GROWTH FACTOR STUDIES WITH SPIRODELA POLYRRHIZA (L.) SCHLEID.

Thesis by

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INTRODUCTION

Leeuwenhoek was one of the first men to record observations upon the peculiar growth habits of members of the family Lemnaceae. Taxonomists and morphologists have long argued about the proper classification and anatomical interpretation of these, most modified of higher plants, while physiologists from the time of Sachs have seen in Lemna and Spirodela a type of plant peculiarly suited for experimental investigations in the field of nutrition. This has been because of their small size and aquatic habit which make them ideal for culture in nutrient solution.

In recent times there has been considerable controversy over the question as to whether organic manures, humus, peat, and soil supply small amounts of organic materials which promote the growth of green plants. Are the beneficial effects often observed upon treatment with these substances simply the result of correcting recognized or unrecognized inorganic deficiencies, or are they the result of accessory growth factors?

In undertaking to answer this question, Spirodela polyrrhiza (L.) Schleid. was chosen as the test plant since careful control of its growth environment is readily achieved. The possibility of freeing the plants of microorganisms and employing sterile culture technique make its use particularly desirable in a study concerned with the effects of organic materials.

The primary aim in the investigation was to demonstrate that

organic additions to a medium of inorganic salts balanced for optimal growth can produce a significant increase in growth. Also, that manures, humus, peat, and soil are sources of these growth promoting substances, and to find out as much about their nature as possible. Growing out of this line of study was a broader one, namely, an attempt to gain some insight into the mechanism of the action and interaction of the many different factors, such as light, carbon dioxide, carbohydrate supply, etc., which are known to affect the growth of Spirodela. Finally, unsuccessful attempts were made to induce flowering of Spirodela, which occurs only rarely in nature and has never been induced experimentally.

CHAPTER I

METHOD

Material for Sterile Culture

Several plants of Spirodela polyrrhiza (L.) Schleid. obtained from a greenhouse aquarium were used in initial experiments to find a means of freeing them from microorganisms and culture them in sterile solution. Several attempts were made to accomplish this by the use of oligodynamic carbon and katadynized sand,[1] particular means of using silver ions for their oligodynamic action. The details of these unsuccessful trials are not necessary. Suffice it to say, the silver-ion concentration needed to kill the microorganisms killed the plants of Spirodela in every instance.

When it became apparent that no success was to be achieved with the use of oligodynamic silver, the use of alcohol and bichloride of mercury was resorted to and thereby two or three viable cultures, free from microorganisms were obtained. The successful technique consisted of taking a plant of only one or two fronds, gently held by means of forceps, submerging it for forty-five seconds in 0.1 per cent $HgCl_2$, following this with a quick rinse in sterile water, then transferring it to 50 per cent ethyl alcohol, there submerging it for thirty seconds. The plant was then passed through two rinses of sterile water (or sterile nutrient solution) and thence into a culture tube of sterile nutrient, and set aside to grow. Two of the four plants given this rigorous treatment survived and were free of contaminating organisms. Severe

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1 Obtained through the courtesy of Dr. Alexander Goetz of the department of physics.

necrosis of the edges of the fronds soon appeared. These necrotic fronds in turn gave rise to unusually small fronds, but after three weekly subcultures normal size and vigor were regained. Whether the cultures were freed from microorganisms by the treatment was tested by transferring plants to medium containing sucrose and noting that no growth of fungus or bacterium occurred. From one of the original plants so-treated, the "strain" of Spirodela polyrrhiza used in all subsequent experiments was derived. Through innumerable subcultures over a period of 2 1/2 years, in media with and without sucrose or other organic additions, these plants have been carried, with only an occasional chance contamination.

The sterilizing technique outlined above, when tried at a subsequent date with Azolla sp. and Lemna minor L. again yielded micro-organism-free cultures that were carried without contamination for a year or longer. At the same time, the use of 8-hydroxyquinoline sulfate was tested. Although it was employed in conjunction with alcohol as well as by itself, using different time intervals of treatment, all cultures showed contaminants after 3 to 5 days.

Culture Conditions

For ease in making sterile transfers, Pyrex culture tubes 175 mm. long, 20 mm. in diameter were used. A 25 ml. aliquot of nutrient medium was pipetted into each tube. After sterilization, each tube of medium was inoculated with Spirodela plants sufficient to give a total of 30 fronds. This number was determined by noting the subsequent growth in one week under the prevailing environmental conditions. About

seventy additional fronds were normally produced in this time, the total of 100 fronds nicely covering the surface of the nutrient in the culture tube, held in a special rack beneath the light source at an angle of 60° from the perpendicular (Fig.1).

Tube racks containing inoculated cultures were placed in the inclined position upon shelves, adjustable for height. Above each pair of such inclined racks was suspended a 40-watt "White" fluorescent lamp. By raising or lowering the shelves upon which the cultures rested, variations in light intensity were made possible. Two sets of aluminum-painted shelves with lamps were located in a windowless room, temperature-controlled at 25 ± 1° C. The walls of the room were aluminum-painted for light diffusion. The sets of shelves were located at equidistance from the reflecting walls and from one another. On each set of shelves hung three lamps, from top to bottom. All six lamps were operated continuously, even though some had no cultures under them at times, so that the diffuse light falling on the culture tubes as a result of reflection from the walls remained relatively constant throughout the experiments. The aluminum-painted bottom of the shelf from which a lamp unit was suspended served as the reflector for the lamp.

The intensity of light falling on the surface of cultures located in an inclined rack beneath one of these lamps was not uniform from one end to the other. Using a Weston Photronic cell and galvanometer, readings were obtained (Table 1) which demonstrate this fact. Not only was there variation from one end of a culture rack to the other, but there was variation in the intensity produced by the different lamps as

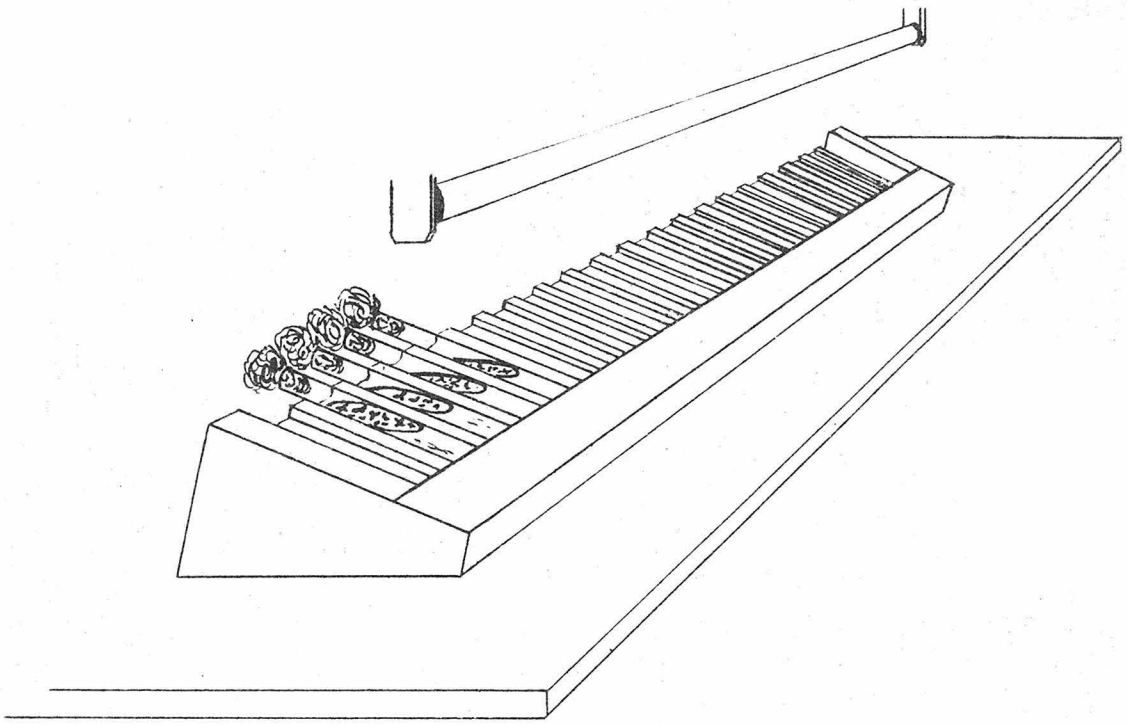

Figure 1. Inclined culture rack beneath fluorescent lamp.

Table 1

Variations in intensity of illumination beneath different lamps as determined with a Weston Photronic cell and galvanometer.

Observation made under lamp	Distance of culture surfaces from lamp	Along 36" culture rack galvanometer readings made at		
		0"	18"	36"
I	16"	30	32	29
II	16"	30	32	29
III	16"	31	32	31
IV	16"	30	34	33
III	28"	16	19	16
V	4"	80	83	80

well. The general practice was therefore adopted of shifting the position of tubes in the racks once a day. Should a test be so large as to be employing more than one lamp, the racks were shifted about under the different lamps at the same time. This shifting was done in such a fashion that each culture, over a period of one week, received approximately the same amount of total light energy. Rather small differences in the amount of light energy Spirodela plants receive make big differences in the rate at which they grow. Practice of this daily shifting procedure caused a substantial decrease in the variability between replicates.

From a typical experiment where a treatment was replicated 10 times the average value for increase in frond number was 223 (σ= 10.7), fresh weight 176 mg. (σ = 14.9 mg.), and dry weight 22.3 mg. (σ= 1.4 mg.).

Growth measurements

As criteria of growth, measurements of the increase in frond number, total frond area, total fresh weight, and total dry weight were made. Cultures were grown for one week, then subcultured and growth measurements made on the material of the subculture, usually one week's growth also. The remaining plants after the inoculation of the subcultures were often used to estimate the area, weight, etc. of the inocula for the subcultures. At the conclusion of a test, fronds were first counted and the hydrogen-ion concentration of the test solutions checked. Immediately thereafter, photographs were taken for area determinations. The fresh weight of the plants was then determined, and after twenty-four hours in a dryer, circulating air at 40° C., the dry weight measurements were made.

Counts--Fronds could frequently be counted without removal from the culture tube. Where conditions of crowding made this impossible, the contents of each tube were removed to a glass plate, separated and counted, then returned to the tube so that they would not dry out before area measurements could be initiated.

Area--The principle employed in making determinations of area was to obtain by photography silhouettes of the plants in each culture as well as of a series of discs (or standards) of known area. By means of a photo-electric photometer these silhouettes were compared with the standards and the total area of the fronds in each culture expressed in terms of square millimeters.

The photographic apparatus consisted of a modified 35-mm. camera with a focal length adjusted by means of an extension tube to 8 inches. This was so constructed that it could travel at fixed focus above a polished brass container, 28 inches long, 1 3/8 inches wide, 1 1/2 inches deep, having a bottom of opal glass. The dimensions of this container, separated into twelve equal compartments, were such that the focussed image of a single compartment occupied the length of one frame. The width, however, had to be considerably less than that of a frame since the resulting pictures could be no wider than the width of the oblong photo-electric cell of the photometer. The compartmenting separations only extended to within one quarter inch of the opal glass bottom, so that when the container was filled to the brim with water, the concave meniscus in each chamber was at the same distance from the camera lens. Attached to the carriage which supported

the camera, and moving with it, but beneath the bottom of the brass container, was an illuminating box containing a 7-watt pilot light and a 250-watt photoflood lamp.

When the apparatus was to be used, the inner walls of the compartments were polished for high reflection. The container was then filled with water to the brim. With a camel's-hair brush, the contents of a culture tube were transferred to a single compartment (where growth was extraordinary, two or sometimes three compartments were used). When all twelve compartments were filled, two or more always being reserved for varying numbers of standard discs, a check was made to see that no fronds over-lapped one another. With only the light from the illuminating box, the travelling camera was then used to record silhouettes of the contents of each compartment. This finished, the plants were transferred to a large covered pan with layers of saturated blotting paper inside, and there reserved for weight determinations.

At the beginning of each film of 36 exposures an identification picture was taken of a glass slide laid across one of the compartments. Written on this in black grease pencil, was information concerning the experiment number, film sequence, cultures photographed, and the date when photographed. At the same time careful note was kept, frame by frame, film by film, of what was photographed and what mistakes, if any, were made in the process. This obviated the chance of there being confusion as to what culture a particular photograph referred to.

Inexpensive positive or sound-recording film of Weston speed

rating of about 1.0 was found very satisfactory, as it could be developed to a suitable degree of high contrast. Exposure at f/4.5, for 1/200th seconds, followed by tank development for 2.5 minutes using Eastman developer, formula D-8, at 18°C., gave contrast such that the image of the plants was transparent, with no details. The background was black and almost completely opaque.

The photo-electric photometer was one built for the particular purpose. The film guide was made adjustable in order to accomodate occasional frames whose sides were not parallel to the edges of the film so that they could be properly oriented over the photo-cell. Slight variations in frame length and width could be corrected for by means of an adjustable mask over one end and side of the oblong photo-cell. For any one film this mask was left at a particular adjustment. Illumination of the photo-cell was provided by a 6-watt, 0.25-ampere lamp operated on a storage battery at 0.25 amperes, carefully controlled by means of variable resistances. This lamp was located on a bracket directly above the photo-cell at a distance of 2 inches. It was housed in such a way that by means of a lens, light was concentrated evenly over the surface of the photo-cell.

The deflection of a sensitive galvanometer attached to the photo-cell was used for determining current, and thus area variation, in most of the experiments. By measuring these current variations potentiometrically, as was done later, the limitations imposed by the range of the galvanometer scale were overcome, and the precision of the measurements somewhat improved. With increasing transparent area over the photo-cell, galvanometer deflection increased essentially

linearly while the increasing e.m.f. actually generated by the photocell, when plotted against increasing area, yielded a decided curve. For comparison, the area determinations of a particular experiment (Fig. 2) were made by both methods. The values so-obtained showed that the discrepancy between the two methods was of not enough significance to necessitate discarding data obtained with the use of the galvanometer.

Previously mentioned was the fact that at the same time silhouettes of the plants were being photographed, silhouettes of varying numbers of floating discs of known area were also pictured. On each film, at regular intervals, were pictures of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 discs. Upon photometry, the values obtained for these known areas provided the means for construction of curves for converting galvanometer or potentiometer readings into terms of square millimeters. The curves obtained from two films developed simultaneously in the same tank did not always coincide. Films developed separately showed even wider differences in total density. However, since a complete set of reference standards was always included on each film, these variations were of no importance.

In order to test how precise a particular area determination actually was, successive photographs were taken of the same number of floating standards, rearranging them and moving them from one compartment to another. Twelve determinations of area were made in this way for 5, 10, and 20 standard discs. Five discs with an area of 135 sq. mm., had a standard deviation of 4.5 sq. mm. Ten discs, with an area of 270 sq. mm. had a standard deviation of 3.2 sq. mm., while 20 discs, with an area of 540 sq. mm. had a standard deviation

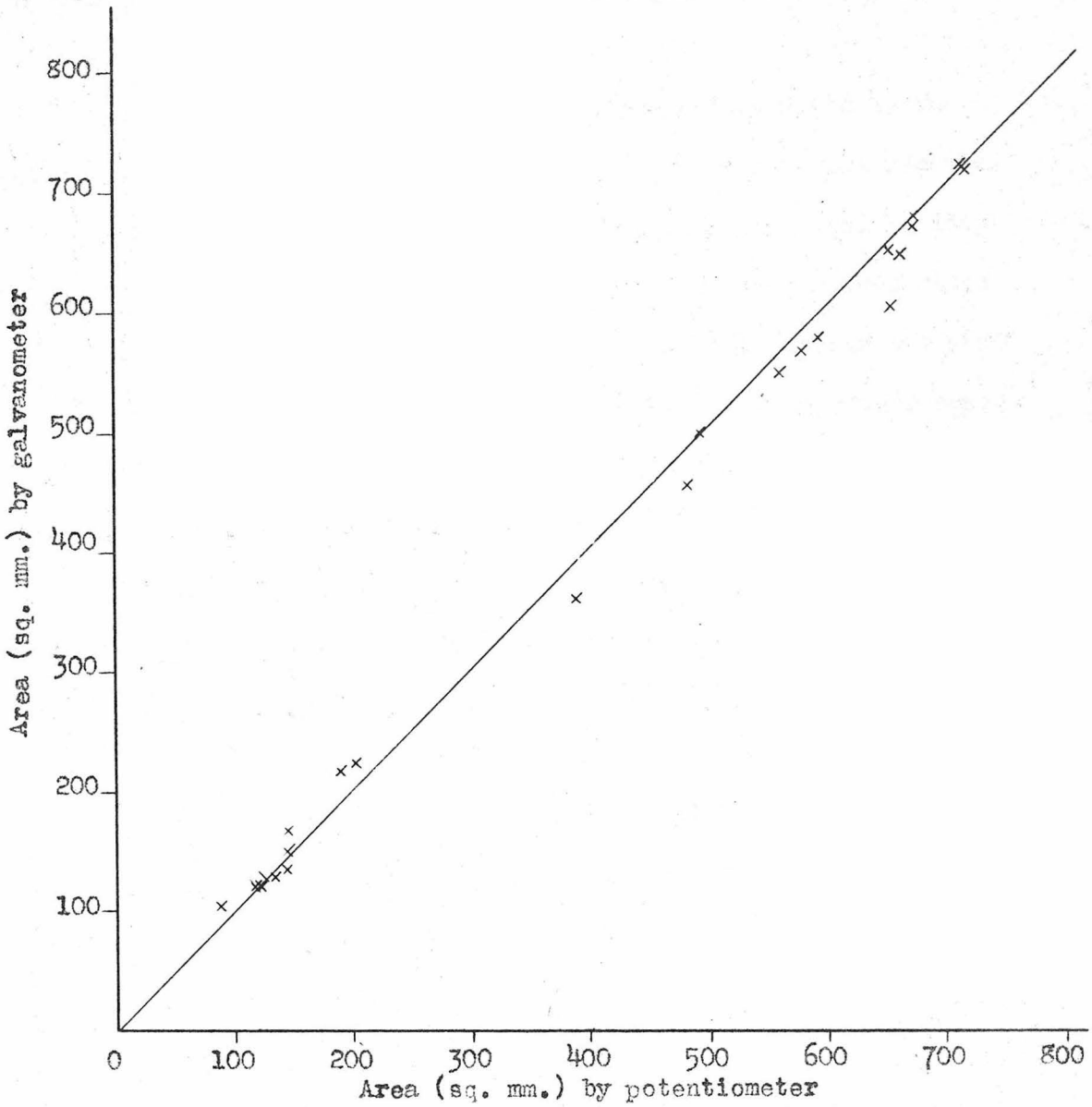

Figure 2. Comparison of the area determinations of a particular experiment made with the use of a galvanometer or with the use of a potentiometer. Line represents ideal agreement.

of 4.2 sq. mm. Since by far the greatest number of determinations were in the range of four to five hundred square millimeters, most of the measurements were quite precise.

Weight--After the area photographs had been taken, the plants saved on moist blotting paper in a covered pan were removed, culture by culture, blotted to remove surface moisture and rapidly weighed to the nearest milligram on a balance of the chain-o-matic type. A glass microscope slide counterpoised with another slide of ~~the~~ similar weight was found more satisfactory than any other type of container for rapid weighing. After recording the fresh weight, the plants of each culture were either discarded or else placed in small folded-paper pockets to be dried at 40° C. for 24 hours in a forced-air drying chamber. The dried plants were then weighed, this time, however, to the nearest tenth of a milligram.

CHAPTER II

PRELIMINARY EXPERIMENTS

Quite a number of experiments were done in the early stages of the investigation, testing different humus extracts to see if any effect they might have on the growth of Spirodela could be attributed to some influence on iron nutrition (Olson, 1930). The results of one experiment clearly indicated that humus extract could stimulate growth, no matter whether iron were supplied to Hoagland's medium[2] in the form of an organic or inorganic salt, although in line with Olson's view, no stimulation should have resulted when humus extract was added to a medium containing iron as ferric tartrate. An attempt to establish the optimal humate concentration in the presence of ferric tartrate gave such inconclusive and inconsistent results that it was judged expedient to devote some time to investigating the major element nutrition, hydrogen-ion concentration, and effects of light intensity. This was to make possible a more intelligent control of environmental factors so that demonstration of growth promoting activity would not be confused with corrected inorganic nutrient deficiencies or suboptimal hydrogen-ion concentrations.

In some respects these initial experiments differed from later ones, which followed the procedure already outlined with but few exceptions. In particular, the best number of fronds to use for

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2 Concentration in grams per liter glass distilled water: $Ca(NO_3)_2.4H_2O$ 1.180; $MgSO_4$ 0.493; KNO_3 0.506; KH_2PO_4 0.136; ferric tartrate 0.005. Minor elements in milligrams per liter: H_3BO_3 2.86; $MnCl_2.4H_2O$ 1.81; $ZnSO_4.7H_2O$ 0.22; MoO_3 0.07; $CuSO_4.5H_2O$ 0.08.

inoculation had not been decided upon. The use of rather small numbers made replicate variation higher than would otherwise have been the case. The best time for subculturing, and whether subculture inocula should be of the same number of fronds or of half the number in each parent culture were details which were standardized as experimental work proceeded. However, the conclusions to be drawn from these preliminary experiments were little affected by these variations in technique. These were discarded in favor of others found more satisfactory from the standpoint of saving in time and effort.

After only a few experiments had been concluded, it was noticed that certain plants in somewhat poorly growing cultures became very abnormal in appearance and ultimately failed to reproduce at the normal rate. These plants appeared saturated with water, and as more and more of the frond surface became affected, rendering it almost transparent, these plants would slowly submerge and finally sink to the bottom of the culture tube. Any appreciable numbers of these "water-soaked" plants in a particular culture affected not only its over-all growth rate, but by their transparency, made photographic area measurements subject to additional error. A little observation and work soon made it seem probable that the cause was nutritional and not disease. This could mean either a deficiency of some particular substance or substances, or else a condition of nutrient unbalance was responsible.

To test whether Hoagland's nutrient was not properly balanced or had too high a salt content for the best growth of Spirodela, a

series of dilutions of the major elements was prepared, minor elements being maintained at the normal concentration in all cases. The question as to whether inorganic magnetite (Fe_3O_4) was as useful a source of iron as ferric tartrate was tested by having half the tubes of the dilution series supplied with 1 p.p.m. iron as tartrate, while the other half had approximately 1 gm. of only slightly soluble magnetite added to each tube.

In Table 2 are given the frond counts for the growth period following subculture, when half the total fronds in each parent culture was used for inoculum. The ferric tartrate and magnetite series show no significant differences. Only the two lowest dilutions tested, 0.05 and 0.10, showed markedly inferior growth. Increasing dilutions also caused the hydrogen-ion concentration to shift over an ever widening range throughout the growth period.

Table 3 shows the effect of dilution upon the per cent of fronds showing "water-soaking" symptoms. It is interesting to note that the dilutions supporting the poorest growth had the greatest number of "water-soaked" fronds, suggesting that nutrient deficiency is the underlying cause.

A repetition of this experiment at different light intensities, thus different growth rates, showed that "water-soaking" was more apparent in fast-growing cultures than in slow-growing ones (Table 4). This is in accord with the notion that a deficiency is responsible. As before, magnetite proved every bit as satisfactory an iron source as ferric tartrate. Where the reaction of the medium changed from acid to alkaline during the long growth period, so that pronounced iron

Table 2

Effect of dilution of major elements on frond multiplication. Figures represent mean of four replicates. Cultures 16" from light source.

Ferric Tartrate

Dilution	0	1	3	5	7	9 days	Init. pH	Final pH
1.0	17	19	28	33	42	53	4.8	6.0
0.75	18	18	27	32	45	54	4.8	6.2
0.50	18	19	29	34	42	54	4.8	6.4
0.25	18	19	29	34	40	52	4.9	6.8
0.10	14	16	23	27	32	39	4.9	7.0
0.05	13	14	19	23	27	32	5.1	7.0

Magnetite

1.0	16	17	25	29	39	49	4.6	6.0
0.75	17	18	30	34	45	56	4.6	6.2
0.50	17	19	29	35	44	55	4.6	6.4
0.25	17	18	26	32	38	51	4.7	6.7
0.10	16	16	26	32	36	43	4.6	6.9
0.05	14	15	21	25	30	36	4.8	7.0

Table 3

Per cent of fronds affected by "water-soaking" at conclusion of 9-day's growth.

	Dilution					
	1.0	0.75	0.50	0.25	0.10	0.05
Ferric tartrate	23.1	22.9	29.7	40.1	58.0	67.7
Magnetite	16.8	15.1	30.4	36.9	76.7	65.0

deficiency could be expected, there was still no apparent advantage in the use of one iron salt in preference to the other. No very noticeable iron deficiency developed in any case.

At both light intensities, Hoagland's nutrient at full strength appeared less suitable for growth than when it was diluted to approximately one half. However, "water-soaking" was least pronounced with full strength, so a triangulation experiment was set up to determine what concentrations of the major salts produced a medium that was optimal as far as the growth of Spirodela was concerned.

Concentrations of magnesium sulfate, monopotassium phosphate, and calcium nitrate were varied simultaneously, in one series with no added potassium nitrate, in another series with potassium nitrate added at a concentration of 0.005 M, as in Hoagland's medium. Each series was tested, using two replicates for each treatment, at 16" distance from the light source. The whole test was run in duplicate to compare the effect of growth under 40-watt "Daylight" fluorescents with that under 40-watt "White" fluorescent lamps. A comparison of the characteristics of these two lamps is given in Table 5.

Growth under the "White" fluorescents was generally better than that under the "Daylight" fluorescents, in approximately the same proportion that the luminous output of the "White" fluorescent exceeds that of the "Daylight." The per cent "water-soaked" fronds was also somewhat higher under the "White" than under the "Daylight" fluorescents, a fact that seemed to be correlated with better growth as a result of more luminous energy supplied rather than the spectral differences of the two light sources.

Table 4

Effect of dilution of major elements on frond multiplication and fresh weight at two light intensities. Ten-frond per tube inoculum. Figures are increases during 19-day growth period and represent means of four replicates. Initial pH = 4.8 in all cases.

Cultures 16" from light source	Dilution 1.0	0.75	0.50	0.25	0.10	0.05
Frond No.	63	66	68	74	62	49
Fresh weight	47	52	57	64	51	44
% "water-soaked"	7	11	12	27	49	58
Final pH	6.2	6.4	6.8	7.1	7.3	7.3
Cultures 28" from light source						
Frond No.	44	48	44	43	39	37
Fresh weight	29	35	33	35	33	28
% "water-soaked"	7	7	9	11	33	30
Final pH	5.9	6.0	6.3	6.5	6.9	6.6

Table 5

Comparison of characteristics of 48" 40-watt General Electric Fluorescent Mazda Lamps.*

Lamp	Region of maximum energy output	Average rated life	Lumen output**
Daylight	Blue, red	2500 hours	1800
"White"	Red	2500 hours	2100

* Mazda Lamps. General Electric Company. Nela Park Engineering Department. 1940. pages 42, 56

** Measured at end of 100 hours operation. Efficiency at 70 per cent of rated life is about 85 per cent of initial rating.

For the sake of brevity only the data from the series under "White" lights is given (Tables 6, 7) since, with the exception of the differences noted above, the plants under "Daylight" lamps behaved in very similar fashion.

In all cases best growth occurred when KH_2PO_4 was supplied at a concentration of 0.001 M. Increase in the concentration of $MgSO_4$ from 0.001 M to 0.006 M caused a slight improvement in growth, whereas increase in the concentration of $Ca(NO_3)_2$ over the same range caused growth to decrease. When KNO_3 was present (0.005 M) growth was generally decreased, but the number of "water-soaked" fronds was greatly reduced. The widest range in pH change during the 8-day growth period was 4.8 to 6.5. This range occurred where growth was best, and was the same in both the series with and the series without added KNO_3. In view of this reduction in number of "water-soaked" fronds, it was decided to retain KNO_3 in the nutrient formula. Hoagland's formula was retained as a basis for further experiments since it differed only slightly from the better one indicated by the triangulation experiment.

Before the suitability of Hoagland's medium was finally decided upon, several additional experiments were performed, mostly to ascertain the causes of the "water-soaking" phenomenon, and also to find out what effect other environmental factors have on the growth of Spirodela. In the preceding experiment best growth was obtained in those salt combinations where the lowest concentration (0.001 M) KH_2PO_4 was present. A test was then made to see if a similar series with only 0.0005 M KH_2PO_4 would produce even better growth. At the same time, the effect of pH of the initial medium, of light intensity,

Table 6

8-day increase in frond number and fresh weight with varying concentrations of salts of major elements. 30-frond inoculum. Figures represent means of duplicates. (Concentrations in millimoles.)

Increase in frond number

No KNO_3

KH_2PO_4	$Ca(NO_3)_2$						$MgSO_4$
			6	40	1		
		5	48	52	2		
	4	42	54	56	3		
3	47			54	4		
2	51	49		54	61	5	
1	48	60	59	53	60	58	6
	6	5	4	3	2	1	

KNO_3 (0.005 M)

KH_2PO_4	$Ca(NO_3)_2$						$MgSO_4$
			6	40	1		
		5	45	55*	2		
	4	39	43	58	3		
3	42			55	4		
2	41	46		54	58	5	
1	39	46	45	49	49	48	6
	6	5	4	3	2	1	

Increase in fresh weight (mg.)

KH_2PO_4	$Ca(NO_3)_2$						$MgSO_4$
			6	33	1		
		5	37	40	2		
	4	34	43	44	3		
3	37			44	4		
2	37	37		42	49	5	
1	32	38	41	41	45	46	6
	6	5	4	3	2	1	

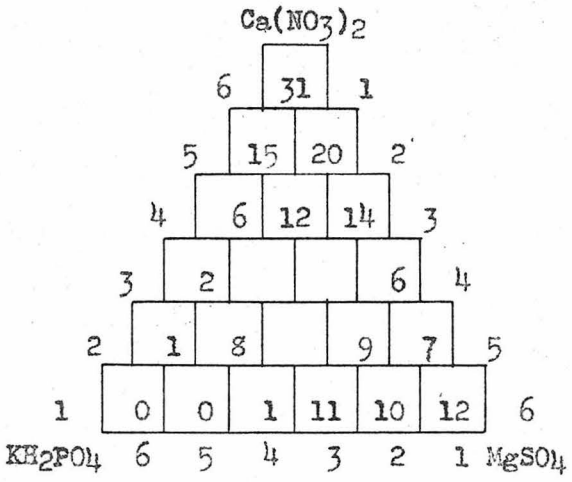
KH_2PO_4	$Ca(NO_3)_2$						$MgSO_4$
			6	28	1		
		5	32	40*	2		
	4	30	33	43	3		
3	31			38	4		
2	29	31		38	41	5	
1	22	28	28	31	33	35	6
	6	5	4	3	2	1	

* Hoagland's formula

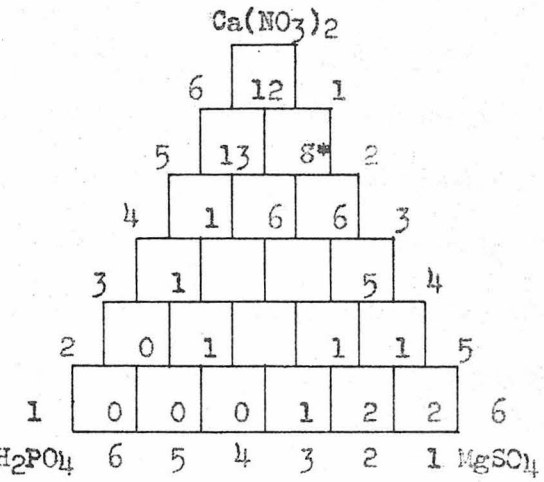
Table 7

Per cent fronds "water-soaked" at conclusion of 8-day growth period. Figures represent means of duplicates.

No KNO_3



KNO_3 (0.005 M)



* Hoagland's

and of photoperiod were studied for their effects upon growth and upon "water-soaking". To achieve a 16-hour day, one of the sets of shelves in the culture room was covered with a heavy black cloth and the lamps were controlled by a timeclock. Unfortunately, heat dissipation from the continuously enshrouded lamps was poor, and only towards the end of the experiment was the temperature inside measured and found to be 33-35°C., instead of the 24-25°C. of the culture room. Undoubtedly this contributed considerably to a reduction in the amount of growth observed when light was given only 16 hours per day. As in subsequent experiments, cultures were grown for 7 to 10 days, then subcultured, using 30 fronds for each inoculum. The growth measurements were made on the subcultured material, it being considered more representative of the type and amount of growth the various media were capable of supporting.

In table 8 the data are given for increase in frond number. Total area and fresh weight measurements were made but since they only reflected changes in frond number, they have been omitted. Varying the concentrations of $Ca(NO_3)_2$, $MgSO_4$, and KH_2PO_4 made little difference in the resulting amount of growth which took place, even when this was tried using different light intensities (and photoperiods) and adjusting the media to different initial hydrogen-ion concentrations; factors which have a significant effect upon growth. The growth conditions causing the greatest change in pH (Table 9) proved to be the best. The conditions were a high light intensity, with a medium whose $Ca(NO_3)_2$ and $MgSO_4$ content was respectively 0.003 - 0.005 M and 0.002 - 0.004 M. KH_2PO_4 was best supplied at 0.001 M concentration, with the reaction of the

Table 8

Effect of varying intensity, photoperiod, initial pH, and major salt concentrations on 11-day increases in frond number. Figures represent means of duplicates. Iron as Fe_3O_4, usual Hoagland minor elements.

			KH_2PO_4 (.001M)						KH_2PO_4 (.0005M)						
	$Ca(NO_3)_2$ (millimoles)		6	5	4	3	2	1	6	5	4	3	2	1	
	$MgSO_4$ (millimoles)		1	2	3	4	5	6	1	2	3	4	5	6	
Initial pH approx. 5.0	Dist. from l.s.	Day length													Mean of both phosphate series
	16"	24 hrs.	78	87	100	93	91	91	87	81	90	95	97	90	90
	16"	16 hrs.	37	46	44	45	45	40	41	44	44	47	48	44	44
	28"	24 hrs.	26	33	32	39	33	35	30	31	33	36	37	35	33
	28"	16 hrs.	18	19	22	21	23	21	27	23	24	23	21	23	22
Initial pH approx. 6.0															
	16"	24 hrs.	50	59	42	34	39	43	37*	38*	28	32	25	32	38
	16"	16 hrs.	37	32	32	30	28	30	34	29	21	16	28	23	28
	28"	24 hrs.	23	24	22	15	22	26	21	22	20	18	21	21	21
	28"	16 hrs.	18	22	21	17	16	16	24	20	11	11	19	11	17
Mean of both pH series			36	40	39	37	37	38	38	36	34	35	37	35	

*Contaminated

Table 9

Change in pH of different media after growth for 11 days.

		KH_2PO_4(0.001M)						KH_2PO_4(0.0005M)					
$Ca(NO_3)_2$ (millimoles)		6	5	4	3	2	1	6	5	4	3	2	1
$MgSO_4$ (millimoles)		1	2	3	4	5	6	1	2	3	4	5	6
Initial pH		4.8	4.6	4.8	4.8	5.2	4.9	4.7	4.7	4.7	4.7	4.8	4.9
Dist. from lrs.	Day length												
16"	24 hrs.	6.1	6.2	6.2	6.2	6.3	6.3	6.4	6.5	6.6	6.6	6.5	6.5
16"	16 hrs.	5.7	5.6	5.6	5.6	5.6	5.6	5.7	5.8	5.8	5.9	5.8	5.8
28"	24 hrs.	5.4	5.5	5.5	5.6	5.6	5.6	5.6	5.6	5.8	5.8	5.8	5.8
28"	16 hrs.	5.0	4.9	5.0	5.0	5.2	5.1	4.9	5.0	5.1	5.2	5.1	5.2
Initial pH		5.2	5.2	5.3	6.0	6.1	6.0	5.7	5.6	6.2	6.3	6.3	6.4
16"	24 hrs.	6.0	6.1	6.3	6.3	6.4	6.5	6.3	6.3	6.4	6.6	6.6	6.5
16"	16 hrs.	5.8	5.9	6.1	6.2	6.3	6.4	6.1	6.2	6.2	6.5	6.5	6.7
28"	24 hrs.	5.9	5.9	6.0	6.3	6.3	6.4	6.1	6.0	6.2	6.5	6.5	6.6
28"	16 hrs.	5.8	5.6	5.8	6.2	6.3	6.4	5.8	5.8	6.2	6.5	6.4	6.7

Table 10

Per cent "water-soaked" plants after 11-day growth period in media of varying salt and hydrogen-ion concentrations, under continuous illumination, 16" from light source. Figures are means of duplicates.

	KH_2PO_4 (0.001M)						KH_2PO_4 (0.0005M)					
$Ca(NO_3)_2$ (millimoles)	6	5	4	3	2	1	6	5	4	3	2	1
$MgSO_4$ (millimoles)	1	2	3	4	5	6	1	2	3	4	5	6
Initial pH approx. 5.0	6	3	2	0	1	2	6	10	5	4	3	3
Initial pH approx. 6.0	4	3	2	4	2	2	5*	4*	28	24	13	11

* Contaminated

medium adjusted to approximately 5.0, for "water-soaking" became more apparent with decreased phosphate and hydrogen-ion concentrations (Table 10). Decreasing the total amount of light energy given to the plants by decreasing the intensity and also the length of day caused the"water-soaking" phenomenon to virtually disappear (thus these data were omitted from Table 10), but growth was too slow for satisfactory experimental purposes (Table 8).

The possibility remained that "water-soaking" might be diminished by aeration of the solution. Four combinations of salt concentration which had produced the greatest numbers of "water-soaked" plants in the triangulation experiment were employed. Magnetite was used as the source of iron and all cultures had the usual complement of minor elements added.

As previously mentioned, the addition of 0.005 M KNO_3 had sharply reduced "water-soaking" in the triangulation experiment. This time a series of 0.01 M KNO_3 was set up to determine the effect of increased concentration both with and without aeration. Measurements were made of the increase in fresh weight, frond number, and area for an 11-day growth period of the first subcultures from 30-frond inocula. Cultures were grown under a special fluorescent lamp (Cooper-Hewitt) such that the intensity was approximately three times as great as the intensity when cultures are placed 16" from a 40-watt "White" fluorescent lamp.

The tubes receiving aeration were sterilized with rubber stoppers through which passed a capillary inlet extending to the bottom of the medium. An outlet tube was also passed through the stopper (Fig. 3) so that a number of tubes could be connected together and air bubbled through at a slow rate.

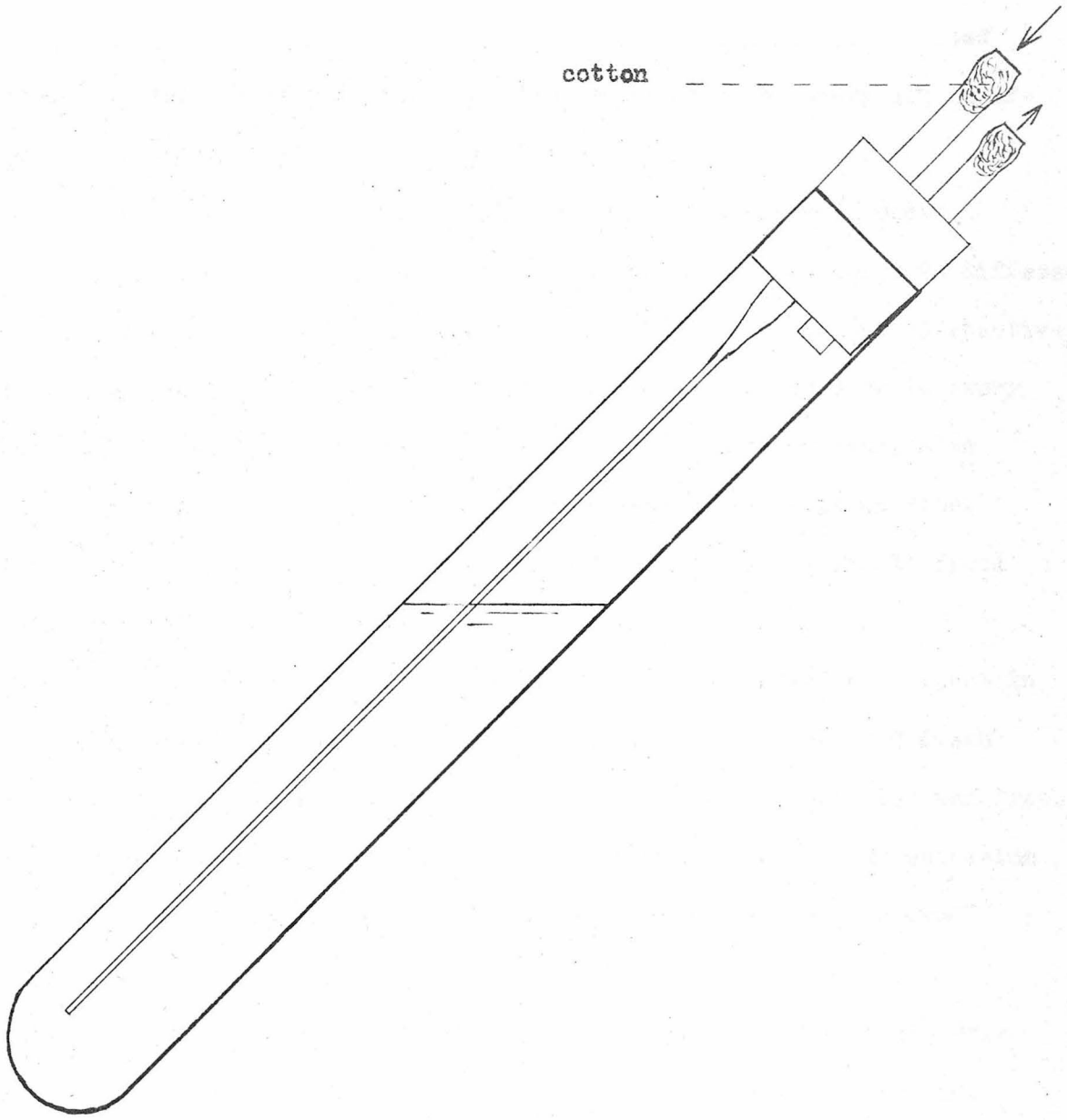


Figure 3. Details of aerated culture tube.

Some of the capillaries snapped above the level of the solution when being replaced after subculture, so the incoming air just wafted across the surface of the culture. This appeared to be every bit as effective as bubbling the air through the solution.

As may be seen in Table 11, growth of the cultures under relatively high light intensity varied considerably in response to different combinations of concentrations of KH_2PO_4, $Ca(NO_3)_2$, and $MgSO_4$. Respective concentrations of 1, 1, and 6 millimoles gave the best results in every case (Tables 11 to 13), while "water-soaking" was at a minimum, also. The effect was not solely confined to increased multiplication rate. Frond size also increased, although the fresh weight of a single frond was little affected (Table 13).

The addition of 0.01 M KNO_3 to the medium caused a decrease in the rate of frond production (reflected in total frond area and fresh weight) of the order of 10 per cent (Tables 11, 12). Frond size and fresh weight per frond were scarcely affected by the addition of the potassium nitrate, but it produced a reduction in the number of "water-soaked" fronds (Table 11).

Aeration of the media, or just circulation of fresh air over the surface of the media, was attended by an approximate 10 per cent increase in frond size and fresh weight per frond, while the number of "water-soaked" fronds increased only slightly (Tables 11, 12). The most striking effect produced by aeration, however, was the 30 per cent increase in frond number.

The beneficial effects of aeration could be attributed primarily to an improved carbon dioxide supply in the environment above the plants

Table 11

The effect of aeration and the addition of 0.01 M KNO_3 upon growth and "water-soaking" of plants in four media composed of different concentration combinations of three major salts. Light intensity 3 times greater than the most intense hitherto employed.

	Non-aerated (means of 4 replicates)								Aerated (means of duplicates)							
	No KNO_3				0.01 M KNO_3				No KNO_3				0.01 M KNO_3			
KH_2PO_4 (millimoles)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
$Ca(NO_3)_2$ "	6	5	4	1	6	5	4	1	6	5	4	1	6	5	4	1
$MgSO_4$ "	1	2	3	6	1	2	3	6	1	2	3	6	1	2	3	6
Inc. in fr. no.	103	103	106	112	91	99	94	106	112	124	139	174	116	121	131	142
Inc. tot. area(sq. mm.)	299	292	450	491	279	338	384	434	414	478	576	718	437	530	523	595
Inc. fresh wt. (mg.)	86	86	94	99	79	76	80	89	111	115	133	162	115	114	114	132
% "water-soaked"	12	10	6	9	6	3	1	1	16	16	5	4	8	3	1	0

Table 12

Sum of the means (Table 11) for comparison of the effect of aeration and the effect of KNO_3 addition.

	Non-aerated	Aerated	% increase
Inc. in fr. no.	814	1059	30
Inc. tot. area	2967 sq. mm.	4271 sq. mm.	44
Inc. fresh wt.	688 mg.	996 mg.	44
Frond size	3.7 sq. mm.	4.0 sq. mm.	8
Fresh wt./fr.	0.85 mg.	0.94 mg.	10

	No KNO_3	KNO_3	% decrease
Inc. in fr. no.	973	900	7
Inc. tot. area	3718 sq. mm.	3520 sq. mm.	5
Inc. fresh wt.	886 mg.	799 mg.	10
Frond size	3.8 sq. mm.	3.9 sq. mm.	-3
Fresh wt./fr.	0.91 mg.	0.89 mg.	2

Table 13

Sum of the means (Table 11) for comparison of the effect of different concentration combinations of KH_2PO_4, $Ca(NO_3)_2$ and $MgSO_4$.

KH_2PO_4 (millimoles)	1	1	1	1
$Ca(NO_3)_2$ "	6	5	4	1
$MgSO_4$	1	2	3	6
Inc. in frond no.	422	447	470	534
Inc. in tot. area (sq. mm.)	1429	1638	1933	2238
Inc. in fresh wt.	391	391	421	482
Frond size (sq. mm.)	3.4	3.7	4.1	4.2
Fresh wt./fr.	0.93	0.88	0.90	0.90

rather than to better salt accumulation by the roots, since it was not necessary that the air bubble through the medium. This was not clearly demonstrated, however, since the volume of solution was small, (only 25 ml.) and the plant roots very near the surface, so that in the cases where air only wafted across the surface of the medium, the oxygen tension near the surface of the solution was quite probably maintained at as high a level as in the cases where the air actually bubbled through.

Whether or not aeration improved growth by maintaining either the carbon dioxide content above the plants or the oxygen content of the solution at a more suitable level was not clear, but in all subsequent experiments the tightly packed cotton plugs used up to that time were replaced by lightly rolled loose-fitting plugs, so that gaseous exchange might be less hindered than it conceivably had been.

From the foregoing preliminary experiments, the following conclusions were reached:

1. Growth is best if KH_2PO_4 is kept at a concentration of 0.001 M, $MgSO_4$ at approximately 0.006 M and $Ca(NO_3)_2$ at approximately 0.001 M (Table 13), using high light intensities. Decreasing the concentration of KH_2PO_4 to 0.0005 M has no effect, but a change of the initial pH of the medium from ca.5.0 to ca.6.0 greatly decreases growth (Table 8), irrespective of differing light intensities. Coincident changes of $Ca(NO_3)_2$ and $MgSO_4$ concentrations over the range of 0.001 - 0.006 M affect growth but the optimal combination varies somewhat depending upon the light intensity at which the plants are grown (Tables 6, 8, 11, 13). Increasing the concentration of KH_2PO_4

from 0.001 to 0.005 or 0.006 M is attended by a reduction in growth. In media having 0.005 M KNO_3 additional, there is a general decrease in growth, particularly in cultures already having a high KH_2PO_4 concentration (Table 6). This decrease seems to be correlated with increasing concentration of potassium ion, for increased nitrate ion (supplied as calcium nitrate) does not have this effect.

Variations in light intensities and day length, or in other words, variations in total luminous energy supplied affect the growth rate profoundly. Finally, aeration of the medium or constant circulation of fresh air above it greatly accelerates growth. A better supply of carbon dioxide may be the reason that growth increases.

2. The per cent "water-soaked" fronds increases with dilution of Hoagland's medium, but is decreased by lowered light intensity (Table 4). Increasing $MgSO_4$ and KH_2PO_4 concentrations tends to decrease the number of "water-soaked" fronds as does decreasing the concentration of $Ca(NO_3)_2$ (Tables 7, 8, 11).

Addition of 0.005 M KNO_3 greatly reduces the per cent "water-soaked" fronds (Table 7), while 0.010 moles KNO_3 reduces the percentage still further (Table 11). A medium, initially too alkaline, will produce more "water-soaked" fronds, even though the final pH may be the same as that of other media initially more acid (Tables 9, 10). Aeration of media tends to increase the incidence of "water-soaked" fronds somewhat.

It seems most logical to attribute "water-soaking" primarily to a deficiency of potassium ion (since it can be largely corrected by

additional potassium). A deficiency of $MgSO_4$ coupled with an excess of $Ca(NO_3)_2$ also results in a greater number of "water-soaked" fronds. Unfortunately no attempts were made to correct these conditions and observe the effect upon the per cent "water-soaked" fronds, but the indications suggest that addition of KNO_3 and the use of even higher concentrations of $MgSO_4$ with possibly an even lower concentration of $Ca(NO_3)_2$ would make the occurrence of "water-soaked" fronds very infrequent indeed.

Not wishing to pursue the study of the inorganic nutrition of Spirodela further, experiments of this sort were discontinued. At the time, what now seems a reasonable explanation of the "water-soaking" phenomenon was not grasped. It was apparent that a medium such as Hoagland's with 0.005 M KNO_3 produced fewer "water-soaked" fronds than one which lacked KNO_3. In all subsequent experiments Hoagland's formula was employed rather than adopt a new variation of it. At the low light intensities used and the general short duration of the experiments, the slightly suboptimal growth and small number of "water-soaked" fronds produced were not so significant as to seriously affect the outcome of later tests.

CHAPTER III

LIGHT, CARBON DIOXIDE, CARBOHYDRATE

The fact observed in one of the preliminary experiments (Table 8) that growth, as measured by increase in frond number, total frond area, or total fresh weight, is proportional to the amount of light energy received, was described by Clark (1925) and by Ashby (1929a). The latter further observed that the average area and dry weight of an individual Lemna frond increased with increasing light intensity. He also noted that fronds then tended to stay attached and at supraoptimal light intensities they became a tangled mass of connected, very-much-thickened fronds, lacking the usual amount of chlorophyll. At low intensities, frond size and weight were unaffected by the duration of light, only rate of multiplication being affected.

These observations were confirmed for Spirodela by the following experiment. A rectangular box divided into seven equal compartments was fitted with a sliding lid, above which were suspended three 40-watt "White" fluorescent lamps mounted side by side on a white board which served as a reflector. By means of a time clock an electric motor periodically rotated a drum. This periodic rotation was sufficient each time to wind the cables attached to the sliding lid just enough so that it was drawn back the distance of one of the compartments. The lights were hung at one side of the box so that there was substantially decreased light intensity on the opposite side of each compartment. By means of the time clock the daily duration of illumination of the seven compartments was respectively 7, 10, 13,

16, 19, 22, and 24 hours. The sliding cover had to be manually returned to the starting position once each day.

Three tubes of sterile Hoagland's medium, inoculated with 30 fronds apiece, were placed in each compartment. One was inclined directly beneath the lights, the other two were inclined parallel to the side of the compartment away from the lamps (Fig. 4). The ratio of intensities thus supplied was 4 : 1.2 : 1. This apparatus (designed for other purposes) was located in an air-conditioned darkroom maintained at night at 19° C. and in the day at 26° C. The circulation of air in the room was rapid and no heating effects from the lamps altered the temperature within the compartments.

In Figure 5 the increase in frond number (Table 14) is plotted against the relative amount of luminous energy daily received by each culture (Table 15). Particularly at the lower intensities, as with Lemna, the rate of frond production is largely controlled by the amount of luminous energy supplied. The two cultures receiving the greatest amount of illumination had fronds whose size and weight were significantly increased (Table 14). Just as noted by Ashby (1929a), these fronds generally failed to separate completely. Clusters of new fronds were interconnected by very apparent filamentous internodes to form chains. All cultures under intensity "a" had roots about five times as long as those under intensities "b" and "c"

This increase in frond size and weight was thought perhaps to be the result of additional carbohydrate, in excess of that needed for new frond formation, which was being used for increasing frond size. As previously noted, aeration of the cultures at high light in-

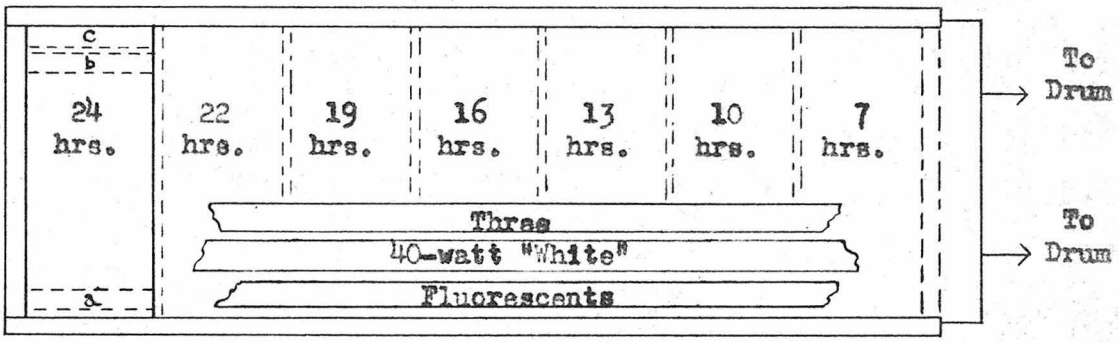


Figure 4. Diagram of apparatus for culturing Spirodela under different light intensities and durations. Ratio of three intensities at a, b, and c respectively 4 : 1.2 : 1.

Table 14

The effect of three light intensities, a, b, and c in the ratio of 4 : 1.2 : 1 for durations of 7 to 24 hours per day upon increase in frond number, frond size and fresh weight. 30-frond inoculum, cultured 7 days. Initial pH 4.8.

Hrs.	Increase in Frond Number			Frond Size (Sq. mm.)			Fresh wt. per Frond (mg.)			Final pH.		
	a	b	c	a	b	c	a	b	c	a	b	c
24	123	27	22	5.8	5.4	4.9	.82	.67	.67	6.8	6.0	5.5
22	90	26	19	5.3	5.0	5.0	.75	.64	.67	6.8	5.9	5.3
19	80	19	15	4.8	4.2	4.5	.67	.69	.58	6.5	5.5	5.2
16	67	16	10	4.9	4.2	4.4	.66	.61	.58	6.4	5.2	5.2
13	60	18	8	5.0	5.0	5.2	.66	.60	.58	6.2	5.1	4.9
10	52	17	8	4.9	5.0	4.9	.57	.57	.63	5.8	5.1	4.8
7	28	3	2	4.2	5.0	5.2	.57	.64	.59	5.5	4.8	4.7

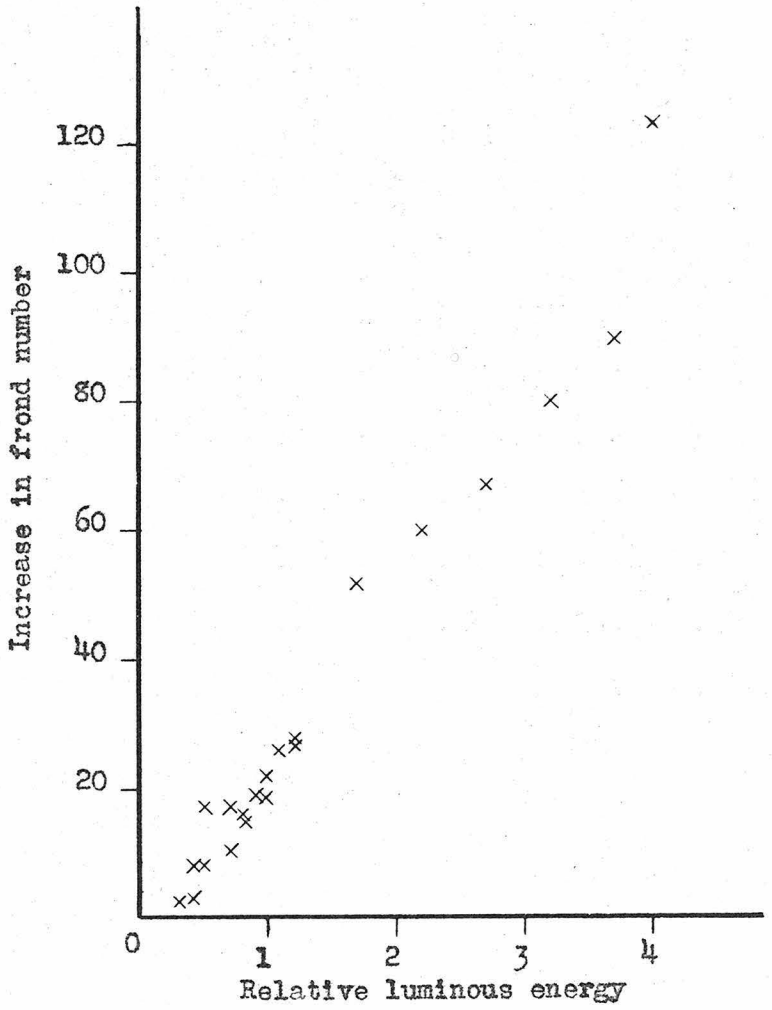


Figure 5. The dependence of increase in frond number upon the amount of luminous energy supplied.

Table 15

Relative amounts of luminous energy received by cultures under different intensities for different lengths of time each day.

Hours Per Day	Intensities (4.0) a	(1.2) b	(1.0) c
24	4.0	1.2	1.0
22	3.7	1.1	0.9
19	3.2	1.0	0.8
16	2.7	0.8	0.7
13	2.2	0.7	0.5
10	1.7	0.5	0.4
7	1.2	0.4	0.3

tensity occasioned a 10 per cent increase in unit frond size and fresh weight. It was suggested that a better carbon dioxide supply, made possible by aeration, perhaps induced more carbohydrate formation, some of which was available for increasing frond dimensions. Steinberg (1941) studying the nutrition of Lemna, reported that glucose (0.5 per cent) added to an otherwise satisfactory inorganic medium greatly promoted growth (frond multiplication). It was observed from his data that the glucose also increased the size of the individual fronds.

Glucose and sucrose were both tested, therefore, and found to have a pronounced effect upon growth of Spirodela. Both sugars were tried at 1 per cent and 0.5 per cent concentrations. The higher concentration was in each case more effective for promoting growth. Sucrose also seemed to be somewhat more effective than dextrose. To check the point as to whether the plants were not growing optimally because of a carbon dioxide deficiency and also to determine the optimal sugar concentration under the usual light intensity (16" from 40-watt "White" fluorescent), and one three times as great (Cooper-Hewitt), the following experiment was performed.

Eight sets of tubes of sterile Hoagland's containing varying amounts of sugar, 0, 1, 2.5, 4, 5, and 6 per cent, were prepared. Four of these sets had provisions for aeration (Figure 3, page 31). Three sets were placed under the Cooper-Hewitt lamp, one set being the unaerated control, the second set having 5 per cent carbon dioxide in air bubbled slowly through it, while the third set had 0.5 per cent carbon dioxide in air bubbled slowly through it. Under the lower

intensity "White" fluorescents was placed a similar set-up, with the following addition, however. The streams of 5 per cent and 0.5 per cent carbon dioxide were conducted to the tops of respective bell-jars, each of which enclosed a set of cotton-stoppered tubes containing media with the various sucrose concentrations. No effort was made to seal the rim of each bell jar. They just rested on one of the shelves beneath a 40-watt "White" fluorescent, and the carbon dioxide-air mixtures were permitted to leak out about the rims. The culture tubes were inclined upon a support inside, but the light intensity reaching the culture surfaces was undoubtedly diminished somewhat by the interposed glass of the bell jar which was not too clear. All cultures were inoculated with 30 fronds apiece and allowed to grow for 7 days. Measurements of the increase in frond number, total area, and total fresh weight were then made.

The experiment was repeated in a somewhat different fashion. All cultures were duplicated. Only a 4 per cent carbon dioxide-air mixture was available. Sucrose was used in concentrations of 0, 0.5, 1, 2.5, 4, and 5 per cent since growth had been found abnormal in 6 per cent sucrose.

From the two experiments it may be seen that the optimal sugar concentration for frond increase lies between 1 and 2.5 per cent (Tables 16, 19). If one compares the growth of the controls under the "White" lamp with the growth under the Cooper-Hewitt lamp in the first experiment (Table 16) and also in the second (Table 19 and Fig. 6), one observes that the effect of increased light is general over the whole range of sugar concentrations. Despite the plentiful

Table 16

The effect upon the 7-day increase in total frond number of 0.5% and 5% carbon dioxide-air mixtures supplied to media containing varying concentrations of sucrose. The gas mixtures were supplied by bubbling through the solution and also by placing the cultures in an atmosphere of the particlular carbon dioxide content.

"White"
Percent Sucrose

	0	1	2.5	4	5	6
Control	38	139	137	122	58	28
5% CO_2 bubbled	35	102	122	86	6	9
0.5% CO_2 bubbled	43	130	121	93	72	32
5% CO_2 atm.	57	127	125	132	92	69
0.5% CO_2 atm.	51	102	117	122	99	82

Cooper-Hewitt
Per cent Sucrose

	0	1	2.5	4	5	6
Control	68	193	158	149	81	41
5% CO_2 bubbled	84	170	216	130	3	29
0.5% CO_2 bubbled	106	160	191	115	119	37

Table 17

The effect of carbon dioxide addition upon (a) frond size and (b) fresh weight per frond.

(a) Frond Size (sq. mm.)	"White" Per cent sucrose						Cooper-Hewitt Per cent sucrose					
	0	1	2.5	4	5	6	0	1	2.5	4	5	6
Control	4.3	4.8	4.2	3.5	3.9	3.6	4.2	4.6	4.0	3.5	3.6	3.2
5% CO_2 bubbled	4.4	4.6	4.5	3.5	3.8	3.3	4.4	4.8	4.7	3.3	2.0	1.9
0.5% CO_2 bubbled	4.7	4.7	4.3	3.2	3.5	3.4	4.3	5.0	3.8	3.2	3.6	3.1
5% CO_2 atm.	4.3	4.6	4.2	3.7	3.8	3.6	---	---	---	---	---	---
0.5% CO_2 atm.	4.3	4.6	4.2	4.1	3.5	3.1	---	---	---	---	---	---

(b) Fresh weight per frond (mg.)	"White" Per cent sucrose						Cooper-Hewitt Per cent sucrose					
	0	1	2.5	4	5	6	0	1	2.5	4	5	6
Control	0.65	0.76	0.70	0.63	0.65	0.67	0.68	0.87	0.76	0.68	0.68	0.76
5% CO_2 bubbled	0.68	0.76	0.70	0.59	0.58	0.69	0.86	0.91	0.84	0.58	0.40	0.71
0.5% CO_2 bubbled	0.67	0.81	0.69	0.56	0.59	0.64	0.80	0.97	0.73	0.65	0.67	0.70
5% CO_2 atm.	0.68	0.88	0.71	0.66	0.71	0.75	----	----	----	----	----	----
0.5% CO_2 atm.	0.70	0.94	0.79	0.72	0.65	0.67	----	----	----	----	----	----

Table 18

Final hydrogen-ion concentration after 7-day growth of Spirodela upon media containing varying sucrose concentrations, supplied with additional carbon dioxide. Initial pH = 4.8.

"White"
Percent sucrose

	0	1	2.5	4	5	6
Control	5.1	6.7	7.0	6.7	6.4	6.0
5% CO_2 bubbled	5.4	6.0	6.1	5.9	4.5	4.9
0.5% CO_2 bubbled	5.4	6.4	6.6	6.3	6.2	5.8
5% CO_2 atm.	6.1	6.6	6.7	6.6	6.6	6.5
0.5% CO_2 atm.	6.1	6.5	6.8	6.8	6.7	6.5

Cooper-Hewitt
Per cent sucrose

	0	1	2.5	4	5	6
Control	6.3	6.5	6.9	6.9	6.5	6.1
5% CO_2 bubbled	6.0	6.2	6.4	6.2	4.0	5.3
0.5% CO_2 bubbled	6.5	6.8	6.8	6.6	6.6	5.9

Table 19

The effect of different sucrose concentrations upon 8-day increase in frond number, grown under two light intensities in an atmosphere of 4 % carbon dioxide-air or with 4 % carbon dioxide-air bubbled through the solution. Inoculum 30 fronds. Initial pH 4.8. Figures are means of duplicates.

"White"

	Per cent sucrose					
	0	0.5	1.0	2.5	4.0	5.0
Frond Number						
Control	45	131	147	143	148	131
4 % CO_2 bubbled	56	---	149	154	152	127
4 % CO_2 atm.	54	144	151	153	156	130
Frond size (sq. mm.)						
Control	4.6	5.5	4.9	4.5	4.3	3.9
4 % CO_2 bubbled	5.5	---	6.7	5.6	5.3	4.5
4 % CO_2 atm.	4.5	5.2	5.3	4.6	4.5	3.9
Final pH						
Control	5.8	6.7	6.8	6.9	6.9	6.9
4 % CO_2 bubbled	5.8	---	6.4	6.4	6.4	6.4
4 % CO_2 atm.	6.0	6.6	6.8	6.9	7.0	7.0

Cooper-Hewitt

Frond Number						
Control	77	181	189	198	171	165
4 % CO_2 bubbled	141	221	239	236	247	226
Frond size (sq. mm.)						
Control	4.7	4.8	5.2	4.9	4.3	3.6
4 % CO_2 bubbled	5.5	5.6	6.0	5.5	5.0	4.8
Final pH						
Control	6.4	6.8	6.8	6.8	6.9	7.0
4 % CO_2 bubbled	6.6	6.8	6.6	6.6	6.6	6.6

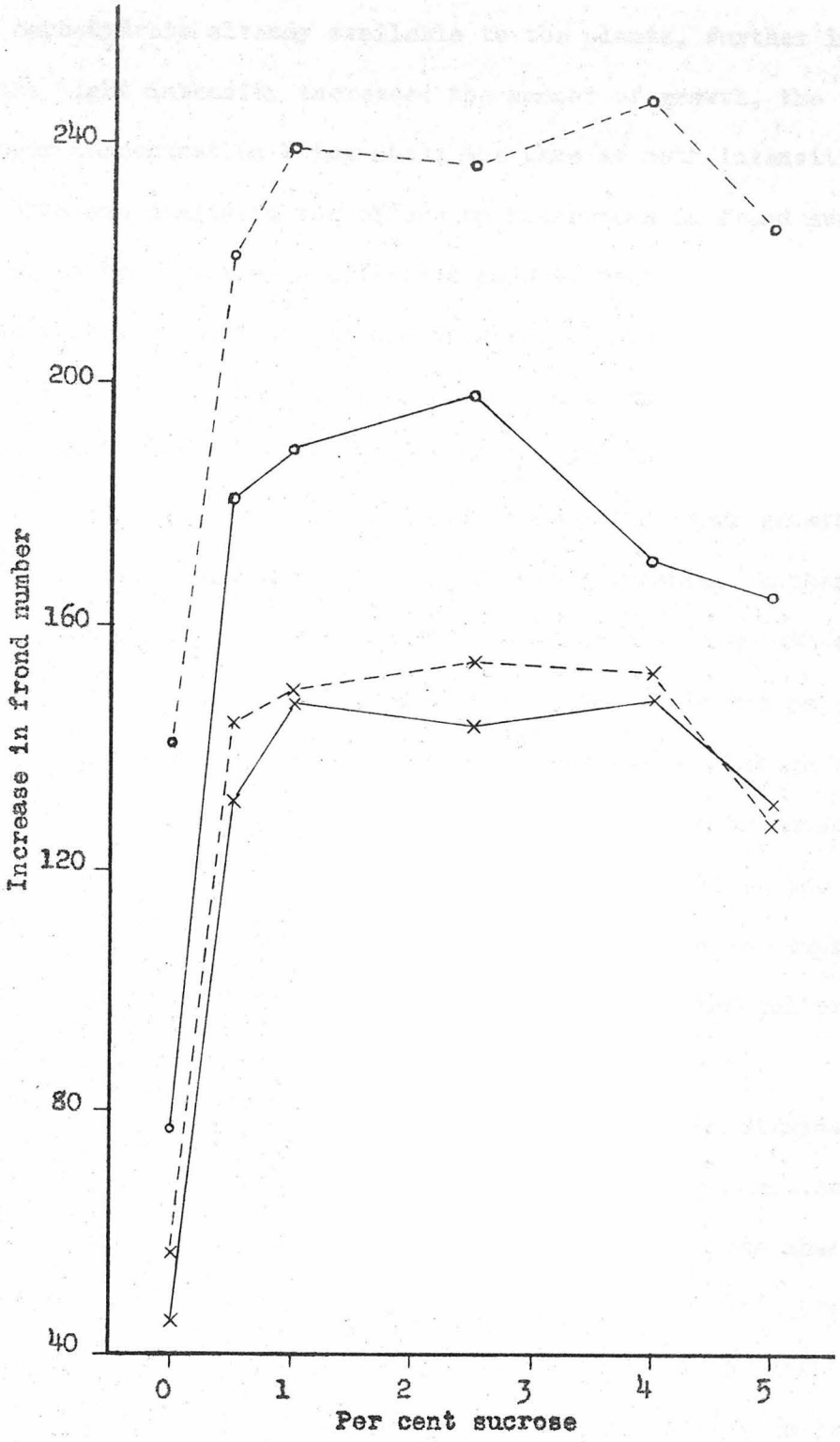


Figure 6. The effect of 4 per cent carbon dioxide-air upon cultures having increasing concentrations of sucrose. Dotted lines indicate carbon dioxide treatment; high light intensity indicated by circles; intensities approximately one-third lower indicated by x's.

supply of carbohydrate already available to the plants, further increasing the light intensity increased the amount of growth, the optimal sugar concentration being about the same at both intensities.

When one considers the effect upon increase in frond number of supplying carbon dioxide in different amounts considerably in excess of the normal content of air one observes (Tables 16, 19) that in non-sucrose medium growth can be promoted by as much as twenty-five to fifty per cent. In the first experiment (Table 16) using 0.5 and 5 per cent carbon dioxide-air mixtures, the effect upon growth in media of various sucrose concentrations was not favorable. Rather, there was a general though not too consistent decrease in growth as a result of treatment with additional carbon dioxide. This was particularly the case if 5 per cent carbon dioxide-air was bubbled through the different media. At the low light intensity, cultures in an atmosphere of 5 per cent carbon dioxide-air grew almost as well as the controls, even doing somewhat better when the sucrose concentration was supra-optimal. Under high light intensity carbon dioxide-treated cultures grew little better than the sucrose controls.

In the second experiment using 4 per cent carbon dioxide in air all treatments were duplicated. The agreement between duplicates was very close, so that the data are more reliable. Here one observes (Table 19 and Fig. 6) that the slight effect of carbon dioxide treatment of cultures having sucrose is of no significance at low light intensity, while at a higher light intensity the carbon dioxide-treated cultures grew 20 to 25 per cent more than did the controls. Here one may also observe that the effect of increasing the sugar concentration

in the medium from 1 to 4 per cent only slightly affected the rate of growth. Beyond 4 per cent the osmotic concentration was high enough to adversely affect growth.

The final pH of media through which gas was bubbled was generally lower than the pH of control media or media maintained in a carbon dioxide atmosphere (Tables 18, 19). Frond size, almost without exception, was greatest in a 1 per cent sucrose medium, becoming smaller with increasing concentration (Tables 17, 19). Particularly in the second experiment, carbon dioxide treatment produced still further increases in frond size in the presence of 1 per cent sucrose. Fresh weight per frond varied with the frond size. A concentration of sucrose greater than 5 per cent caused weight to increase even though the fronds became smaller (Table 17).

From the foregoing experiments one can draw the following conclusions concerning the light and carbohydrate relations of Spirodela. Under a moderate light intensity plants are not able to produce all the carbohydrate they can utilize for growth. A supplement of sucrose in the medium can correct this "deficiency," and growth is thereby accelerated. Probably there is a limit to the amount of sucrose that can be assimilated, for growth changes but little over a range of concentration from 1 to 4 per cent. Concentrations of 5 and 6 per cent sucrose then begin to decrease growth, osmotic concentration of the medium being too high.

The favorable effect of aeration upon growth at high light intensity was thought attributable to the provision of a better carbon dioxide supply, so that photosynthesis of carbohydrate was increased.

Treatment with carbon dioxide increases the growth rate to a marked degree. A sugar analysis of aerated and of carbon-dioxide-treated plants showed them to contain very nearly the same amounts which were 100 per cent higher than the sugar content of the controls. Since cultures in a carbon dioxide-enriched atmosphere grow every bit as well as those which have carbon dioxide-air bubbled through the solution, it is apparent that the carbon dioxide supplied by aeration is responsible for the additional growth and not the oxygen bubbled through the solution about the roots.

Increasing the light intensity is accompanied by a proportional increase in growth rate. What was not expected, however, was that plants upon medium containing sucrose in the optimal concentration range would grow still more rapidly when placed under a higher light intensity. Such, however, proved to be the case. From evidence deduced from these tests and from experiments conducted in the absence of light it is concluded that growth is controlled not only by the sugar supply available, but is further limited by an unknown factor (or factors) whose formation requires light. If light of high enough intensity is supplied, carbon dioxide treatment of cultures growing on sucrose medium produces a further increase in growth. The products of photosynthesis are distinguishably different from the sucrose coming from the medium, since their effects upon growth are essentially additive.

CHAPTER IV

COCONUT MILK, PEA DIFFUSATE

One of the chief problems of this investigation with Spirodela polyrrhiza was to show that there are substances, both in a crude and in a pure state, which when added to sterile inorganic medium balanced for optimal growth will cause the plants to grow at an increased rate.

In a preliminary experiment nicotinic acid and yeast extract were tested for growth promoting activity in concentrations of 15, 75, and 150 mg. per liter. The two highest concentrations of nicotinic acid decreased growth. The fronds were very abnormal in appearance for they were small, dark green, with a satiny gloss to the upper surface. At 150 mg. per liter growth was much arrested, and the fronds instead of separating grew in a clump, one on top of the other. Many fronds died. At the lowest concentration growth was only slightly diminished.

Decreasing growth and smaller fronds were associated with increasing yeast extract concentration, also. The symptomatic appearance was similar, though less pronounced, than was the case with nicotinic acid. This points to the nicotinic acid content of the yeast extract as being partly if not wholly responsible. In all yeast extract concentrations root length was 12 mm. With nicotinic acid root length diminished with increasing concentration from 12 to 7 to 4 mm. The controls had roots 5 to 6 mm. long. Root growth was stimulated by a suitable concentration of nicotinic acid, while frond growth, both in size and multiplication rate, was adversely affected.

In the cultivation of very young embryos of Datura, van Overbeek, Conklin, and Blakeslee (1942) reported that non-autoclaved coconut milk promoted growth when added to an agar containing inorganic salts plus dextrose. If, besides the non-autoclaved coconut milk, there was a supplement of growth factors[3] (concentrations fixed arbitrarily) in the medium, growth was promoted even more.

Coconut milk was tested for its effect on the growth of Spirodela in the following manner. Hoagland's medium at five-thirds normal strength was tubed and sterilized, 15 ml. per tube. Sterilized separately was a concentrated mixture of the growth factors, made up in such a way that 0.5 ml. in 25 ml. of final culture solution gave the proper concentrations. Fresh coconut milk was drawn sterilely from a carefully opened nut and added (10 ml. per 25 ml. culture) to the concentrated Hoagland's plus growth supplement. For comparison, old coconut milk rendered sterile by Seitz filtration was also tested, along with a control having only the growth supplement, and a control without any additions. Six replicates of each treatment were inoculated with 30 fronds apiece, allowed to grow one week, then 30 fronds apiece subcultured upon freshly prepared media. At the time of subculturing, all plants which had received the filter-sterilized coconut milk were dead, although a few new fronds were formed before death. One culture receiving fresh coconut milk was also dead. These cultures were discontinued. Measurements were therefore made on the 7-day growth of five cultures having fresh coconut milk plus supplement, six cultures having the

3 Concentrations in mg. per liter: glycine 3.0, thiamin 0.15, ascorbic acid 20.0, nicotinic acid 1.0, vitamin B_6 0.2, adenine 0.2, succinic acid 25.0, pantothenic acid 0.5.

supplement only, and six controls. Cultures were grown at 16" distance from the 40-watt "White" fluorescent.

Although the supplement of growth factors caused a very slight increase in number of fronds (Table 20) their size and weight were diminished. The addition of coconut milk increased the frond number by more than 20 per cent and fresh weight to an even greater degree, but frond size was diminished. Those plants treated only with the supplement of mixed growth factors were distinctly different in appearance from the controls. The satiny gloss of the upper surface, coupled with the diminished size, strongly suggested the effect produced by too much nicotinic acid. The amount present was only 1.0 mg. per liter and previously this effect had been observed with 15.0 mg. per liter nicotinic acid, so that some other component of the mixture of growth factors may have been jointly or solely responsible.

Through the courtesy of Dr. van Overbeek several different concentrates and purified extracts of coconut milk which had shown activity when tested with Datura embryos were obtained. In many instances only a few milliliters were available so that testing in replicate was not possible. The test was conducted using 1 per cent sucrose Hoagland's as basal medium, so that the sugars known to be in coconut milk could not be credited with all the activity. The extracts tested, together with the description furnished with them and the amounts used are summarized in Table 21.

Thirty-frond inoculum was used. Subcultures were made after one week and measurements made on the subsequent week's growth. Cultures with I, II, III, and VII developed bacterial growth in this time so have

Table 20

Effect of a mixture of growth substances, with and without the addition of coconut milk (10 ml./25 ml.) upon 7-day growth of Spirodela. Initial pH 4.0. Figures represent means of 5 or 6 replicates.

	Increase in frond number	Increase in total area (sq. mm.)	Increase in total fresh weight (mg.)	Final pH
Fresh coconut milk plus growth supplement	68	266	50.2	5.6
Growth supplement	56	225	28.4	4.5
Control	52	298	30.7	6.0

Table 21

Different coconut milk preparations, together with the descriptions furnished, the amounts added to each 25 ml. culture, and their relative equivalence in terms of milliliters of fresh coconut milk.

Code	Description	Ml. used per 25 ml. culture	Equivalence in ml. fresh coconut milk
I	#8	1.0	1.0
II	Fuller's earth treated(5 x conc.)	1.5	7.5
III	#7	1.0	1.0
IV(a)	#3 filtrate 50° 1 hr.	1.0	1.0
(b)	" " " "	2.0	2.0
(c)	" " " "	5.0	5.0
V	Combined #7 and #8	2.0	2.0
VI	Combined #7 and #8 (5 x conc.)	0.75	3.75
VII	#6 (5 x conc.)	1.0	5.0
VIII	Charcoal, fuller's earth treated	1.5	1.5
IX	Combined #4 and #5 activated charcoal 5 hrs.	1.5	1.5

not been included in Table 22. Cultures with I and II grew very little, while with III growth was almost exactly the same as the 1 per cent sucrose control. With VII growth was 50 per cent better than this control, and despite bacterial contamination, was about on a par with the culture having VI (Table 22). Preparations IV, V, VI, VIII, and IX possessed the ability to stimulate growth in excess of that in the 1 per cent sucrose control, the activity being proportional to the concentration. Although frond size increased 10 per cent in cultures V and VI (combined #7 and #8; 5 x concentrate of same combination) there was little significant change in other cultures. The most striking effect was the great increase in frond weight which exceeded that of the 1 per cent sucrose control by as much as 30 to 50 per cent. All treated plants had some anthocyanin pigmentation on the underside indicating excess carbohydrate.

The two preparations VIII and IX which had undergone a charcoal adsorption supported the greatest amount of growth, even though used at comparatively low concentrations (1.5 ml. for both). Either these preparations were more active to begin with or treatment with charcoal removed inhibitory material from them.

From analyses that have been made upon milk from ripe coconuts, it is known that it contains about 4.5 per cent sugar (almost entirely sucrose). In this experiment, then, the cultures treated with coconut milk had added to the 1 per cent sucrose in the basal medium an additional 0.18 to 0.90 per cent sugar, composed largely of non-reducing sugar but with a certain small amount of reducing sugar. If one looks back (Table 19, page 50) to the experi-

Table 22

Growth of Spirodela on 1 per cent sucrose Hoagland's to which varying amounts of coconut milk preparations have been added. Measurements upon 7-day growth of subcultures from 30-frond inocula.

	CONTROLS % sucrose			Coconut milk preparations with equivalent amounts tested						
				IVa	IVb	IVc	V	VI	VIII	IX
	0	0.5	1.0	1.0	2.0	5.0	2.0	3.75	1.5	1.5
Inc. in fr. no.	43	96	129	131	162	178	183	196	217	228
Fr. size (sq. mm.)	3.7	3.9	4.6	4.5	4.8	4.2	5.1	5.1	4.5	4.4
Fresh wt./fr. (mg.)	0.60	0.71	0.82	1.18	1.22	1.17	1.26	1.19	1.08	1.13
Final pH	5.9	6.5	6.9	5.8	5.7	5.6	6.3	6.0	5.8	5.6

ment on the effect of sugar concentration under the "White" lamp upon increase in frond number, one sees that the additional increment caused by coconut milk is far greater than the increment that would occur if only sugar concentration were increased from 1 to 2 or even 2.5 per cent.

Although it did not seem very likely that the beneficial effects of coconut milk were to be ascribed solely to its sugar content, the possibility was tested by analyzing a coconut milk preparation for reducing and non-reducing sugars; then simultaneously testing this coconut milk and a preparation of sucrose and dextrose having the same concentration of sugars, using the same dilutions. For the Datura embryo effect the coconut milk must not be autoclaved. In this experiment a series using autoclaved coconut milk was compared with a series using filter-sterilized coconut milk as well as a series of the sucrose-dextrose combination. It was thought that if part of the activity was not caused by the sugars and was destroyed by heat, then the activity of the sugars as they really are in coconut milk could be plainly assessed.

A 4.3 times concentrated coconut milk preparation upon analysis revealed 21.1 per cent non-reducing sugar and 5.6 per cent reducing sugar. In terms of unconcentrated coconut milk that equalled 4.9 per cent non-reducing, 1.3 per cent reducing sugar. Twenty-five milliliters of 21.1 per cent sucrose, 5.6 per cent dextrose were prepared. Expressing concentrations as milliliters unconcentrated coconut milk per 25 ml. culture, four strengths were tested in each of the three series. Measurements were made after seven days had been

given the subcultures to grow from 30-frond inocula. During that period the "White" lamp burned out and was replaced only after twenty-four hours or more, so that the plants actually received six days or less of full illumination. Thus the figures in Table 23 are all lower than normally would have been the case.

Contrary to expectation only the lowest concentration of coconut milk, which had been autoclaved, produced a significant increase in growth. None of the cultures which had filter-sterilized coconut milk grew as well as the sugar controls, although frond weight practically doubled. This increase in frond weight was even more pronounced in cultures with autoclaved coconut milk. Frond size was greatly increased with the two lowest concentrations of autoclaved coconut milk.

This test gave every indication that the factor or factors in coconut milk responsible for accelerated growth were not only heat-stable, but in this particular preparation at least, were associated with inhibitors partially destroyed by autoclaving. It may be argued that this was a more active preparation than used hitherto, so that the range of concentrations was supraoptimal. With autoclaving, the activity was partially destroyed so that the lowest concentration tested more nearly approached the optimum. However, the inhibitor interpretation seems more reasonable in the light of the evidence to follow. In the previous test (Table 22, page 61) growth was not the same when similar concentrations of different filter-sterilized preparations were used, indicating that activities do differ. This may be explained on the basis of different amounts of active substance or of inhibitor being present. More probably it represents different ratios of active

Table 23

The effect of different concentrations of Seitz-filtered or autoclaved coconut milk when compared with similar concentrations of a sucrose-dextrose mixture of the same per cent composition as the sugars in the coconut milk. Measurements made after 7 day's growth of subcultures from 30-frond inocula. Figures represent means of closely agreeing duplicates. ($V_a$lues small because burned-out lamp not replaced for 24 hours or more.)

	Ml. unconcentrated prep./25 ml.				
	Control	2.5	5.0	7.5	10.0
Increase in frond number					
Sucrose-dextrose	23	90	97	104	101
Filter-sterilized	--	72	60	9	10
Autoclaved	--	137	88	12	7
Frond size (sq. mm.)					
Sucrose-dextrose	4.0	5.0	5.4	4.9	4.5
Filter-sterilized	---	5.2	4.2	3.4	3.3
Autoclaved	---	6.1	6.5	3.6	3.0
Fresh weight per frond (mg.)					
Sucrose-dextrose	0.47	0.79	0.84	0.76	0.72
Filter-sterilized	-----	1.4	1.4	1.2	1.0
Autoclaved	-----	1.5	2.1	1.4	1.0

substance to inhibitor since van Overbeek (1942) has found that certain treatments of coconut milk preparations may release inhibitory substances which mask the Datura embryo factor activity. He particularly showed how ether extraction and to a lesser degree, autoclaving, cause a release of active auxin. He concluded that auxin is responsible for the root inhibition of the embryo cultures. Spirodela plants which were markedly inhibited by treatment with coconut milk showed pronounced epinasty, just as Lemna plants show with high concentrations of indoleacetic acid (Gorham, 1941). There is a strong likelihood that supraoptimal auxin concentration is at least one of the inhibiting factors for Spirodela as well.

Although the active principle in coconut milk may not be the same for Spirodela as for Datura embryo, since autoclaved coconut milk is active as regards the former, the difference in response is probably a reflection of the different behavior of Spirodela and Datura embryo towards the concentrations and types of masking inhibitors, of which there appears to be more than one.

Through the courtesy of Dr. D. M. Bonner a sample of pea diffusate (70 mg. per ml.) was obtained. Bonner, Haagen-Smit, and Went (1939) and Bonner (1940) have reported pea diffusate to be a rich source of leaf growth factors. More recently Went and Bonner (1943), by means of a special test with tomatoes, have shown that pea diffusate, along with coconut milk, possesses caulocaline as well as leaf growth activity. There was good reason, therefore, to suppose pea diffusate would promote growth of Spirodela.

A test was made using four concentrations, 0, 0.05, 0.5, and

5.0 mg. per ml. in four sucrose concentrations, 0, 0.01, 0.1, and 1.0 per cent. The pea diffusate was toxic at 5.0 mg. per ml. and inhibitory at 0.5 and 0.05 mg. per ml. in all sucrose concentrations. Unfavorable hydrogen-ion concentration was not responsible for the inhibitory and toxic effects for pH was checked at the beginning and end of the experiment. It is not unlikely that some inhibitor completely masked any growth promoting activity the pea diffusate may have had, but no solvent extractions were made to test this possibility.

In summary, therefore, it can be said that nicotinic acid and yeast extract both diminish growth over a range of concentration from 15 to 150 mg. per liter. Pea diffusate likewise decreases growth at concentrations ranging from 0.05 to 5.0 mg. per ml. Coconut milk possesses growth promoting activity, partly the result of sugars, partly attributable to unknown materials. Too high concentrations of coconut milk are inhibitory, autoclaving removing the inhibitory effects somewhat. Autoclave temperatures do not destroy, or only partially destroy growth promoting activity. The factors promoting growth of Datura embryos may be similar to those which promote growth of Spirodela, the presence of inhibitors accounting for differences in response.

CHAPTER V

HUMUS, SPHAGNUM, AND DUNG EXTRACTS

Bottomley (1914, 1917a) was one of the earlier workers who investigated the role of organic growth promoters as they affect higher plants. He first reported work with wheat seedlings grown in both sand and solution culture. A 20 to 25 per cent increase in growth (in terms of dry weight) resulted when these seedlings were treated with an ethanolic extract of peat in which certain soil microorganisms had been growing (bacterized peat) or fractions separated by phosphotungstic acid or silver nitrate.

He used Detmer's formula,[4] which has a very high total salt content, as the basis for his solution culture experiments. Neither wheat seedlings nor Lemna minor would long maintain growth in a medium having only inorganic constituents. The presence of soluble organic matter appeared essential for their normal growth. With Lemna minor, increase in number and size of fronds was proportional to an increase in the amount of growth promoting substance ("auximone," as he called it) added to the culture solution. He suggested that some of the organic material acted directly as an organic nutrient, while the remainder might be in the nature of accessory food substances.

Because bacterized peat had been observed to affect nuclear development, Bottomley (1917b) undertook to analyze this material to see if it contained nucleic acids. He found no nucleic acid as such,

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4 Concentrations in grams per liter: KNO_3 2.33, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $CaSO_4.2H_2O$ 1.67, $FeCl_3$ trace.

but all the constituents which were present he concluded to have been broken down by bacterial activity. Derivatives from nucleic acid which had undergone decomposition in decaying peat were tested (1919) and found to promote the growth of Lemna minor in Detmer's solution. An autoclaved suspension of Azotobacter chroococcum (previously grown 14 days on mannite-agar) also promoted growth to about the same extent. An enormously increased growth resulted when the nucleic acid derivatives were combined with the Azotobacter preparation. This large increase was equivalent to that obtained by the use of bacterized peat. Neither the ash from the nucleic acid derivatives nor that from the Azotobacter preparation had any activity.

The preparation of nucleic acid derivatives was made by extracting peat with 1 per cent sodium bicarbonate until no longer colored. The combined extracts were neutralized with hydrochloric acid and concentrated. An "adenine-uracil dinucleotide" was separated from this which also showed activity. An autoclaved preparation of Bacillus radicicola grown on maltose-agar likewise showed activity. Bottomley noted that dry weight increased 30 per cent if growth were promoted, but decreased 20 to 30 per cent in the controls or the ash treatments during a growing period of 7 weeks.

In subsequent experiments (1920a, 1920b) Bottomley made a comparison of the growth of Lemna minor and Lemna major (Spirodela polyrrhiza) in Detmer's solution, Knop's solution,[5] and pond water, with and without added bacterized peat, to see if its promotive

5 Concentrations in grams per liter: KNO_3 0.167, KH_2PO_4 0.167, $MgSO_4.7H_2O$ 0.167, $Ca(NO_3)_2.4H_2O$ 0.5, $FeCl_3$ few drops.

effect were only demonstrable in the presence of an unsuitable combination of inorganic materials. Neither species grew normally for any length of time on either Detmer's or Knop's solutions. L. minor produced fewer fronds but was normal in appearance when grown on pond water. Increased growth and healthy appearance occurred in cultures of either Detmer's or Knop's to which bacterized peat was added.

Extracts from an autoclaved growth of Azotobacter chroococcum, crude nucleic acid derivatives from raw peat (bicarbonate extraction), and a water extract of bacterized peat promoted growth of Lemna major, Salvinia natans, and Azolla filiculoides.

Bottomley's methods were used by Mockeridge (1920) to show that aqueous extracts of fresh and well-rotted stable manure, manured soil, and leaf-mold all promoted growth of Lemna major upon Knop's medium. Plants without additions diminished in weight and became unhealthy. All these extracts were found upon analysis to contain varying amounts of nucleic acid derivatives. The greater the effect shown, the greater was the proportion of purine and pyrimidine bases. From Azotobacter chroococcum Mockeridge (1924a) also obtained the essential constituents of nucleic acid: carbohydrate, phosphoric acid, purine and pyrimidine bases. She found(1924b) that with varying concentrations of crude nucleic acid derivatives from peat, growth of Lemna minor was approximately proportional. With a preparation derived from Azotobacter growth was promoted but the increase was not proportional to concentration. An autoclaved preparation of Saccharomyces promoted growth to a greater extent than an autolysed (35°C. for 72 hours) preparation. This she attributed to the greater decomposition of nucleic acid which

would occur at autoclave temperatures (120°C. for 1 hour), for both Mockeridge and Bottomley came to the conclusion that the growth promoting effects of their different preparations were somehow connected with the observed occurrence therein of purine and pyrimidine bases, since both cell and nuclear size increased upon their addition.

The results obtained by Bottomley and Mockeridge and the conclusions they drew from them were at once disputed. Criticism centered about the use of Detmer's solution upon which Lemna failed to grow normally. This had led Bottomley erroneously to believe organic supplements essential for the maintenance of active, vigorous growth. This subnormal growth of the controls made the increases resulting from treatment with organic materials appear questionable. Could not a suitable inorganic medium be devised in which these organic additions would have no effect? A further criticism was levelled at their work, namely, that they had used frond number as a criterion of growth. As this increases exponentially with time, the differences between treated and untreated cultures after a long growth period (7 to 10 weeks) became greatly exaggerated. From their data it was observed that treated and control cultures sometimes did not differ for several weeks, then as the controls began to fail, the treated cultures appeared to be stimulated to increased multiplication.

It was not long before Clark (1924) showed that Lemna major could be grown for a period of 5 months without the addition of any organic materials. Throughout this period normal vigor was maintained. He therefore concluded that Bottomley's "auximones" cannot be considered as essentials for the growth of green plants in the sense that vitamins

are necessary for the growth of animals. Their function must be accessory in nature.

Using what was essentially Bottomley's technique Saeger (1925) found that neither Detmer's nor Knop's solution was suitable for growth of Spirodela polyrrhiza, but in Knop's solution, diluted one part to ten, he successfully grew S. polyrrhiza and Lemna valdiviana Phillipi for nearly half a year. An extract of autolyzed yeast and a preparation of "crude nucleic acid derivatives" from peat by 1 per cent sodium bicarbonate extraction (as outlined by Bottomley) stimulated growth of Spirodela polyrrhiza both in 1/10 Knop's solution and in pond water. The greatest effect was caused by their addition to the 1/10 Knop's solution. Like Clark, Saeger concluded that the necessity for organic foods could not be accepted as an established fact.

Because Ashby (1929b) considered the experimental error so great as to seriously discredit the results obtained by Bottomley and Mockeridge, he undertook a careful investigation of the effect of aqueous extracts of both fresh and well-rotted horse dung upon the growth of Lemna minor. He endeavored to keep bacterial contamination at a minimum by frequent changes of solution, which was sterilized before organic materials were added. The pH was adjusted to 4.8 with sulfuric acid. The cultures were grown, under controlled conditions of light and temperature for only 11 or 12 days using Clark's formula[6] as a basal medium.

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6 Concentrations in grams per liter: $CaHPO_4.2H_2O$ 0.07, KNO_3 0.80, $MgSO_4.7H_2O$ 0.25, $FeCl_3.6H_2O$ 0.0027.

Unlike Mockridge, he found no difference in the activity of extracts from fresh and from well-rotted manure. Both caused a pronounced growth increase. The ash of these extracts had no appreciable activity, but the active materials withstood autoclaving. Frond weight was unaffected by the extracts, butfrond size increased with increasing concentration. From a determination of the solid matter content of the extracts he was able to determine that activity was maximal at a concentration of approximately 2 parts per million organic material added. Increase in concentration up to 20 parts per million had no further effect. At high concentrations (2,000 p.p.m.) Ashby noted some growth inhibition. He observed an increase in cell size and chloroplast number in plants whose growth rate was stimulated so he attributed the increased growth rate to increased photosynthesis.

Olsen (1930) tested a watery extract of bacterized peat (prepared according to Bottomley's Danish patent No. 18966) upon Helianthus annuus growing in solution culture[7] but found that growth was not affected. He repeated the test using Lemna polyrrhiza (Spirodela polyrrhiza) grown in Knop's solution with pH adjusted to about 6.0. Throughout subsequent growth the pH was never permitted to exceed 7.7. Solutions were changed once a week, the experiment being continued for about a month. Olsen compared the effect of iron supplied as chloride (50 to 100 mg. of iron per liter) and as citrate

7 Concentrations in grams per liter: $(NH_4)_2SO_4$ 0.26, $Ca(NO_3)_2.4H_2O$ 0.80, KNO_3 0.25, $MgSO_4.7H_2O$ 0.25, KH_2PO_4 0.25. (Boiled and filtered tap water. Iron as ferric citrate).

(5 mg. of iron per liter), both with and without addition of bacterized peat extract (15 mg. of dry matter per 5 ml. of extract added per liter). Pronounced stimulation occurred when peat extract was added to solutions having iron as ferric chloride, for the plants without this addition soon became chlorotic and multiplied feebly. Treated plants were twice as large and multiplied rapidly. Treated plants upon ferric citrate medium were the same as treated plants on ferric chloride medium, but so also were those untreated plants on ferric citrate medium. Olsen, therefore, concluded that since the effect of ferric citrate was quite similar to that of humus extract, the effect of the latter is to make iron more readily available to the plants.

Finally, Clark (1930), and Clark and Roller (1931) tested extracts from soil, alfalfa, and manure upon Lemna major in both sterile and non-sterile culture, using Clark's formula for their basal medium, and growing the plants in cotton-stoppered Erlenmeyer flasks in sunlight. In sterile culture, the addition of organic substances had no effect or else a depressing effect upon the rate of reproduction, when optimal concentrations (1 to 60 p.p.m., effective range in non-sterile culture) were tested. If non-microorganism-free plants were introduced into sterilized medium having organic additions, a stimulation in growth rate occurred. Upon inorganic medium the growth of cultures free from microorganisms was faster than those not so freed. Pure cultures of bacteria sometimes increased, at other times did not increase, and sometimes decreased growth when added to sterile inorganic cultures. Decreased growth was not so pro-

nounced if organic matter was present. They further tested a number of recognized organic compounds such as urea and creatinine and found no stimulation in either sterile or non-sterile cultures.

With so much conflicting evidence concerning the growth-promoting properties of extracts from peat, soil, and manure, there was clear need for a reinvestigation of the problem. First of all, an attempt was made to decide to what extent Olsen's hypothesis was correct. It was noticed from his data that when the cultures received iron as ferric chloride the concentration was so high as to prove toxic, and indeed, such cultures grew poorly. However, the pH range at which they were grown was also so high as to have precipitated the iron as hydroxide, thereby rendering it virtually unavailable to the plants. Poor growth and chlorotic condition was thus caused, perhaps, by an almost complete lack of iron, which condition could be corrected by adding extract of bacterized peat or using a medium with iron supplied as the citrate.

Olsen used Knop's formula for his basal medium, adjusting the pH to 7.7. With an adequate supply of iron, such as was furnished by ferric citrate, an extract of bacterized peat produced no growth stimulation. Several other investigators have found a pH of 4.8, as used by Ashby and Clark, to be about optimal for the growth of Lemna. Possibly Olsen's failure was occasioned by his use of such a sub-optimal hydrogen-ion concentration.

In preliminary experiments, previously mentioned, a humus extract was found to stimulate growth of Spirodela polyrrhiza in sterile culture under constant illumination of moderately high intensity. Iron

was supplied as the tartrate and as four inorganic salts. At a pH of about 5.0 growth was the same in all five types of cultures. A 20 per cent increase in growth attended the addition of humus extract to all five types of cultures. Repetition of the experiment to try and establish the optimal concentration of humus extract was not successful. The problem was therefore dropped while the inorganic nutrition was being studied, to be picked up when it and the effect of other environmental factors upon the growth of Spirodela were better understood.

Two extracts of humus, composed largely of rotting leaves of scrub oak (Quercus dumosa), were prepared by repeated aqueous extraction of 250-gram samples until no more color remained. One extract was made by daily replacing the colored solution with distilled water adjusted to pH 8.5 with ammonium hydroxide. The other extract was prepared under alkaline conditions achieved by the use of 5 per cent sodium bicarbonate solution. The daily aliquots of extract were collected over a period of nearly 3 weeks. Each day's aliquot was centrifuged then added to those of previous days, and kept at 3° C. to hinder growth of microorganisms. After collection ceased, the two extracts were concentrated to a volume of 250 ml. under vacuum at a temperature which did not exceed 60° C.

The ammonium extract was tested over a wide range of concentrations (extract from 0.001 to 20 gms. humus per liter). Both autoclaving and Seitz filtration were employed in sterilizing the extract. In either case, growth of Spirodela was completely

unaffected by any concentration tested.

The sodium bicarbonate extract was similarly tested over a range of concentrations (extract from 0.001 to 16 gms. of humus per liter). The test with autoclaved extract was a failure because of neglect to neutralize its alkalinity. This was done before the test was repeated using filter-sterilized extract. With sulfuric acid the pH of the concentrated extract was adjusted to approximately 7.0. The following concentrations were used: extract from 0, 0.001, 0.01, 0.1, 1.0, and 10.0 grams humus per liter, tested in two series, one in which no sucrose was added to Hoagland's medium, the other in which there was the addition of 0.5 per cent sucrose. The growth made in the subcultures, inoculated with 25 fronds apiece, at 16" from the "White" fluorescent lamp was measured after 7 days.

With no addition of sucrose to the medium, the lowest concentrations of oak humus extract promoted an increase in frond number (Table 24). Frond size was somewhat increased, while frond weight was affected but little. The highest concentration was inhibitory. In 0.5 per cent sucrose medium the beneficial effect of the extract upon increase in frond number was no longer apparent. At concentrations of 0.1 and 1.0 gm. per liter frond size was considerably greater, but the extract had little or no effect upon the fresh weight per frond. Hydrogen-ion concentration changed over a greater range where the greater amount of growth had taken place, namely, in the 0.5 per cent sucrose series. This experiment showed, therefore, that humus extracts can promote growth in the presence of ferric tartrate provided the pH is not too high. It also indicated a relationship between

Table 24

The effect of different concentrations of filter-sterilized oak humus extract with and without the addition of 0.5% sucrose. Growth of subcultures measured after 7 days. 25-frond inoculum. Figures represent means of 4 replicates.

No sucrose

	Extract from the following amounts of humus (gm./l.)					
	0	0.001	0.01	0.1	1.0	10.0
Inc. in fr. no.	38	47	48	47	43	27
Frond size(sq. mm.)	4.6	4.8	5.4	5.1	5.1	4.2
Fresh wt./fr.(mg.)	0.58	0.63	0.56	0.53	0.60	0.62
Initial pH.	5.2	5.0	5.1	5.5	6.0	6.3
Final pH.	5.6	5.7	5.8	5.9	6.3	7.6

0.5% sucrose

Inc. in fr. no.	133	124	134	131	87	69
Fr. size (sq. mm.)	5.9	6.1	5.8	6.5	7.2	5.7
Fresh wt./fr.(mg.)	0.87	0.87	0.86	0.93	0.91	0.91
Initial pH.	4.8	4.9	5.0	5.4	5.9	6.4
Final ph.	6.8	6.8	6.8	6.9	6.9	7.7

carbohydrate supply and the type of growth which would occur.

Five differing soil samples from locations in Maine, New Hampshire, and Vermont were extracted with water by autoclaving, centrifuging, and making up to standard volume (extract from 50 gms. soil per 100 ml.). Eighty-seven grams of sphagnum peat were dried, extracted with water at pH 8.5 in a Waring blender, centrifuged, the clear liquid concentrated, neutralized with hydrochloric acid, and made up to 250 ml. Samples of fresh horse and cow dungs were dried, 100 grams of each autoclaved in 500 ml. of distilled water, centrifuged, and made up to 500 ml. volume.

These extracts were tested for activity by adding 2.5 ml. per 25 ml. culture to medium containing 1 per cent sucrose. The tests were made using three concentrations of iron, 1, 10, and 100 parts per million, supplied in one series as the tartrate, in another as the chloride.

Several cultures became contaminated with molds, but from the incomplete data gathered the following observations could be made.

1. Under the experimental conditions employed, no extract promoted growth significantly.

2. Several extracts inhibited growth to a certain extent.

3. Ferric tartrate and ferric chloride serve about equally well as iron sources provided the concentration is not too great.

4. An iron concentration of somewhat more than 1 part per million may benefit growth slightly.

5. One hundred parts per million of iron as chloride was immediately toxic because the hydrogen-ion concentration was too high. The same concentration of iron as tartrate

produced nearly complete inhibition, although hydrogen-ion concentration was not seriously affected.

6. In 100 parts per million of iron as tartrate the growth inhibition was overcome to quite a degree in the presence of all extracts.

7. In point of increase in frond number, and frond weight, though not completely with respect to frond area, additional iron overcame the growth inhibition caused by sphagnum peat extract.

8. Frond size and weight were significantly increased in the presence of extracts of horse and cow dungs.

It was realized that in the foregoing experiment the more or less arbitrarily chosen concentrations of extract might be too high or too low for growth promotion to be apparent. There was also the possibility that the preparation of the extracts by autoclaving was destroying substances which might have had activity. Since, as noted, extracts of sphagnum peat, horse dung, and cow dung produced the most unusual response, the same extracts of these materials were next tested over a range of concentrations of 0, 0.01, 0.1, 0.5, 1.0, 2.5, and 5.0 ml. extract per 25 ml. culture. That additions of sugar via the extracts might not become a complicating factor in the analysis of any growth promotion which might occur, the test was conducted using 2.5 per cent sucrose Hoagland's. An actual control test was carried out simultaneously, using 1.0 ml. of each extract per culture in 0, 1, 2.5, 4, and 5 per cent sucrose media. Were an effect caused by sugar added via the extract, then at high sugar concentrations the inhibition caused by supraoptimal amounts of sugar in the controls should be less than in the treated cultures.

The test followed the usual pattern. Growth of the sub-cultures from inocula of 30 fronds was measured after a period of 7 days. The effect of two light intensities was compared, one series being placed under the Cooper-Hewitt lamp, which provided approximately three times the intensity of the "White" fluorescent at a distance of 16 inches.

An examination of the data in Tables 25 and 26 reveals the fact that a particular extract promotes an increase in frond number if a) the concentration is within the proper range, b) there is sucrose in the medium at a suitable concentration, and c) the illumination is of sufficiently high intensity. It further shows that different factors in different proportions control increase in frond number and increase in frond size, for one can have increased frond number with decreased size, or vice versa, as in the case of the sphagnum and horse dung treatments respectively. Sometimes, as in the case of the cow dung treatments, increased frond size accompanies increased frond number.

The effects of extracts from cow dung and horse dung were very similar, both differing from the effects produced by sphagnum extract. While the medium remained essentially physiologically alkaline with additions of extracts from cow or horse dung, over a certain range of sphagnum extract concentrations it became physiologically acid (Tables 27, 28).

New extracts of the dried cow dung, horse dung, and sphagnum peat were prepared by grinding 50 grams of each in a Waring blender for 20 minutes. The aqueous mixtures were allowed to stand in the

Table 25

The effect of different concentrations of sphagnum, cow dung, and horse dung extracts upon the growth of Spirodela in 2.5 per cent sucrose Hoagland's under two light intensities. Measurements on 7-day growth of subcultures from 30-frond inocula.

"White"

		Ml. extract per 25 ml. culture					
	Control	0.01	0.1	0.5	1.0	2.5	5.0
Sphagnum							
Inc. in fr. no.	137	141	178	189	175	126	107
Fr. size (sq. mm.)	4.2	3.9	3.3	2.9	2.6	2.8	2.8
Fresh wt./fr. (mg.)	0.70	0.63	0.55	0.47	0.49	0.54	0.62
Cow							
Inc. in fr. no.	137	130	128	118	112	74	62
Fr. size (sq. mm.)	4.2	4.2	4.5	4.5	3.9	4.7	4.4
Fresh wt./fr. (mg.)	0.70	0.66	0.75	0.73	0.72	1.04	1.04
Horse							
Inc. in fr. no.	137	138	144	102	134	124	57
Fr. size (sq. mm.)	4.2	4.2	4.3	4.5	4.5	4.7	5.5
Fresh wt./fr. (mg.)	0.70	0.62	0.61	0.63	0.68	0.75	1.26

Cooper-Hewitt

Sphagnum							
Inc. in fr. no.	158	213	242	258	227	137	113
Fr. size (sq. mm.)	4.1	4.5	4.3	3.3	2.4	2.7	2.7
Fresh wt./fr. (mg.)	0.76	0.39	0.69	0.59	0.55	0.59	0.62
Cow							
Inc. in fr. no.	158	187	190	191	181	126	90
Fr. size (sq. mm.)	4.1	4.4	4.7	4.8	4.4	4.8	5.5
Fresh wt./fr. (mg.)	0.76	0.77	0.90	0.87	0.86	1.01	1.23
Horse							
Inc. in fr. no.	158	197	168	191	205	206	118
Fr. size (sq. mm.)	4.1	4.5	4.9	4.8	5.1	5.0	5.3
Fresh wt./fr. (mg.)	0.76	0.75	0.88	0.80	0.84	0.85	1.09

Table 26

Effect of four sucrose concentrations upon growth of Spirodela in Hoagland's medium to which 1.0 ml. per 25 ml. culture of sphagnum peat, cow dung, or horse dung extract has been added. Measurements on 7-day growth under two light intensities of subcultures from 30-frond inocula.

"White"

	Per cent sucrose				
	0	1.0	2.5	4.0	5.0
Sphagnum (1 ml./25 ml.)					
Inc. in fr. no.	32	150	175	128	82
Fr. size (sq. mm.)	3.1	3.0	2.6	2.7	2.5
Fresh wt./fr. (mg.)	0.58	0.52	0.49	0.49	0.58
Cow (1 ml./25 ml.)					
Inc. in fr. no.	33	110	112	112	110
Fr. size (sq. mm.)	4.0	4.7	3.9	3.4	2.7
Fresh wt./fr. (mg.)	0.62	0.75	0.73	0.70	0.61
Horse (1 ml./25 ml.)					
Inc. in fr. no.	29	107	134	143	141
Fr. size (sq. mm.)	4.8	5.0	4.5	3.3	3.1
Fresh wt./fr. (mg.)	0.81	0.76	0.68	0.54	0.58
CONTROL					
Inc. in fr. no.	38	139	137	122	58
Fr. size (sq. mm.)	4.3	4.8	4.2	3.5	3.9
Fresh wt./fr. (mg.)	0.65	0.76	0.70	0.63	0.65

Cooper-Hewitt

Sphagnum (1 ml./25 ml.)					
Inc. in fr. no.	67	200	227	158	100
Fr. size (Sq. mm.)	3.9	2.9	2.4	2.9	2.8
Fresh wt./fr. (mg.)	0.60	0.51	0.55	0.54	0.62
Cow (1 ml./25 ml.)					
Inc. in fr. no.	55	168	181	155	158
Fr. size (sq. mm.)	5.1	5.1	4.4	4.1	3.5
Fresh wt./fr. (mg.)	0.82	0.80	0.86	0.71	0.70
Horse (1 ml./25 ml.)					
Inc. in fr. no.	71	157	205	222	186
Fr. size (sq. mm.)	5.5	4.9	5.1	3.8	3.3
Fresh wt./fr. (mg.)	0.82	0.83	0.84	0.61	0.56
CONTROL					
Inc. in fr. no.	68	193	158	149	81
Fr. size (sq. mm.)	4.2	4.6	4.1	3.5	3.6
Fresh wt./fr. (mg.)	0.68	0.87	0.76	0.68	0.68

Table 27

Initial and final pH of 2.5 per cent sucrose media used for ›wth of Spirodela in different concentrations of sphagnum, cow ıg, or horse dung extracts.

"White"

	Ml. extract per 25 ml. culture						
	0	0.01	0.1	0.5	1.0	2.5	5.0
SPHAGNUM							
Initial	4.8	4.3	4.7	4.2	4.8	5.0	5.0
Final	7.0	6.9	6.4	3.3	3.1	3.2	3.8
COW							
Initial	4.8	4.5	5.0	5.5	5.4	5.7	6.5
Final	7.0	6.8	6.6	6.6	6.5	6.0	6.6
HORSE							
Initial	4.8	4.4	4.4	5.0	5.2	5.5	5.6
Final	7.0	7.0	7.1	6.8	5.8	5.3	5.3

Cooper-Hewitt

SPHAGNUM							
Initial	4.8	4.3	4.7	4.2	4.8	5.0	5.0
Final	6.9	6.4	6.4	3.6	3.0	3.2	3.7
COW							
Initial	4.8	4.5	5.0	5.5	5.4	5.7	6.5
Final	6.9	6.5	6.4	6.1	5.3	5.3	5.3
HORSE							
Initial	4.8	4.4	4.4	5.0	5.2	5.5	5.6
Final	6.9	6.5	6.6	6.4	6.3	6.2	6.0

Table 28

Initial and final pH of media used for growth of Spirodela in different sucrose concentrations with and without addition of 1.0 ml. per 25 ml. culture of extracts from sphagnum, cow dung, or horse dung.

"White"

	Per cent sucrose				
	0	1.0	2.5	4.0	5.0
SPHAGNUM (1 ml./25 ml.)					
Initial	4.8	4.7	4.8	4.6	4.6
Final	4.7	3.2	3.1	3.1	3.2
COW (1 ml./25 ml.)					
Initial	5.4	5.3	5.4	5.2	5.2
Final	6.5	5.3	6.5	5.2	6.3
HORSE (1ml./25 ml.)					
Initial	5.3	5.0	5.2	5.0	4.9
Final	6.2	6.4	5.8	6.5	6.5
CONTROL					
Initial	4.8	4.8	4.8	4.8	4.8
Final	5.1	6.7	7.0	6.7	6.4

Cooper-Hewitt

SPHAGNUM(1 ml./25 ml.)					
Initial	4.8	4.7	4.8	4.6	4.6
Final	3.8	3.1	3.0	3.1	3.2
COW (1 ml./25 ml.)					
Initial	5.4	5.3	5.4	5.2	5.2
Final	6.6	5.6	5.3	5.3	5.5
HORSE (1 ml./25 ml.)					
Initial	5.3	5.0	5.2	5.0	4.9
Final	6.2	6.3	6.3	6.2	6.4
CONTROL					
Initial	4.8	4.8	4.8	4.8	4.8
Final	6.3	6.5	6.9	6.9	6.5

laboratory for 12 to 18 hours before centrifuging. The cow dung and horse dung extracts were made up to 1.5 liters, while the sphagnum extract was made up to 1.0 liter. These three extracts were then tested over a range of concentrations in 2.5 per cent sucrose Hoagland's and in Hoagland's having no sucrose added (2 instead of 1 p.p.m. iron used in Hoagland's henceforth), to see whether plants allowed to grow long enough at high light intensity, manufacturing their own carbohydrate, would respond favorably to the presence of the extracts.

Each extract was tested in concentrations of 0, 0.001, 0.01, 0.1, 1.0, and 5.0 ml. per 25 ml. culture. (5 ml. per 25 ml. culture is equivalent to the extract from 0.167 grams dried cow or horse dung, or from 0.25 grams dried sphagnum per liter of nutrient solution.) Sterilization was by means of autoclaving.

The subcultures in the sucrose series from 30-frond inocula were grown for 10 days beneath a "White" fluorescent lamp at a distance of 4 inches (giving very nearly the same light intensity as was previously obtained with the Cooper-Hewitt lamp). Under the same conditions the non-sucrose series required 14 days before the number of fronds in the control cultures equalled the number attained by the sucrose controls in 10 days.

The peculiar ability of sphagnum extract to promote an increase in frond number at the expense of frond size and weight was equally apparent in both the sucrose and non-sucrose series (Table 29). In the latter this condition was somewhat accentuated. The horse dung extract did not affect increase in frond number except, perhaps, to inhibit it slightly. Apparent in the sucrose series, however, was

Table 29

Effect of different concentrations of sphagnum, cow dung, or horse dung extracts upon the growth of Spirodela in Hoagland's medium with or without the addition of 2.5 per cent sucrose. Measurements on growth of subcultures from 30-frond inocula under "White" fluorescent lamp at a distance of 4". Figures represent means of duplicates, except controls which are means of 6 replicates.

	No sucrose (14-day growth) Ml. extract per 25 ml. culture					
	Control	0.001	0.01	0.1	1.0	5.0
SPHAGNUM						
Inc. in fr. no.	267	263	272	258	307	329
Fr. size(sq. mm.)	4.4	3.9	4.3	4.7	4.1	3.1
Fresh wt./fr.(mg.)	0.67	0.65	0.63	0.66	0.58	0.44
Dry wt./100 fr.(mg.)	4.5	4.6	4.8	5.0	4.2	3.7
COW						
Inc. in fr. no.	267	263	264	255	277	289
Fr. size(sq. mm.)	4.4	4.4	4.2	4.7	4.3	3.6
Fresh wt./fr.(mg.)	0.67	0.67	0.70	0.72	0.64	0.54
Dry wt./100 fr.(mg.)	4.5	4.6	4.9	5.1	4.3	4.5
HORSE						
Inc. in fr. no.	267	267	261	236	233	244
Fr. size(sq. mm.)	4.4	4.3	3.9	4.4	4.5	4.6
Fresh wt./fr.(mg.)	0.67	0.75	0.63	0.72	0.71	0.70
Dry wt./100 fr.(mg.)	4.5	4.8	4.6	5.5	5.6	5.3
	2.5 per cent sucrose (10-day growth)					
SPHAGNUM						
Inc. in fr. no.	298	262	254	255	335	498
Fr. size(sq. mm.)	4.5	4.5	4.5	4.4	4.4	4.3
Fresh wt./fr.(mg.)	0.89	0.90	0.92	0.91	0.84	0.69
Dry wt./100 fr. (mg.)	11.4	12.6	12.2	12.5	10.1	6.8
COW						
Inc. in fr. no.	298	274	288	308	364	491
Fr. size (sq. mm.)	4.5	4.6	4.4	4.6	5.1	5.0
Fresh wt./fr.(mg.)	0.89	0.96	0.96	0.87	0.92	0.89
Dry wt./100 fr.(mg.)	11.4	11.7	11.8	10.7	9.9	8.6
HORSE						
Inc. in fr. no.	298	291	310	294	270	330
Fr. size(sq.mm.)	4.5	4.6	4.7	5.0	5.3	5.2
Fresh wt./fr.(mg.)	0.89	0.92	0.95	0.92	0.96	0.88
Dry wt./100 fr.(mg.)	11.4	12.0	11.9	11.9	11.1	8.5

an increase in frond size and weight which was a function of the concentration. The cow dung extract showed no significant growth promoting effects in the absence of sucrose, but in its presence, a considerable increase in frond number, frond size, and to a lesser degree frond weight occurred.

To ascertain what part the inorganic constituents of these three extracts played in the effects produced on Spirodela, 100 ml. of each was evaporated to dryness and the residue ashed. A few drops of concentrated hydrochloric acid were used to dissolve the ash, then water was added to make up to 25 ml. in each case. The following amounts of each ash solution were added to each 25 ml. culture of 2.5 per cent sucrose Hoagland's: 0, 0.1, 1.0, and 5.0 ml. (5 ml. being equivalent to the ash of the extract from 0.67 grams dried cow or horse dung, or from 1.0 gram dried sphagnum). With a few drops of dilute sodium hydroxide the pH of the prepared media was adjusted to 5.2-5.5. After sterilization by autoclaving, each culture was inoculated with 30 fronds, then placed for 7 days at 4" distance from a "White" fluorescent lamp.

A feature which had been noted in previous experiments was very apparent in this test, namely, the deepened green, glossy fronds which were always associated with the presence of one of these organic extracts in proper concentration. In the 5.0 and 1.0 ml. treatments, and to a somewhat lesser extent in the 0.1 ml. treatment, the plants were a beautiful uniform green, the upper surfaces being somewhat glossy. Root length (10-12 mm.) was unaffected by the ash treatments.

Increase in frond number was not significantly affected by

treatment with ashed extracts of cow or horse dung (Table 30). The ashed extract of sphagnum, at the lowest concentration, produced an increase in the rate of frond multiplication which may have significance. The inhibition produced by the highest concentration of sphagnum ash and of cow dung ash must have been caused by something quite toxic for the plants so-treated looked decidedly abnormal.

Since the ash from the extract of cow dung showed no appreciable growth promoting activity, solvent extraction of dried cow dung was undertaken, to find out whether the material responsible could be separated. For a period of 40 hours dried cow dung was extracted in Soxhlets. Fifteen grams were extracted with peroxide-free ether, another 15 grams with methanol, and another 20 grams with acetone. After storage in the cold room for several days, the solvents were distilled off under vacuum and the residues dissolved as much as possible in water. Insoluble material was centrifuged off, and each of the three extracts made up to 250 ml.

Meanwhile, the residues from the solvent extractions were extracted with water in a Waring blender for 15-minute periods. Each mixture of about 250 ml. then was placed in the cold room for a week. As a control, 20 grams of dried cow dung were water-extracted in the same way. All four mixtures were centrifuged and the extracts made up to 500 ml. volume.

The three solvent extracts, the three residue extracts, plus the control were each tested immediately in concentrations of

Table 30

Effect of different concentrations of ash from extracts of sphagnum, cow dung, and horse dung upon growth of Spirodela for 7 days in 2.5 per cent sucrose Hoagland's, 4" from "White" fluorescent lamp. 30-frond inocula. Initial pH approx. 5.2. Figures represent means of duplicates, except control which is mean of 6 replicates.

	Ml. ash solution per 25 ml. culture			
	Control	0.1	1.0	5.0
SPHAGNUM				
Inc. in fr. no.	160	191	162	134
Fresh wt./fr.(mg.)	0.69	0.67	0.64	0.60
Dry wt./100 fr.(mg.)	7.9	7.6	7.5	8.6
COW				
Inc. in fr. no.	160	175	176	107
Fresh wt./fr.(mg.)	0.69	0.65	0.69	0.58
Dry wt./100 fr.(mg.)	7.9	7.0	7.3	8.4
HORSE				
Inc. in fr. no.	160	155	181	166
Fresh wt./fr.(mg.)	0.69	0.73	0.70	0.66
Dry wt./100 fr.(mg.)	7.9	8.4	7.6	7.2
Final pH				
Sphagnum	6.9	7.1	7.0	6.9
Cow	6.9	7.0	7.0	6.3
Horse	6.9	7.0	6.9	6.4

0, 0.1, 1.0, 5.0, and 10.0 ml.[8] per 25 ml. of 2.5 per cent sucrose Hoagland's. Growth measurements were made upon the subcultured material from 30-frond inocula after a period of 8 days at 4" distance from the "White" fluorescent. The solvent extracts proved to be quite acid, so the pH of the media having the highest concentrations had to be adjusted to 5.3-5.5 with dilute sodium hydroxide.

The data in Table 31 reveal the fact that dried cow dung contains not only material which promotes growth, but also material which causes pronounced inhibition. The data further indicate that by solvent extraction these substances having opposite effects can be separated from one another. In Figure 7 the values for increase in frond number have been plotted against the amount of dried cow dung (or residue after solvent extraction) which must be extracted by each method to produce the indicated effect. From this graph it can readily be seen that acetone and methanol (solid lines) remove mostly inhibitor, while ethyl ether removes both. The residues from the acetone and ether extractions (broken lines) contain the promoter substance. The residue from the acetone extraction in particular, appears to be largely freed of inhibitor and to have lost only a small amount of promoter substance by the extraction. A feasible method, therefore, for obtaining the growth promoting material from dried cow dung for its isolation and

8 Ten milliliters of extract is equivalent to the extract from the following amounts of dried cow dung:

	Solvent extracts	Residue extracts
Acetone	0.8 grams	0.4 grams
Ether	0.6 "	0.3 "
Methanol	0.6 "	0.3 "
Water	0.4 "	

Table 31

Assay of the ability of ethyl ether, methanol, and acetone to serve as solvents for the growth-promoting principle in dried cow dung. Aqueous extracts of the residues after solvent extraction, and of dried cow dung also tested as controls. Basal medium 2.5 per cent sucrose Hoagland's. Initial pH 5.0-5.5. Measurements upon 8-day growth of subcultures at 4" distance from "White" fluorescent lamp. Figures represent means of duplicates, except control which is mean of 10 replicates.

Solvent extracted

	Ml. aqueous soln./25 ml. culture				
	Control	0.1	1.0	5.0	10.0
ACETONE					
Inc. in fr. no.	223	262	264	197	152
Fresh wt./fr.(mg.)	0.79	0.76	0.78	0.77	0.72
Dry wt./100 fr.(mg.)	10.0	9.6	9.8	14.2	12.5
ETHER					
Inc. in fr. no.	223	270	253	313	279
Fresh wt./fr.(mg.)	0.79	0.77	0.81	0.75	0.75
Dry wt./100 fr.(mg.)	10.0	9.5	10.2	10.1	10.5
METHANOL					
Inc. in fr. no.	223	206	209	157	0
Fresh wt./fr.(mg.)	0.79	0.89	0.81	0.77	0
Dry wt./100 fr.(mg.)	10.0	11.8	12.0	14.5	0
WATER CONTROL					
Inc. in fr. no.	223	210	275	316	237
Fresh wt./fr.(mg.)	0.79	0.81	0.79	0.82	0.82
Dry wt./100 fr.(mg.)	10.0	9.8	9.2	8.6	9.3

Residue

ACETONE					
Inc. in fr. no.	223	248	247	293	320
Fresh wt./fr.(mg.)	0.79	0.77	0.85	0.72	0.78
Dry wt./100 fr.(mg.)	10.0	9.3	9.8	7.9	8.2
ETHER					
Inc. in fr. no.	223	232	234	248	273
Fresh wt./fr.(mg.)	0.79	0.78	0.79	0.75	0.78
Dry wt./100 fr.(mg.)	10.0	9.7	9.2	8.6	9.3
METHANOL					
Inc. in fr. no.	223	219	228	265	261
Fresh wt./fr.(mg.)	0.79	0.80	0.81	0.79	0.71
Dry wt./100 fr.(mg.)	10.0	10.1	8.6	7.9	7.3

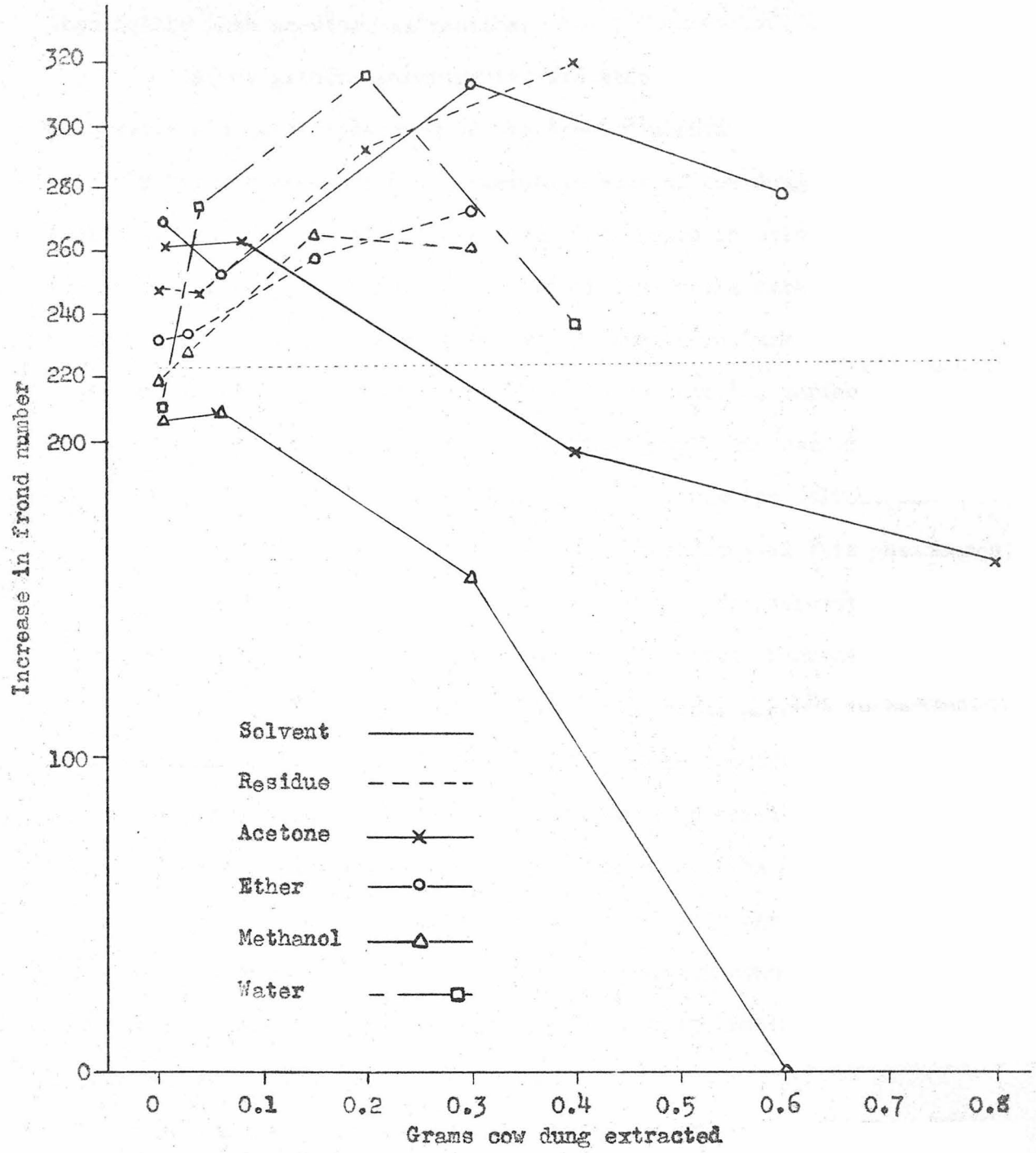


Figure 7. Comparative effect of different means of extraction of dried cow dung as they affect frond number. Dotted line at 223 is mean of 20 untreated replicates.

study would be first to extract with acetone to remove the inhibitor, then follow with an ether extraction.

Before growth measurements were made in this experiment some observations on the appearance of the treated plants were taken. With a fairly high concentration of aqueous extract of cow dung it had long been observed that the plants were very dark green in color and if the concentration was high enough to be inhibitory to the rate of multiplication the tips and edges of the fronds curled up, making an angle of as much as 30 degrees with the plane of the solution surface. This ability to induce the formation of more chlorophyll has been mentioned as a property of the ash. This was further confirmed by solvent extraction, for in every case only the residue tests showed this phenomenon, while plants treated with the fractions removed by the solvents had the same rather yellowish-green color of the plants which remained untreated.

The curling phenomenon, on the other hand, appears to be caused by some ether-soluble substance, for at no concentration tested did the ether-soluble fraction cause the plants to display the curling symptom. On the other hand the ether residue caused the plants to curl as much as 30 degrees in the highest concentration tested. The material responsible for upturned edges is almost completely removed upon extraction with methanol, and partially removed with acetone, so it is concluded that it is organic in nature.

In a 2.5 per cent sucrose culture a chance contamination (that appeared to be a short rod type upon microscopic examination) was discovered which produced a 40 per cent increase in frond number, a 10 per cent increase in frond size, but a 6 per cent decrease in fresh weight

per frond and a 27 per cent decrease in dry weight per frond. The plants were larger, greener, and less sturdy-looking than the controls. This contaminant has been saved and cultured in the hope of investigating it further at some later date.

Asparagine (2.5 mg. per 25 ml.), when tested in conjunction with a series of concentrations of sphagnum, horse dung, and cow dung extracts, affected growth by increasing frond number an additional 10 to 20 per cent. However, the replicate variation was unusually large so that until asparagine can be tested over a range of concentrations its growth promoting ability remains uncertain. That asparagine should affect the rate of frond multiplication is not surprising since it is very active in so-doing when Spirodela is grown in the dark.

The present investigation has shown that there are substances in sphagnum peat, dried cow dung, and dried horse dung which are thermostable and promote the growth of Spirodela polyrrhiza in sterile culture provided conditions of illumination and carbohydrate supply are suitable. The substances in cow dung responsible for this effect have been shown to be organic in nature. Ash from an extract of horse dung has little or no activity so the substances in horse dung are presumed to be organic compounds similar to those in cow dung. The active materials present in sphagnum appear to be different from those in dung. An aqueous extract of oak humus showed a certain measure of growth promoting activity of a distinctive nature. The type and/or amount of growth inhibitors present in crude extracts of these materials may play a large part in determining differences of expression. A bacterial contaminant induced an increase in growth of a type very different from

that produced by the dungs, sphagnum, or oak humus.

With this information, an attempt can be made to explain Clark's inability to demonstrate growth promotion with soil and dung extracts when tested in sterile culture. Perhaps the illumination the cultures received was not adequate, and since no additional carbohydrate was furnished, the extract in sterile culture produced no effect or only one of inhibition. The growth promotion he observed upon contaminating an unresponsive sterile culture, or when sterile medium was inoculated with plants not freed from microorganisms, might have been caused by something totally different from the potential growth promoting substances supplied in the extracts. As observed here, and noted by Clark, the presence of certain bacteria can cause a stimulation of growth to occur. There is the possibility, therefore, that in non-sterile culture, either the microorganisms excreted some growth substance into the medium or else broke down some of the extract constituents to a more readily utilizable form, or even a combination of both alternatives. Something of this sort, incidentally, is what Bottomley and Mockeridge believed to take place.

Ashby, on the other hand, demonstrated growth promotion by an extract from manure, using Clark's medium without the addition of carbohydrate. His culture conditions were aseptic, the complete medium being sterile before plants not freed from microorganisms were introduced. Culture solutions were changed so frequently that over the short period of an experiment contamination presumably was not even apparent. Since the cultures were not completely sterile, perhaps some such explanation as that advanced for Clark's case is also applicable to Ashby's. However,

he found that growth promotion was a function of the concentration of the extract present. At the very much higher light intensities employed by Ashby for the growth of his cultures it is quite possible that growth promotion of the type observed in this investigation was demonstrable in the absence of additional carbohydrate. This did not appear to be the case in the one experiment designed to check this point which was made during the course of the work. However, a different outcome might have attended the use of more intense illumination. The additional chlorophyll noted by Ashby in treated plants was doubtless caused by some of the inorganic constituents of the extracts which do not significantly promote growth. His idea that the extract promoted growth by increasing photosynthesis is incorrect inasmuch as increased growth was dependent on more photosynthesis as a result of there being more chlorophyll.

CHAPTER VI

DARK CULTURE EXPERIMENTS

Attempts to analyze the factors which determine the growth of green plants are beset with complications. One of the most important factors that complicates such analyses is light, whose role in determining growth is little understood. The influence of light upon the photosynthesis of carbohydrates and upon phototropic and photoperiodic effects has received considerable investigation. That light influences other reactions as well is clear from the different type of growth one observes if, say, a potato tuber sprout or a pea seedling grows in darkness or in light. The characteristic growth in length which occurs in darkness is much diminished by illumination. On the other hand a mature tomato plant when placed in darkness and fed additional carbohydrate by special means soon stops growing. Very little is known concerning these aspects of the influence of light upon growth.

If it were possible to culture plants successfully in the dark, a more rigidly controlled environment would be furnished for evaluating the effects of other growth factors. Since indefinite growth of higher plants in darkness has not been achieved by simply supplying carbohydrate, investigation has first to reveal what other necessary growth materials besides carbohydrate are produced by light, before other factors influencing growth can be studied.

With the discovery that in light, plants of Spirodela are able to assimilate and utilize carbohydrate supplied in the medium, a test

was made to find out what they would do in darkness, both in the presence and absence of sucrose or dextrose. It was not expected that growth would occur at the same rate as in light, or that the plants would make any appreciable growth at all. A supplement of growth factors (cf. page 56), therefore, was tested in conjunction with the carbohydrate to find out if any of its components might improve growth in the dark.

Test cultures were inoculated with 30 fronds each, taken from stock cultures maintained on sucrose medium at 16" distance from a "White" fluorescent lamp. They were placed in a darkroom used for Avena testing for a period of 7 days, then transferred to fresh media. After 7 more days, and again after 18 days, a count of the increase in frond number in these subcultures was made. The dextrose subcultures were contaminated so only the counts for the sucrose series were recorded. From the growth of the cultures during the first 7 days, however, sucrose appeared to be somewhat superior as a carbohydrate source.

The presence of adequate amounts of sucrose proved to be essential since the controls made no growth at all (Table 32). Furthermore, the supplement of growth factors was far from beneficial in its effects. Besides reducing the number of fronds produced, the size of the new fronds was much smaller than normal. Fronds produced in the dark lacked chlorophyll, but the chlorophyll in fronds of the original inoculum appeared unchanged. The plants not treated with sugar were still capable of growth when placed on sucrose medium, and did not seem harmed in any way by three or more weeks of comparative dormancy.

At the time of the final frond count, all cultures were transferred to 2.5 per cent sucrose Hoagland's. After another 18 days the

Table 32

Increase in frond number after 7 days and after 18 days in darkness upon sucrose Hoagland's with and without a supplement of growth factors.

	Control		0.1% sucrose		1.0% sucrose		0.1% sucrose & supplement		1.0% sucrose & supplement	
	1	2	1	2	1	2	1	2	1	2
7 days	0	0	19	13	37	34	7	5	28	29
18 days	0	0	35	32	133	123	25	24	121	120

fronds which had previously had growth supplement were still multiplying, while those which had had no growth supplement had virtually ceased. At the time this was interpreted as an indication that some component of the growth supplement was useful after all, so other substances were tested in an endeavor to ascertain the requirements for indefinite growth in darkness. A parallel objective was to duplicate or surpass in darkness the growth rate which can be attained in light.

In this initial experiment, observation and transfers were made under the red safelight used for work with etiolated Avena seedlings. It was soon observed, however, that red light of such low intensity was able to stimulate growth during the relatively short periods of time (10 to 20 minutes) necessary for such operations. A light, which would have to be used as little as possible, of such low intensity and restricted transmission as to render its stimulation effect negligible was therefore sought.

Cultures of etiolated plants were placed in a light-tight box with windows on top, and glass filters transmitting bands of different wavelength were inserted in the window frames. Over the row of filter-windows was hung a 40-watt "White" fluorescent lamp. The cultures were allowed to grow for 14 days, then they were removed and the number of fronds counted. The filters were of different thicknesses so that the light intensities differed.

From the results obtained (Table 33) the violet filter was indicated as best for use in the safelight. However, the necessity of having enough light to carry out operations made even the signal blue filter unsatisfactory. In all subsequent work, therefore, a sextant

Table 33

Increase in frond number over a period of 14 days in different sucrose concentrations, beneath filters transmitting different wavelengths. 30-frond inocula of etiolated plants. Figures represent means of duplicates.

Filter	Transmission(A°)	Per cent sucrose			
		0	0.5	1.0	2.5
Dark red	>6100	68	236	275	270
Sextant green	>4600 < 6000	56	166	186	142
Signal blue	<5800	41	134	164	141
Sextant green & dark blue green	>4600 < 5700	40	114	147	127
Violet	<4700	41	124	114	124

green filter was used in a safelight employing only a 40-watt Mazda lamp. Operations were carried out at least 3 feet distant from the safelight to one side of the direct rays, while cultures awaiting transfer were placed 6 feet away. In this way the best possible compromise was arrived at.

The first test was of a number of pure substances and crude extracts which are known from other work to have growth promoting properties. The concentration of each, most likely to be effective, was determined by analogy with the amounts found necessary under other circumstances. In addition, dilutions of one tenth and one hundredth were tested. Hoagland's medium containing 2.5 per cent sucrose was used as the basal medium. Each tube was inoculated with ten fronds which had been in the dark on a sucrose medium for more than a month. Cultures were allowed to grow for 53 days, while periodic counts of frond number were made.

In the concentrations tested, glycine, vitamin B_6, vitamin B_1, pantothenic acid, and adenine proved to be the most effective materials tried (Table 34). The controls were accidentally disposed of before the conclusion of the experiment, but comparison with the extrapolated value and the 19-day count shows that growth was considerably better in the presence of the materials just listed. These substances do not appear to be too specific in their effect, but seem to function only as sources of organic nitrogen. Of the other materials tested, some had little or no effect, while others might have produced more of an effect had different concentrations been employed.

Cultures removed from the light and placed in darkness appear

Table 34

Periodic frond counts of cultures containing different growth substances, maintained in darkness. Inocula of 10 fronds from cultures in darkness for more than a month.

Substance	Amount per 25 ml.	Days 8	15	19	53
Vitamin B_1	250 γ	34	43	54	201
	25	26	36	42	189
	2.5	29	39	44	151
Vitamin B_6	250 γ	30	41	46	212
	25	27	35	39	102
	2.5	29	34	44	147
Nicotinic acid	250 γ	18	20	22	53
	25	20	24	30	105
	2.5	19	33	33	132
Pantothenic acid	250 γ	28	39	44	135
	25	32	44	49	201
	2.5	28	36	43	169
Indoleacetic acid	250 γ	18	28	41	100
	25	19	35	47	148
	2.5	27	36	46	162
Adenine	250 γ	30	33	48*	200
	25	31	37	39	194
	2.5	21	26	32	144
Glycine	2500 γ	25	35	50*	282
	250	31	37	40	128
	25	27	31	33	116
Yeast extract (from 10 gm./l.)	1.0 ml.	30	41	58	183
	0.1	26	31	38	136
	0.01	32	36	41	112
Coconut milk (10xconcentrated)	1.0 ml.	18	cont.	--	---
	0.1	36	*	*	188
	0.01	37	51	56	152
Sphagnum extract (from 348 gm./l.)	1.0 ml.	39	50	63	176
	0.1	28	36	40	74
	0.01	25	32	cont.	---
Cow dung extract (from 200 gm./l.)	1.0 ml.	25	25	26	64
	0.1	26	30	31	78
	0.01	19	19	19	23
Horse dung extract (from 200 gm./l.)	1.0 ml.	21	23	25	76
	0.1	30	32	35	91
	0.01	35	40	46	107
$(NH_4)_2SO_4$	264 mg.	11	12	12	13
	132	13	14	14	19
	66	15	15	15	20
CONTROLS	(a)	25	32	40	
	(b)	25	31	34	
	(c)	29	32	33	120 **
	(d)	25	30	32	
	(e)	31	34	37	

cont. = contaminated

* Fronds so clumped together as to make accurate count impossible while plants remained in culture tube.

** Estimated by extrapolation of growth curve.

to possess enough reserve materials essential for growth to grow very slowly for quite some time as long as they are supplied with carbohydrate. Plants on sucrose medium tend to clump, i.e., they fail to absciss from one another. Subculturing, where a definite number of fronds has to be introduced into each tube, is next to impossible under conditions of dim green light if the fronds are clumped. For this reason the following change in procedure was adopted. Plants taken from the light were placed, 30 fronds per tube, on sucrose medium in the dark for a period of two weeks in order to deplete their reserves of growth materials. During this time some growth occurred. All the fronds (not just 30) in each tube were then transferred to tubes containing test media and allowed to grow for 2 to 3 weeks, after which measurements were made.

Using this method, the effect of a number of growth substances in different combinations was investigated. The concentrations used in milligrams per liter were: vitamin B_1 0.1, vitamin B_2 0.1, vitamin B_6 0.1, p-aminobenzoic acid 0.1, calcium pantothenate 0.1, adenine 0.1, inositol 10.0, asparagine 100.0, glycine 3.0, biotin 0.0004. The increase in frond number and in dry weight was measured after three weeks on the test media. Table 35, in the form of a checkerboard, gives the data for increase in frond number.

All cultures containing asparagine had nearly twice as many fronds as had the controls. Total dry weight was likewise doubled. All cultures containing p-aminobenzoic acid (except those with asparagine) grew less well than the controls. Cultures containing biotin, inositol, glycine, or adenine averaged about the same as the controls, while those

Table 35

Five week's (2 without treatment) increase in frond number, as a result of treatment with ten growth substances in various combinations. Control (mean of 5 replicates) = 79. (Means exclude those with asparagine or p-aminobenzoic acid.)

	B_1	B_2	B_6	Pab.	Inos.	Pant.	Bio.	Asp.	Glyc.	Aden.	MEAN
B_1B_2	--	--	93	70	105	100	109	156	72	92	95
B_2B_6	93	--	--	58	90	84	64	146	84	73	81
B_6Pab.	41	58	--	--	57	64	52	164	60	80	
Pab.Inos.	67	73	57	--	--	73	38	149	70	74	
Inos.Pant.	85	68	102	73	--	--	72	163	83	79	82
Pant.Bio.	96	87	85	59	72	--	--	158	83	98	86
Bio.Asp.	216	140	152	169	150	158	--	--	169	166	
Asp.Blyc.	181	180	170	162	175	195	169	--	--	137	
Glyc.Aden.	75	126	88	66	72	108	73	137	--	--	90
Aden.B_1	--	92	100	45	77	86	95	183	75	--	88
MEAN	87	93	94		83	94	83		79	85	

having vitamins B_2, B_6, or pantothenic acid showed small effects of growth promotion of possible significance.

Although it looked as though one master reaction, such as the response to asparagine, could largely obscure others, interaction effects were tested further with asparagine, pantothenic acid, the vitamin B complex (B_1, B_2, B_6), indoleacetic acid, and biotin. Two concentrations of each substance were used and all possible combinations of concentrations of the five materials tested. Without presenting the actual data the outcome was as follows:

1. Pantothenic acid (1.0, 4.0 mg. per 1.), the B complex (1.0, 4.0 mg. per 1. each constituent), and indoleacetic acid (0.2, 2.0 mg. per 1.) showed no promotive or inhibitory effects at the concentrations tested.

2. Biotin at 0.0004 mg. per liter was slightly inhibitory, while at 0.002 mg. per liter inhibition was pronounced, largely overcoming the effects of asparagine.

3. Asparagine at 100, 400, and 1000 mg. per liter produced a stimulation of frond production which was proportional to concentration (Table 36).

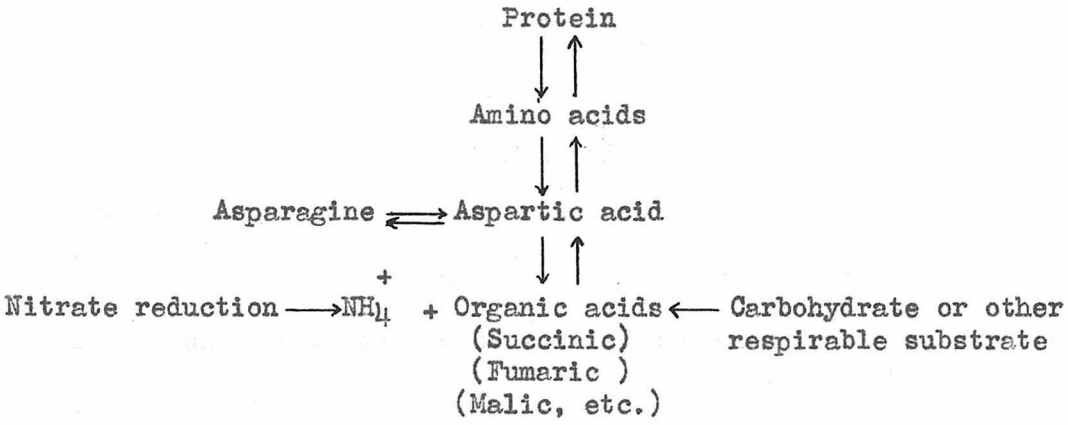
Aspartic and succinic acids were also tested to see if they would replace asparagine. Nitrogen metabolism in plants is believed by many to follow the course indicated in the accompanying scheme[9] in which aspartic acid occupies a key position, and asparagine serves as a storage compound.

9 Simplified from scheme presented by P. W. Wilson in The Biochemistry of Symbiotic Nitrogen Fixation. Madison: The University of Wisconsin Press. 1940. p. 167.

Table 36

The effect of different concentrations of asparagine, aspartic acid, or succinic acid upon frond multiplication. Inoculation with approximately 75 fronds. Test period 14 days. (Number of replicates in parentheses.)

		Asparagine(mg./l.)			Aspartic(mg./l.)		Succinic(mg./l.)	
	Control (3)	100 (3)	400 (3)	1000 (2)	100 (1)	400 (1)	100 (1)	400 (1)
Final fr. no.	97	201	367	444	73	145	72	77
Fresh wt./fr. (mg.)	0.74	0.85	0.74	0.80	0.68	0.52	0.60	0.71
Dry wt./100 fronds (mg.)	19.6	18.1	13.4	15.6	22.6	13.1	21.6	23.4



Both concentrations of succinic and the lower concentration of aspartic inhibited growth completely (Table 36), while the higher concentration of aspartic acid produced a significant increase in number of fronds which were, however, abnormal in appearance. It would be of interest to test aspartic acid in lower concentrations which the plant would be more likely to tolerate. It should then be possible to duplicate the asparagine effect if the proposed mechanism is essentially correct.

With the higher concentrations of asparagine the fronds most recently formed tended to become successively smaller and less vigorous in appearance. In Table 36, the figures showing a general decrease in dry weight give some indication of this as they are averages of the dry weights of the many normal and increasing numbers of sub-normal fronds. In comparison with the greatest rate of growth ever achieved when the plants were grown in the light upon sucrose medium with accessory growth promoters, the rate attained with 1000 mg. per liter asparagine is about one half to two thirds as great. A search for still other factors influencing growth in darkness, particularly

frond growth, was therefore carried on.

Another test was made in an endeavor to find out if any other amino acid might replace asparagine. Concentrations of 10, 100, and 1000 mg. per liter were used in testing 16 amino acids in groups rather than individually. To gain some idea of the size of inocula being transferred to test media two stock cultures were saved and measurements made upon them.

Although several of the combinations of amino acids slightly promoted the rate of frond multiplication at the lower concentrations, they were either toxic, inhibitory, or without effect at the highest concentration tested (Table 37). In no case did they behave as substitutes for asparagine. The series of dilutions of cystine-methionine looked the most promising inasmuch as the lowest concentration produced the greatest increase in frond number. Lower concentrations of either or both of the consituents may prove more effective. Combined with asparagine still greater increases in frond number may result.

Glutamic acid and glutaric acid are not substitutes for asparagine, either, which suggests that Spirodela is an "asparagine plant" like the legumes, grasses, and asparagus. The death of the plants at the highest concentration of glutamic acid was partly the result of hyperacidity because of failure to properly adjust the initial pH.

Because thus far it appeared that asparagine was a specific requirement for increase in frond number, a test was begun to determine how long cultures could be maintained with successive transfers on

Table 37

Final frond counts and pH measurements (parentheses) of cultures grown for 17 days on sucrose medium to which various groups of amino acids have been added. Initial inoculum approximately 205 fronds. Initial pH approximately 5.0.

Amino acid	Amounts of each acid present (mg./l.) 10	100	1000
Cystine Methionine	488 (6.6)	376 (3.5)	280 (4.3)
Serine Phenylalanine Tyrosine Histidine Tryptophane	370 (7.0)	349 (6.6)	210 (5.0
Valine Leucine iso-Leucine nor-Leucine	311 (7.0)	387 (5.9)	256 (5.2)
Ornithine Lysine	383 (6.8)	411 (6.6)	379 (5.3)
Proline Hydroxyproline	438 (6.8)	445 (6.6)	261 (6.1)
Glutamic acid	401 (6.6)	403 (6.1)	200(dead) (2.9)
Glutaric acid	---	288 (5.6)	---

Asparagine 1500 mg./l.	642 (5.9)	CONTROL: 373 (6.8)	324* (6.7)
" 500 "	561 (6.5)		
" 20 "	413 (6.6)		
" 10 "	406 (6.7)		

* Of this number 37 were dead, thus the count is low.

medium having only sucrose and asparagine added to it. Not being convinced as to just what was the optimal asparagine concentration, a series containing 0, 0.4, 1.0, 2.0, and 3.0 grams per liter was prepared. A comparable series with controls contained vitamin B_1 (1 mg./l.). The anthocyanin observed in asparagine-treated plants clearly indicated that carbohydrate was being accumulated, perhaps because of a lack of vitamin B_1 for its utilization.

Plants were taken from non-sucrose medium in the light, inoculated directly into the test media and placed in darkness. This was because such plants are less clumped together than are those growing on sucrose medium. Since weekly subcultures, entailing the counting of an exact number of fronds, had to be made in dim green light, it was desired to have the initial inocula contain as few clumped fronds as possible.

The cultures were examined after one week, but practically no growth had occurred, so they were put back and not subcultured until the end of the following week. At that time the growth made in two weeks was measured. The subsequent week's growth of the subcultures was also measured, then the test was discontinued.

Instead of producing many times the number of fronds formed in the control cultures (Table 38), both asparagine series (the presence of vitamin B_1 made no difference) made one or two normal fronds, followed by very tiny fronds which separated from the parent plant and rapidly became necrotic. Both controls appeared normal, growing slowly, so that the behavior was not traceable to some error made in the preparation of the basal medium.

Table 38

Increase in frond number in the presence and absence of asparagine and vitamin B_1. Initial inoculum 30 fronds from the light where they had been on Hoagland's medium without sucrose. Figures represent means of duplicates.

		Asparagine(gm./l.)			
	Control	0.4	1.0	2.0	3.0
After 2 weeks					
No B_1	30	39	52	18	6
B_1 (1mg./l.)	31	50	38	12	3
After 1 weeks growth of subculture					
No B_1	14	16	6	1	1
B_1(1 mg./l.)	13	6	1	4	0

The experiment differed from preceding ones in that the inoculum was derived from illuminated cultures having no sucrose. It also differed in having the inoculum come directly from the light to darkness, where it was placed immediately upon asparagine-sucrose medium without first growing for some time in darkness on just a sucrose medium. The asparagine with which all these tests were conducted was known to have impurities. By way of comparison a sample of much higher purity was tested (1.0 gm./l.) in this last experiment. However, it too produced exactly the same results.

This experiment provides convincing proof that more than asparagine is needed for growth in darkness on a sucrose medium if growth is to be maintained for an indefinite period of time. The effect of asparagine is to accelerate meristematic activity and thus the rate of frond multiplication. If, as in this experiment, the fronds lack the necessary materials for growth in size, successively smaller fronds are produced which are so lacking in vigor that necrosis soon sets in.

In view of the fact that frond size increases considerably when plants are transferred to sucrose medium in the light, one must conclude that such plants possess quite a reserve of frond growth materials which is gradually used up in the dark. Depletion is hastened by asparagine treatment (note reduction in dry weight, Table 36, and the mention that fronds tended to become smaller and less vigorous in appearance). Only when there is a very low reserve, as in plants without previous sucrose treatment in the light, do the plants multiply

themselves to death in a very short space of time.

The fact that the controls are able to grow slowly, but normally, irrespective of whether or not they have a previous history of sucrose treatment suggests that frond growth materials can be made by the plants in darkness. Such plants, however, may well have sufficient reserves accumulated while in the light with which they continue to grow. Since they multiply so slowly, a critical shortage would not become apparent except over a longer period of culture than has thus far been observed.

Barker (1935, 1936) has studied the metabolism of the colorless alga Prototheca zopfii Krüger. This organism, unlike Spirodela grown in darkness, was unable to develop in the absence of complex organic materials such as are present in yeast autolysate. Although glucose and other monosaccharides, as well as fatty acids and certain alcohols could be utilized, sucrose, or other di- and polysaccharides could not serve as a source of carbon. Neither were any of the keto-, hydroxy-, or dibasic acids tested assimilable. He concluded that the assimilation of these compounds proceeds in two experimentally distinct stages. The primary process is the oxidative conversion of the substrate into a carbohydrate which is stored in the cell as glycogen. The formation of this carbohydrate is rapid compared to its subsequent decomposition in the process of cell synthesis, which constitutes the second stage of assimilation. He tested a number of nitrogenous compounds, including asparagine, which were without effect.

Experimental culture of Spirodela in darkness has not been

extensive, but there are clear indications that its metabolism differs from that of the colorless alga, Prototheca. The only parallel lies in the ability of the two organisms to store reserves which are slowly used up in the process of cell formation. Further experiments may be able to reveal the nature of these reserves in the case of Spirodela.

CHAPTER VII

FLOWER INDUCTION

Flowering in the family Lemnaceae is very rare, and the factors which influence the vegetative form to undergo sexual reproduction are little investigated and very imperfectly understood. For a long time it was believed that only three of the four genera comprising the family still retained the capacity for flower production, species of the genus Wolffiella never having been known to flower. However, Mason (1938) reported the flowering of Wolffiella lingulata (Hegelm.) Hegelm. found at Roberts Island in the delta of the San Joaquin River, California. In June, 1937, he discovered flowering plants in the marshes. In January, 1938, he brought some of the plants that were not then flowering into the laboratory and put them into an aquarium. These flowered after three weeks and continued doing so for six weeks. In June, 1938, the plants at Roberts Island flowered again and produced germinable seeds. Since that time no flowers have been observed at that place, nor has the aquarium material flowered in subsequent years. This case history is typical of others reported when Lemna, Spirodela, or Wolffia have been found flowering.

Hicks (1930, 1932) has investigated the question of flowering in the Lemnaceae most thoroughly to date, actually succeeding in experimentally inducing some of the species to flower. Saeger (1929) found that Lemna minor growing in dilute Knop's solution sometimes produced flowers while other species of Lemna never did so. Hicks, however, was the first to undertake the problem of flower induction

experimentally. He conducted several surveys of duckweed populations throughout the state of Ohio in the summers of 1928-1931. With the exception of 1930, the annual surveys revealed only a few scattered instances of flowering, and then the number of plants which did so was small. During the months of May to September, 1930, an unusual drouth occurred and many small bodies of water supporting duckweed populations were greatly decreased in size or dried up completely. All aquatic vegetation was subjected to most unusual growth conditions. In many cases duckweeds were left stranded with a steady but limited water supply upon mud flats or draped about stems of other aquatic plants. Lemna minor and L. trisulca were found in abundant flower in numerous localities during this unusual season.

Following are statements made by Hicks describing his observations:

> "Flowering plants, without exception, were never found in shaded areas, even though abundant flowering material might be found in better illuminated areas a few feet distant. In most cases, flowers were most abundant in areas most exposed to sunlight. Correlated with this was the observation that flowering plants were invariably found abundantly only in water areas of unusually high temperatures and were absent in colder portions. In addition, since Lemna minor flowered in only about 8% of all of the localities visited, and since the visible environmental characteristics of many localities appeared to be almost the same, it was suggested that the chemical nature or mineral content of the water medium might inhibit or be an important factor in promoting flower production."

Hicks succeeded in experimentally inducing flowering in four species and one variety of the genus Lemna and in Wolffia columbiana. He outlines the following conditions as being necessary for flower production:

"Healthy mature plants making good vegetative growth and with

an accumulation of a food reserve.

Some environmental influence which will rather suddenly check normal vegetative growth with the possible diversion of the accumulated reserve to flower production.

In some species, such as Lemna trisulca, which undergo a marked transformation of the vegetative form at the time of blooming, plants should be selected for treatment which are as near as possible in appearance to the flowering form as such transformations do not come about rapidly. Not all growth forms are equally responsive to experimental treatments."

No success attended Hicks' experiments to induce flowering by means of various nutrient salt combinations or deficiencies. Different photoperiods or different light intensities likewise were ineffective. Approximately 60 common chemical substances were added to the media in varying quantities. Only with dilute sodium hydroxide were flowers of Lemna minor and Lemna trisulca produced in abundance. None of the other species studied flowered under such treatment, and these species often failed to produce flowers when the experiment was repeated. Dilute sodium hydroxide was added only until signs of injury were noted, then treatment was stopped and the cultures allowed to grow. The medium became gradually less alkaline, and at the time of flowering the pH was between 7.2 and 7.4. With this treatment from 10 to 30 per cent of the plants flowered. The controls never flowered.

By the use of ultraviolet rays from a 110-volt quartz mercury vapor lamp at 30-inch distance, for varying lengths of time, four species of Lemna were made to flower. Spirodela polyrrhiza, Wolffia columbiana, Wolffia punctata, and Wolffiella floridana failed to flower, although vegetative growth was checked. Spirodela was severely burned by the rays and gradually died after production of normal vigorous fronds which carried on. The time of treatment which would cause the greatest number

of plants to flower varied with the different Lemna species. He was able to make up to 55 per cent of the plants of responsive species flower, while the controls remained vegetative. A repetition of the experiment using the ultraviolet lamp at 38-centimeter distance so that the plants received 105 ergs per second per square millimeter, was not quite so successful from the standpoint of numbers of plants induced to flower. Destructive effects were more apparent than in the previous experiment. Hicks was still unsuccessful in inducing Spirodela polyrrhiza, Wolffia punctata, and Wolffiella floridana to flower, but did make four Lemna species flower, as well as two plants of Wolffia columbiana which received the treatment for 15 minutes.

With the co-operation of Dr. G. F. Smith and Dr. H. Kersten of the departments of botany and physics, University of Cincinnati, a further attempt to induce Spirodela polyrrhiza to flower was made along the lines suggested by Hicks' investigations.

Single plants of 4 fronds, selected for uniformity, were placed upon agar slants beneath the specially constructed window of cellophane in the wall of small culture tubes (Fig. 8). The inoculated tubes were packed and mailed by air to the University of Cincinnati for ray treatment. Three days later, raying completed, the cultures were returned, but were delayed in transit for several days for plant inspection. As a result the plants were under adverse conditions for a week. At the time of receipt the plants were immediately removed from the already drying agar and placed upon sterile Hoagland's medium under the "White" fluorescent at a distance of 16 inches.

This test was intended as a preliminary one to ascertain

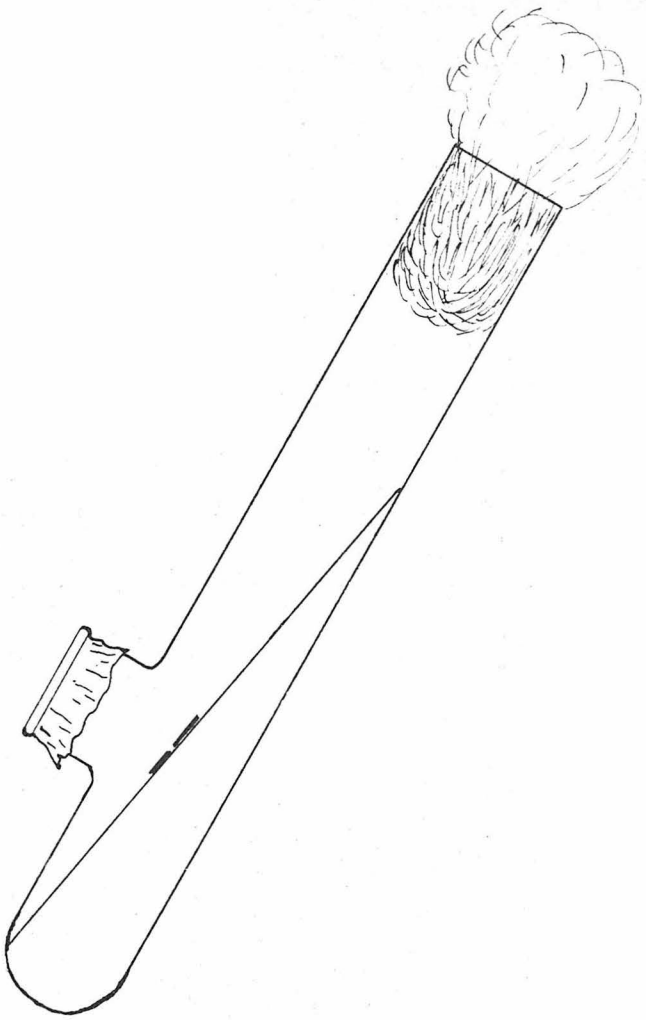


Figure 8. Single plant of four fronds upon agar slant, rayed through cellophane window of modified culture tube.

whether soft X ray (30-k.v., 10-m.a., 5 cm.) or ultraviolet ray (110-v., 20 cm.) would induce flowering and, if so, the approximate dosage necessary. Treatment with ultraviolet was given for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 32, 45, 60, 70, 92, 100, 107, 120, 150, and 175 minutes. Treatment with X ray was given for 1, 2, 3, 5, 6, 7, 9, 10, 13, 15, 20, 25, 30, 35, 40, 45, 60, and 180 minutes. There were five untreated controls.

After three weeks the controls were growing normally, those treated with ultraviolet up to 32 minutes grew less well, while those treated longer failed to survive. X-rayed plants receiving up to 10 to 15 minutes exposure grew a small amount, while those having longer treatments did not survive. In no case was flowering observed at this time or 6 to 8 weeks later.

Because of the small amount of material receiving each ray treatment one might not have obtained any plant which flowered, even though some of the treatments might have been effective in inducing flowering in a small percentage of plants. Also, the plants' food reserves were doubtless lowered while being shipped in darkness to Cincinnati. It does not appear that ultraviolet and soft X-ray treatments offer too much promise as agents for inducing Spirodela to flower, but this one experiment with its attendant adversities is insufficient evidence for complete condemnation.

The conditions which Hicks has described as correlated with occurrence of flowering plants of Lemna sp. suggested the following experiment, namely, to try the combined effects of very high light intensity, high temperature, varying amounts of additional carbohydrate,

and hydrogen-ion concentrations of pH 5, 6, and 7. Two sets of cultures of 12 tubes each were suspended in a waterbath about a 1000-watt Mazda lamp, controlled by a timeclock to provide illumination 16 hours per day. The water in the bath was heated by the lamp and the rate of flow through the bath controlled so that with the light on the temperature was approximately 32°C. and with it off from 15 to 20°C. Sucrose was supplied in concentrations of 0, 3, 6, and 8 per cent. Hoagland's medium with a pH of 4.8 was adjusted by addition of dilute sodium hydroxide to about pH 5 and pH 6. However, upon sterilization the pH range instead of being approximately 5, 6, and 7, was 4.8, 5.2, and 5.4, so that hydrogen-ion concentration was scarcely a variable at all.

Each set of 12 tubes consisted of the four sugar concentrations at the three pH's. Each tube was inoculated with 20 fronds. The inocula for one set of cultures was derived from the X-rayed material from the preceding experiment, while the inocula for the other set came from normal stock cultures.

The cultures were observed over a period of more than 8 weeks but no flowering was induced. The tubes became completely choked with small, light-green fronds with purple margins and undersides (anthocyanin) which had long roots. The test was then discontinued. Apparently the combination of environmental conditions provided, which might have induced flowering of Lemna, had only the effect upon Spirodela of changing its morphology somewhat. Spirodela plants are known to flower naturally upon occasion, but what environmental changes induce this response still remains undisclosed.

CHAPTER VIII

CONCLUSIONS AND SUMMARY

The foregoing chapters have considered a number of factors which influence the growth of Spirodela polyrrhiza (L.) Schleid. Many of these factors undoubtedly influence the growth of other more typical higher plants in much the same manner, although their relative importance and degree of influence probably differs from plant to plant. Conclusions drawn concerning the growth of Spirodela are only to be taken as indications, therefore, of what one might expect when dealing with other higher plants. Particular attention is called to the important role that the supply of carbohydrate plays in the expression of the effects of most other factors influencing growth.

Spirodela plants upon an inorganic medium balanced for optimal growth, when receiving moderate light intensity, are limited in their growth by carbohydrate supply. When this "deficiency" is corrected by the addition of sucrose, some other product of photosynthesis than sucrose then limits the growth. Aeration, by making a better supply of carbon dioxide available, causes an increase in growth rate. A carbon dioxide concentration of no more than 5 per cent is effective in promoting growth. This it can do even when growth is presumably no longer limited by the amount of carbohydrate available. Just how the additional carbon dioxide is utilized is not clear, except that the process is dependent on light.

When grown in the dark, Spirodela plants are able to multiply slowly if only they are supplied with carbohydrate. Supplements of

asparagine greatly increase the rate of frond multiplication but the new fronds produced soon become necrotic, indicating that factors produced in the light (in seemingly greater amount if additional carbohydrate has been supplied) which are necessary for normal frond development become depleted. Dark culture experiments offer a promising means for the study of these factors.

When plants are grown at a particular light intensity and are no longer limited by available carbohydrate, the addition of preparations from coconut milk stimulates growth. Inhibition attends the use of too much coconut milk. The presence of growth inhibitors is suspected, but perhaps the active material which is wholly or at least partially heat stable, becomes inhibitory at high concentrations. Nicotinic acid and yeast extract both cause roots to become elongated in concentrations which are inhibitory to frond growth.

Crude aqueous extracts from humus, sphagnum, cow dung, and horse dung can be shown to promote growth if light intensity and carbohydrate supply are suitably adjusted. Extracts from cow dung and horse dung demonstrate their greatest effect when carbohydrate supply is not a limiting factor. By solvent extraction of cow dung the presence of organic growth inhibitors as well as growth promoters can be clearly demonstrated. Because the ash of the extracts from cow dung and horse dung has no growth promoting activity and the two extracts produce similar effects, it is presumed that the active growth promoting substance in horse dung is quite similar to that in cow dung. Observation of the effect of crude extracts of humus or sphagnum suggest that different substances are responsible for their growth promoting

activity. A rod-shaped bacterial contaminant has been discovered which produces still another distinct type of growth promotion.

Finally, the inability to induce Spirodela to flower experimentally is probably the result of ineffective treatments, although the manner in which they were applied may be partially responsible.

SUMMARY

Growth of Spirodela polyrrhiza (L.) Schleid. upon inorganic medium balanced for optimal growth is limited at moderate light intensities by carbohydrate supply. A supplement of sucrose in the medium helps to correct this deficiency.

Some product of photosynthesis other than sucrose limits growth when sucrose is supplied in the optimal range of 1 to 4 per cent.

Aeration promotes increased growth, as does an additional supply of carbon dioxide. Carbon dioxide treatment even promotes increased growth of cultures treated with 1 to 4 per cent sucrose provided the light intensity is sufficiently high.

Slow growth for quite long periods can take place in darkness if carbohydrate is supplied in the medium, but no growth occurs without it.

Asparagine greatly accelerates frond multiplication in darkness, but rapid depletion of reserves of frond growth materials formed in the light then occurs.

With adequate carbohydrate supply growth is stimulated by some relatively heat stable substance present in coconut milk.

Aqueous extracts of humus, sphagnum, horse dung, and cow dung promote growth under suitable conditions of light intensity and carbohydrate supply.

Extracts of cow dung and horse dung are similar in their effects, which differ from those produced by extracts from humus or sphagnum, or by a rod-shaped bacterium.

The active material in horse dung is presumed to be similar to that in cow dung, shown by solvent extraction to be organic in nature. An organic growth inhibitor substance also occurs in cow dung which can be removed by solvent extraction.

Means of experimentally inducing Spirodela to flower have yet to be discovered.

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