of auxin may be the cause in the abnormal types of growth sometimes met in nature.

Of course, it is naive to assume a priori that only one or a few substances are necessary for the organized growth of embryos. The embryo factor as used in this thesis, is a generic term to include a number of as yet unidentified substances. And as in the case of auxin, an optimum concentration of each of these factors may probably be necessary. Beyond the optimal range, abnormalities in growth or inhibitions of the effects of the other substances may result. Furthermore, accompanying changes in the pH of the endosperm may be necessary. As shown in figure 9, there is a shift in the pH optimum from pH 6.8 to pH 5.5 after 4 days of culture. Whether this shift actually takes place in nature, however, needs to be determined.

SUMMARY

1) The results of van Overbeek *et al* (47) on the successful culturing of small immature *Datura* embryos *in vitro* on media containing coconut milk were confirmed.

2) The growth of *Datura* embryos *in vitro* in the 1 per cent dextrose medium, as given in table 1, was supported also by extracts of coconut meat, *Datura* ovules, wheat germ, almond meal, and yeast.

3) A satisfactory bio-assay for the substances necessary for the growth of *Datura* embryos was developed. This bio-assay was based on the growth of *Datura* embryos cultured aseptically *in vitro* in a medium containing the solution to be tested relative to the growth of *Datura* embryos cultured in the control medium in the absence of the test solution. The quantity of embryo factor was expressed in *Datura* units. One *Datura* unit was the quantity of embryo factor which had to be added to the standard medium to give a relative growth difference in one *Datura* embryo of 0.5 over that of the control in 2 days.

4) The optimum temperature for the growth of *Datura* embryos *in vitro* was found to be 32° C.

5) pH 6.8 was the optimum for the growth of small *Datura* embryos during the first 4 to 5 days of culture *in vitro*. After 4 to 5 days the optimum shifted to pH 5.5.

6) Besides the ingredients in the control medium (vitamin B_1, vitamin B_6, ascorbic acid, nicotinic acid, adenine,

succinic acid, calcium pantothenate, glycine, inorganic salts, and agar) sucrose was found to be necessary.

7) Other sugars, amino acids, purines, and inorganic salts were inactive in the concentrations tried.

8) A stimulation in embryo growth and in root formation was found with the addition of 10 gammas of indole-3-acetic acid or naphthalene acetic acid per liter of a 2 per cent sucrose medium (2 per cent sucrose in place of dextrose in the 1 per cent dextrose medium (given in table 1), containing a somewhat purified embryo factor preparation. Concentrations of 100 gammas or over per liter induced callus formation.

9) Fractionations of coconut milk for the remaining growth factors revealed their insolubility in the usual organic solvents. They were not adsorbed by the usual adsorbents and not precipitated by the usual precipitants.

10) The root inhibitor in coconut milk was removed through extractions with ethyl alcohol and diethyl ether. A probable identity with auxin was suggested.

11) A growth inhibitor was shown to be formed upon heating coconut milk at high temperatures or at low temperatures for a prolonged period. This was removed through ethyl and butyl alcohol extractions and precipitation with lead acetate.

12) Purification of coconut milk yielded preparations with a detectable activity in a dilution of 1:19,000, which represents an enrichment of 170 times. Because of the

removal of the root inhibitor in these preparations, root growth was not suppressed, as was the case in media containing coconut milk.

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