A study of the uptake of several forms of nitrogen and the effect of iron on the assimilation of nitrogen by Spirodela polyrhiza (L.) Schleid.

A STORY OF THE UPTAKE OF SEVERAL FORMS OF NITROGEN and the EFFECT OF IRON ON THE ASSIMILATION OF NITROGEN BY SPIRODELA POLYRHIZA (L.) SCHLEID

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A STUDY OF THE UPTAKE OF SEVERAL FORMS OF NITROGEN AND THE EFFECT OF IRON ON THE ASSIMILATION OF NITROGEN BY *SPIRODELA POLYRRHIZA* (L.) SCHLEID

By

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INTRODUCTION

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The importance of nitrogen in the nutrition of green plants and the role of iron in the development of green plants have been long established. The interrelationship between iron and nitrogen in the metabolism of green plants is not clearly understood, however. A clue to this interrelationship might be obtained by determining the effect of iron on the assimilation of nitrogen.

Most investigations concerning the uptake of different forms of nitrogen were made in non-sterile media so that the results observed were often of doubtful accuracy as to form of nitrogen actually assimilated by green plants. Much work has been done in studying the uptake of various forms of nitrogen, particularly ammonium and nitrate. Relatively little research has been done, however, in studying the uptake of organic forms of nitrogen. It would be definitely of fundamental interest to establish what forms and what amounts of nitrogen are assimilated by green plants.

In this series of experiments, the absorption of urea, guanidine, ammonium, and nitrate forms of nitrogen were studied, as well as the effect of iron on the assimilation of ammonium and nitrate.

HISTORICAL

Beaumont (8) and others have reported the toxicity resulting from the use of ammonium compounds. According to Prianishnikov (48,50) plants absorb ammonium* energetically as long as the reaction of the medium does not hinder its immediate transformation into organic nitrogenous combinations. In neutral or slightly alkaline media, ammonium produces better growth results, while in acid media nitrate is a better source of nitrogen for plants. Prianishnikov (49) claims that ammonium enters plants faster than nitrates, and is more easily assimilated. With excess ammonium, injury appears more readily than with an excess of nitrates. Thus nitrification in the soil regulates the concentration of ammonium and counteracts the excess of acidity produced by ammonium salts so that ammonium toxicity can only rarely occur in soils.

The carbohydrate content of the plant plays the most important role in the synthesis of organic nitrogen compounds, according to Prianishnikov (50). The greater the carbohydrate content, the larger is the amount of ammonium which can be tolerated by the plant. Anything which interferes with the synthesis of organic matter within the plant

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* For the sake of convenience "ammonium" will be used to refer to ammonium ion, and "nitrate" to nitrate ion.

can produce ammonium toxicity. Ammonium is the principal material serving for the synthesis of organic nitrogenous materials by combining with the organic acids which arise from the oxidation of carbohydrates. Nitrates must first be reduced before the nitrogen can be utilized in forming organic nitrogenous combinations.

Beaumont (9), Klein (39), and others have established that plants may directly assimilate such forms of nitrogen as nitrate, ammonium, urea, and certain amino acids. Various amino acids are absorbed by plants, but are not equally utilizable. Thus, asparagine, aspartic, and glutamic acids are more utilizable than glycine and alanine, according to Klein (39). Schreiner and Reed (53) made a study of the toxic actions of certain compounds which occur naturally in plant tissues, and found that amino acid compounds display relatively low toxicity, but that other organic compounds such as guanidine carbonate are toxic even at extremely low concentrations. Brigham (15) and Kawakita (38) reported that concentrations as low as 2 grams of guanidine per liter were toxic to the growth of barley plants and corn plants. Brigham (15) pointed out, however, that organic compounds of high complexity in composition are much more effective for plant growth after ammonification.

The rate of photosynthesis is proportional to the chlorophyll content, which is itself dependent on the amounts of available nitrogen and iron. According to Bennett (12) chlorosis is due to a disturbance of the nitrogen metabolism as well as of iron metabolism in the plant. The physiological functions of the two elements are intimately related. The total amount of iron present in the leaves is not the important factor in chlorophyll production; only the amount of "active" iron present is effective. It was reported as early as 1895 (30) that organic sources of iron produced better results in correcting chlorosis than inorganic sources of iron. Gile and Carrero (29) reported in 1916 that ferrous sulphate, ferric citrate, and ferric tartrate afforded sufficient iron for plant growth in acid, neutral, and alkaline solutions when used in proper quantities, but that ferric chloride was an inferior source of iron. Fly (28) reported that if ferric chloride were used as a source of iron for the growth of Lemna plants in solution cultures, and the pH of the media varied from 4.8 to 8.8, after five days the content of iron was almost one mg. per liter in all the solutions; the remainder was precipitated as $Fe(OH)_3$. When ferric chloride was used as the iron source, there was

not sufficient iron available for plant growth above pH 5. However, when ferric citrate was used, there was sufficient iron in solution to support the growth of Lemna up to pH 8.

Many investigators have reported that the use of organic acid salts of iron, such as citrate, tartrate, and oxalate, increased the amount of iron in solution, particularly in the alkaline range. Hopkins and Mann (35,36) found that the addition of sodium citrate to culture solutions maintained sufficient iron in solution even at alkaline reactions. Sieling (56) also reported that a humic acid solution of iron was effective in preventing chlorosis of Lemna in neutral and alkaline solutions. Olsen (47) offered the hypothesis that iron is taken up from neutral or alkaline solutions and is precipitated in the vascular bundles as $FePO_4$, but that ferric citrate does not give up its iron to the phosphate, and the iron thus passes through the plant as citrate.

EXPERIMENTAL

EXPERIMENTAL

In this series of experiments, Spirodela polyrhiza (L.) Schleid, (also called Lemna major) was used to study the uptake of several forms of nitrogen. This common pond plant may be easily grown under aseptic conditions, thus eliminating all possibility of erroneous results due to the presence of extraneous organisms. The precise control of the environment permits exact quantitative work capable of duplication. According to Steinberg (58,59) the nutrient requirements of Lemna (Spirodela) appear to be quite similar to those of other green plants, and the results obtained through its use may be directly checked with different crop plants.

Ashby (5) and Clark (17) have described the method of reproduction of Spirodela polyrhiza. The plant reproduces by the development of a new individual frond from a cleft on its side. When this new frond is fully grown, it breaks away, and a second frond is produced from a similar cleft on the other side of the parent. By the time the second frond has broken away and becomes a separate plant, a third frond appears in the empty cleft. This is followed by a fourth frond in the cleft formerly occupied

by the second frond. Thus the original frond produces four or five new fronds, after which it dies.

When conditions are suitable for growth and are kept uniform, the rate of increase of Spirodela at any time is proportional to the number of plants present, according to Clark (17) and White (64).

Thus, $N = N_o e^{kt}$ where N = number of plants at any time t.

Thus, $\frac{dN}{dt} = kN$,

Integrating, $\int \frac{dN}{N} = \int k dt$

Therefore, $\log_e \frac{N}{N_o} = k(t-t_o)$

or $\log_{10} \frac{N}{N_o} = K(t-t_o)$

When the log of the number of plants at any time is plotted against time, the result is a straight line of slope K. This is illustrated by Figure I, which was obtained in one of the experiments.

The Spirodela plants used in these experiments were obtained locally from a pond in Hadley, Massachusetts. The plants were sterilized by soaking them in a 10% solution of Chlorox (solution of 5.25% NaOCl) for 60 seconds, following which they were rinsed twice with sterile distilled water. The plants were checked for sterility by incubating

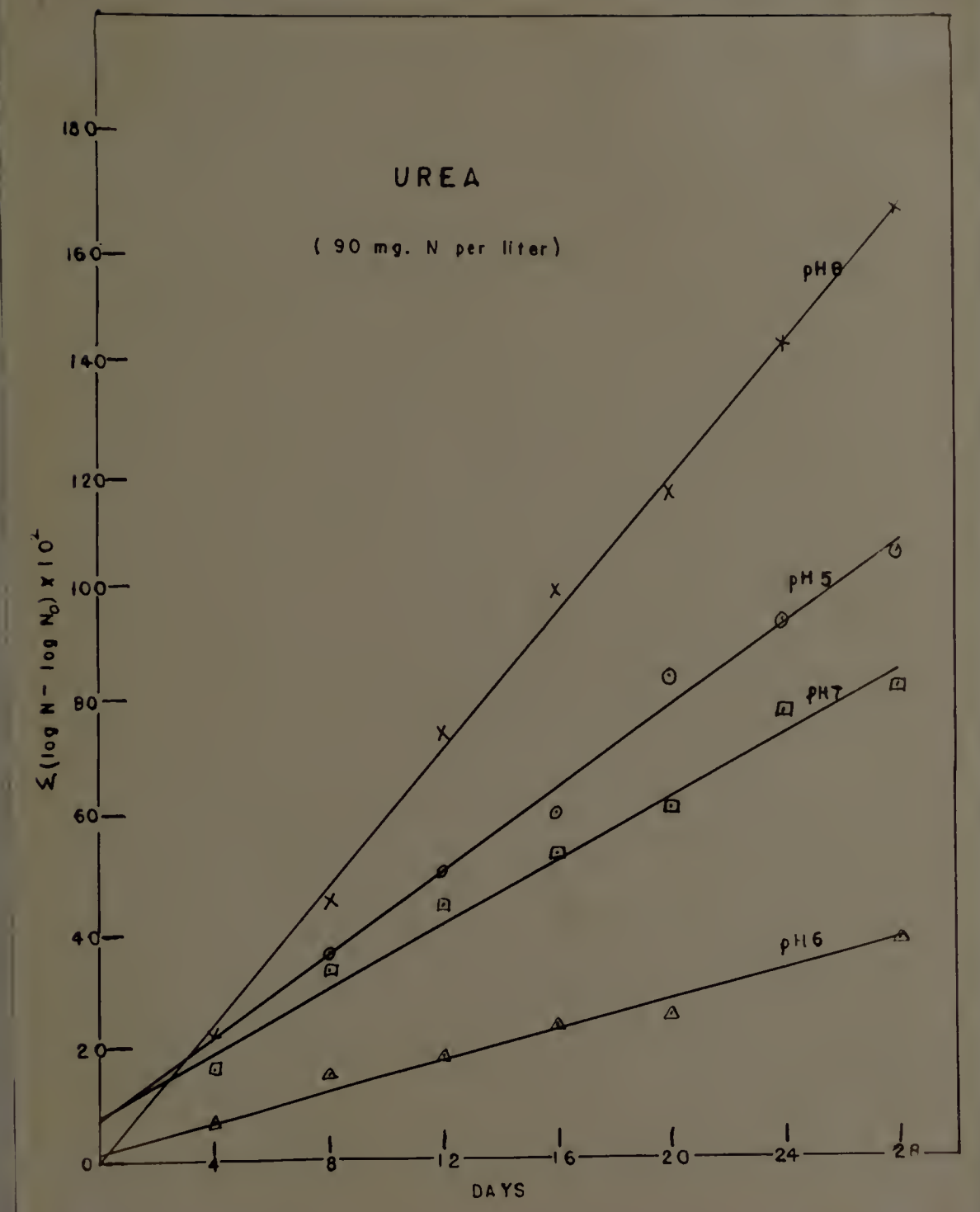


Figure 1. ($\log N - \log N_0$) Values for Solutions Containing 92 mg. Nitrogen per Liter as Urea.

one plant from each culture in Bacto-nutrient broth at 37°C. for 24-48 hours, and checking for the presence of contaminant organisms as shown by turbidity.

The Spirodela plants were grown in a Growth Chamber especially constructed for the purpose (19). The plants were grown in 300 ml. Erlenmeyer flasks containing complete nutrient solutions. The flasks were placed in a copper water bath which was maintained at 25° ± 1°C. by thermostat. Constant illumination of 500 ft.-candles at the surface of the plants was provided by six 40 Watt fluorescent lamps for 15 hours a day. According to Naylor and Gerner (46) plants grown under fluorescent light grow very rapidly and sturdily, and appear more like plants grown in summer sunlight than under any other form of artificial illumination. Fluorescent lamps give off negligible amounts of heat, and are more efficient and economical to operate. They can also be arranged so as to give varying qualities and intensities of light.

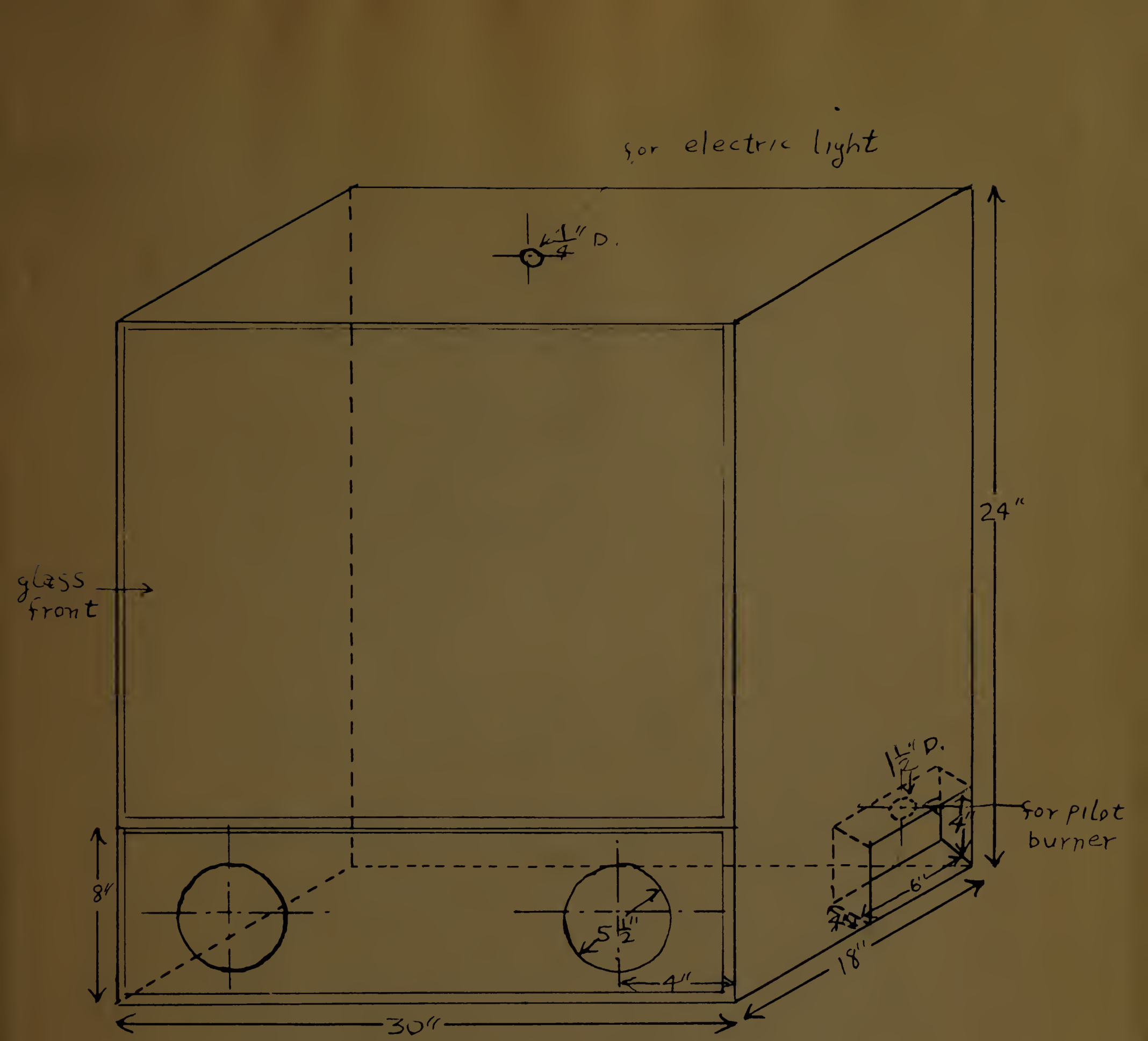
Transfers of plants from one flask to another were performed by means of a platinum loop in a sheet metal Transfer Cabinet which was sprayed with a saturated solution of phenol at least one hour before transferring.

The plants were transferred to fresh solutions every fourth day.

The accompanying diagrammatic sketches in Figures II, III, and IV illustrate the Growth Chamber, Constant Temperature Bath, and Transfer Cabinet used in these experiments. The manner in which the Constant Temperature Bath was operated was rather unusual. The thermostat was connected to an electric relay which functioned physically to switch cooling water from the cooling tube to the overflow and drain. Thus, when the thermostat was "On", cooling water passed out the drain. When the thermostat was "Off" the relay shunted the cooling water into the cooling tube. As a result, there was no heating of the bath by the heater blades and cooling by flowing water at the same time.

In Figure V are photographs of the Growth Chamber and the Constant Temperature Bath showing the arrangement of the temperature regulating mechanisms. Figure VI is a photograph of the Growth Chamber showing the flasks in place. Figure VII is a photograph of the Transfer Chamber.

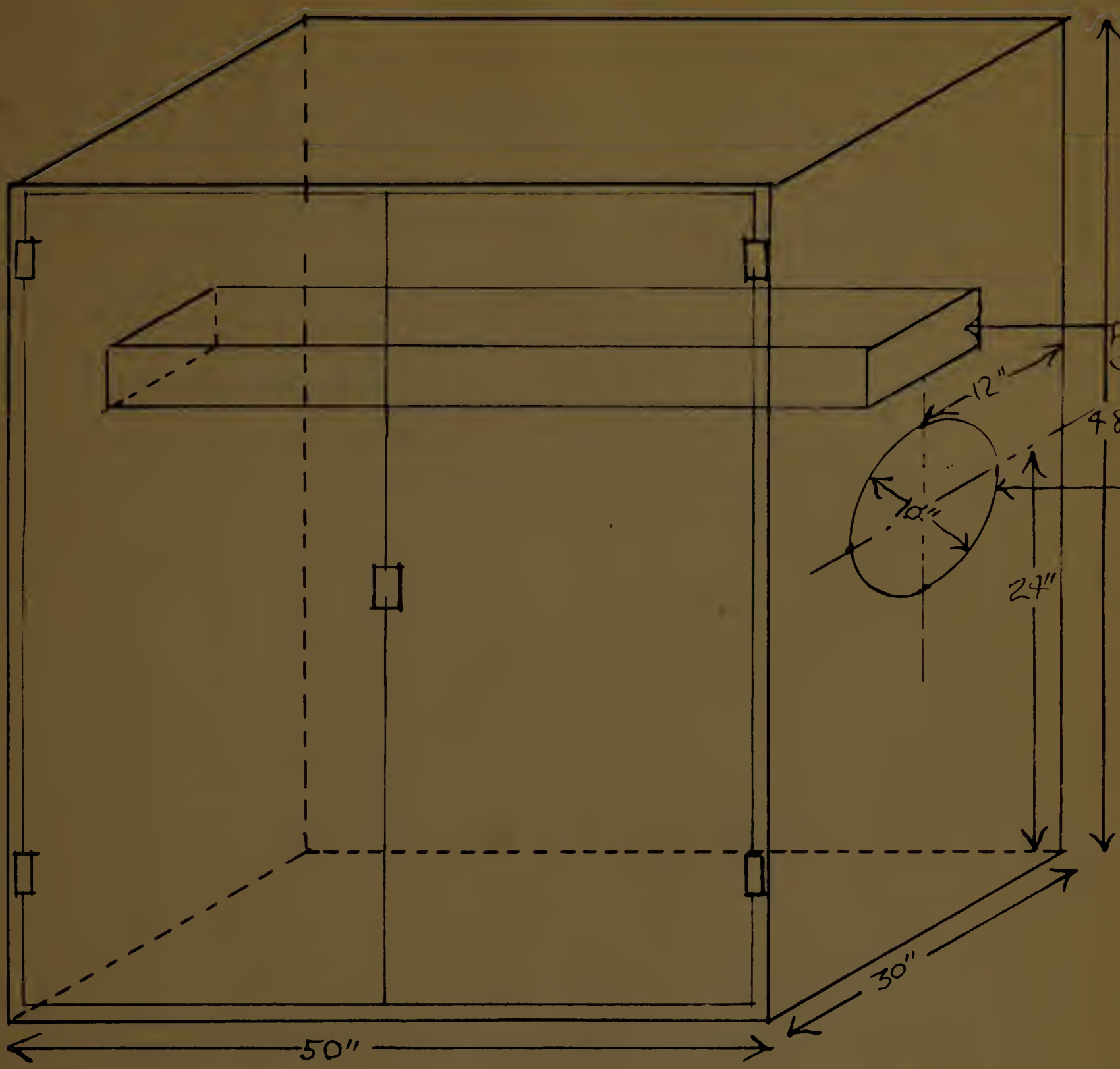
Complete nutrient solutions were used in which the source of nitrogen was varied in each solution. Table I shows the compositions of the nutrient solutions used. It was found necessary to sterilize the urea, the ammonium



TRANSFER CABINET

Figure 2.

E.H.S.



GROWTH CHAMBER

Figure 3.

E.H.B.

CONSTANT TEMPERATURE BATH

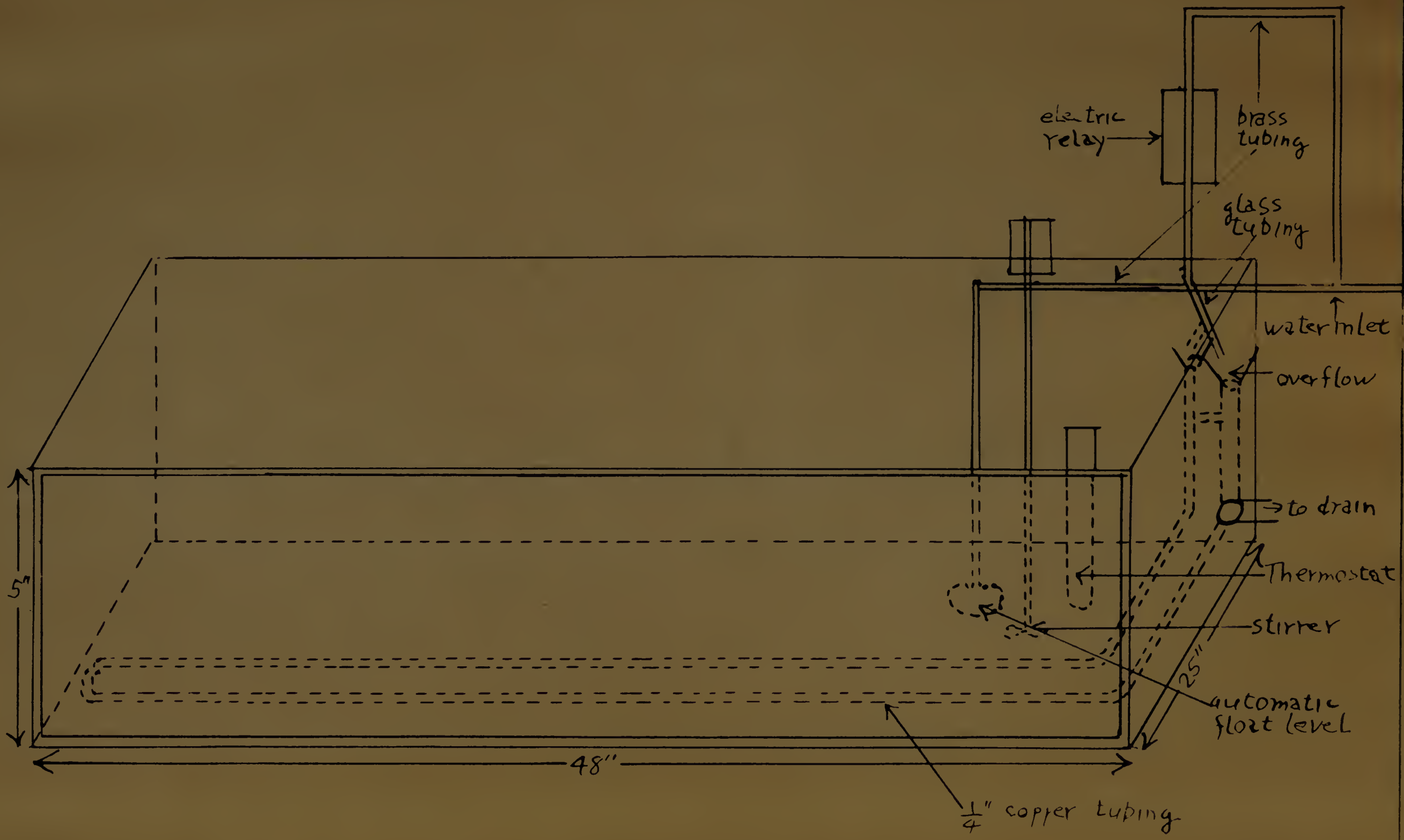


Figure 4.

E.H.B

Figure 5. The interior of the Growth Chamber showing the Constant Temperature Bath and the stirrer, heater blades, thermostat, fan, automatic level, electric relay, and cooling tube.

Figure 6. The Constant Temperature Bath and the flasks containing sterile nutrient solutions and Spirodela polyrhiza plants.

Figure 7. The Transfer Cabinet showing the openings for admitting flasks.

TABLE I

Composition of the Nutrient Solutions*

	Molarity	gm./l.	mg. N/l.	ml. 0.01M/ 100 ml.
Nitrate Solution				
$CaH_4(PO)_2H_2O$	.0002	.0504		2
KNO_3	.0065	.6565	92	13 (.05M.)
$MgSO_4 \cdot 7H_2O$	.0005	.123		5
Ammonium Solution				
$(NH_4)_2SO_4$	.0033	.430	92	6.5(.05M.)
K_2SO_4	.0033	.575		6.5(.05M.)
$MgSO_4 \cdot 7H_2O$	.0005	.123		5
$CaH_4(PO_4)_2 \cdot H_2O$	.0002	.0504		2
Urea Solution				
Urea	.0033	.197	92	6.5(.05M.)
K_2SO_4	.0033	.575		6.5(.05M.)
$MgSO_4 \cdot 7H_2O$	.0005	.123		5
$CaH_4(PO_4)_2 \cdot H_2O$	.0002	.0504		2
Guanidine Solution				
Guanidine HCl	.0022	.209	92	4.4(.05M.)
K_2SO_4	.0033	.575		6.5(.05M.)
$MgSO_4 \cdot 7H_2O$	.0005	.123		5
$CaH_4(PO_4)_2 \cdot H_2O$	.0002	.0504		2

* Stock solutions of each of the salts were provided in .05M. and .01 M. concentrations. Boron and manganese salts, sufficient to provide 1 p.p.m. of these elements in the final volume, and enough of Hoagland's A-Z solution to provide .01 to .02 p.p.m. of the minor elements were added before dilution. Five p.p.m. iron as ferric citrate was provided.

Hoagland's A-Z Solution

LiCl	0.0278 g/l.	$MnSO_4 \cdot H_2O$	0.332 g/l.
$CuSO_4 \cdot 5H_2O$	0.0556	$NiCl_2 \cdot 6H_2O$	0.0503
$ZnSO_4 \cdot 7H_2O$	0.099	$Co(NO_3)_2 \cdot 6H_2O$	0.0556
H_3BO_3	0.611	TiO_2	0.0556
$Al_2(SO_4)_3 \cdot 18H_2O$	0.108	KI	0.0278
$SnCl_2 \cdot 2H_2O$	0.0278	KBr	0.0278

One hundred ml. of the above solution was diluted to 1000 ml., and one ml. of the latter was added to each 100 ml. of nutrient solution.

sulphate, and the potassium hydroxide solutions separately from the remaining constituents of the nutrient solutions in order to obtain the desired pH values after sterilization. Sterilization was accomplished by placing the flasks in an autoclave which was heated to 248°F. and a steam pressure of 15 lbs., which was maintained for 15 minutes.

RESULTS

RESULTS

Part I - Effect of Concentration of Nitrogen

EXPERIMENT ONE

The first experiment was a comparison of the effects of four sources of nitrogen - urea, guanidine, ammonium, and nitrate - at pH values 5.0, 6.0, 7.0, and 8.0 on the growth of Spirodela polyrhiza. The concentration of nitrogen used in the experiment was 92 mg. per liter. The results in Table II indicate that at this concentration of nitrogen, Spirodela grew best with nitrate. The plants of the urea, guanidine, and ammonium series grew poorly at this concentration of nitrogen, the ammonium being so toxic that no growth data could be obtained. These series produced plants which were small, chlorotic, yellowish, white or scorched at the edges of the fronds in different cases. The roots dropped off the fronds in some solutions, and the fronds in others, particularly the ammonium series, existed as individuals, rather than in colonies of two, four or more fronds as in the normal healthy plants. It is interesting to note that for the nitrate series, the plants grew almost equally well at pH values 6.0, 7.0, and 8.0, and less well at pH 5.0. It was not possible to maintain growth in any solution at pH 4.0.

TABLE II

Values for $\Sigma(\log N - \log N_o)$ for Twenty-fourth Day for Solutions Containing 92 mg. Nitrogen per Liter

pH	Σ Value	Remarks
Nitrate		
5	2.05	pale green
6	2.24	normal green
7	2.26	normal green
8	2.26	yellowish green
Urea		
5	.70	tiny, pale
6	.25	tiny, chlorotic
7	.49	small, pale
8	1.19	normal size, pale
Guanidine		
5	.85	tiny, unhealthy dark green, speckled purple undersides
6	.24	small, chlorotic, speckled purple undersides
7	.36	normal size, chlorotic, speckled purple undersides
8	.17	normal size, chlorotic, speckled purple undersides

EXPERIMENT TWO

In order to determine the effect of combining two forms of nitrogen on the growth of Spirodela polyrhiza, concentrations equivalent to 92 mg. nitrogen per liter were prepared, half the nitrogen (46 mg. per liter) coming from each of two different nitrogen sources. The following solutions were thus prepared:

Nitrate-Ammonium
Nitrate-Urea
Nitrate-Guanidine
Ammonium-Urea
Ammonium-Guanidine
Urea-Guanidine

The results of Experiment Two, summarized in Table III show that the best combination at all pH values was the nitrate-ammonium and that the best growth with that combination took place at pH 6.0. It was noted that the ammonium-guanidine series produced better growth than when either form was used alone at this concentration. However, the nitrate-urea series were poorer in growth than when either form was used alone. Except for the nitrate-ammonium series, none of the other combinations of nitrogen produced good growth at the nitrogen concentration used.

TABLE III

Values for $\Sigma(\log N - \log N_o)$ for Twenty-fourth Day for Solutions Containing 92 mg. Nitrogen per Liter from Two Different Sources

pH	Σ Value	Remarks
Nitrate-Ammonium		
5	[illegible]	wrinkled fronds, green
6	[illegible]	normal size, green
7	[illegible]	large, green
8	1.92	large, light green
Nitrate-Urea		
5	.82	tiny, chlorotic
6	.70	small, yellowish
7	.91	small, pale green
8	1.37	normal size, pale green
Nitrate-Guanidine		
5	.30	tiny, unhealthy dark green
6	.20	tiny, unhealthy dark green
7	.30	small, chlorotic
8	.69	small, yellowish
Ammonium-Urea		
5	.77	tiny, yellowish
6	.47	small, yellowish
7	1.25	small, pale green
8	1.38	small, pale green
Ammonium-Guanidine		
5	1.15	small, wrinkled fronds
6	2.53	small, unhealthy dark green
7	1.10	normal size, pale green
8	.40	normal size, yellowish
Urea-Guanidine		
5	.24	tiny, chlorotic
6	.16	small, chlorotic
7	.30	small, chlorotic
8	.13	small, chlorotic

EXPERIMENT THREE

In order to find out whether decreasing the concentration of nitrogen in the nutrient solutions would render the ammonium, urea, and guanidine more effective for promoting plant growth, the concentration of nitrogen was reduced to 46 mg. per liter, one-half that of Experiment One. All the other constituents of the nutrient solutions were maintained constant. The results of Experiment Three are shown in Table IV. Nitrate was still the best source of nitrogen at this concentration, but the growth of the urea and ammonium series plants appeared better than at 92 mg. nitrogen per liter. Although the $\mathcal{E}$ value for the nitrate solution at pH 8 is higher than at pH 7, the plants appeared to be healthier at pH 7 than at pH 8. The guanidine series were as poor at this concentration as in Experiment One.

It was noted that there was a marked degree of purpling on the undersides of the fronds of the guanidine series in Experiment One. This purpling increased noticeably in Experiment Three. The purpling appeared first as specks or flecks, and then increased in size as the plants were continued in the guanidine series.

Although the ammonium series plants reproduced well,

the appearance of the plants was rather poor as compared with the nitrate series. The urea series plants appeared better than the ammonium series, but were also poor, except at the higher pH's, 7.0 and 8.0. It was noticed that the growth of the urea series was poorest at pH 6.0 both in Experiment One and Experiment Three.

TABLE IV

Values for $\Sigma(\log N - \log N_o)$ for Twenty-fourth Day for Solutions Containing 46 mg. Nitrogen per Liter

	pH	Σ Value	Remarks
Nitrate			
	5	2.32	normal, green
	6	2.45	normal, green
	7	2.35	large, green
	8	2.71	large, pale green
Ammonium			
	5	2.22	small, wrinkled fronds
	6	2.50	small, wrinkled fronds
	7	2.22	normal size, yellowish
	8	1.20	normal size, yellowish
Urea			
	5	1.07	tiny, unhealthy dark green
	6	.25	small, chlorotic
	7	1.54	small, pale green
	8	1.89	normal size, green
Guanidine			
	5	1.34	tiny, speckled purple undersides
	6	.20	small, chlorotic, speckled purple undersides
	7	.28	normal size, speckled purple undersides
	8	.21	normal size, speckled purple undersides

EXPERIMENT FOUR

The results in Experiment Three indicated that a lower concentration of nitrogen as urea, ammonium, or guanidine might be more effective in producing growth. Therefore it was decided to try a concentration of 9 mg. nitrogen per liter, which was about one-tenth of the concentration used in Experiment One. The composition of the balance of the nutrient solutions was as in the other experiments. The results in Table V indicate that this concentration of nitrogen was much more effective for plant growth than at higher concentrations, for ammonium, urea, and guanidine. However, it was noted that the nitrate series plants did not appear as well as in Experiments One and Three, but were somewhat paler. In fact, the ammonium series plants seemed better than the nitrate series. It was also noted that pH 7.0 was the best pH for both the nitrate and ammonium series. The urea series plants, as in Experiments One and Three, grew least well at pH 6.0. The guanidine series grew better than in Experiments One and Three, but the degree of purpling increased considerably over that in the higher nitrogen concentrations, so that the undersides of the fronds were often entirely purpled.

TABLE V

Values for $\Sigma(\log N - \log N_o)$ for Twenty-fourth Day for Solutions Containing 9 mg. Nitrogen per Liter

pH	Σ Value	Remarks
Nitrate		
5	1.74	small, green
6	1.75	normal size, green
7	1.92	normal size, green
8	1.82	normal size, pale green
Ammonium		
5	1.72	small, wrinkled fronds
6	2.12	normal size, individual fronds
7	1.86	normal size, light green
8	1.82	large size, light green
Urea		
5	1.92	tiny, pale green
6	.99	tiny, yellowish green
7	1.75	small, pale green
8	1.70	normal size, pale green
Guanidine		
5	1.05	chlorotic, speckled purple undersides
6	1.45	chlorotic, speckled purple undersides
7	1.27	yellowish, speckled purple undersides
8	1.23	unhealthy dark green, speckled purple undersides

Part II - Effect of Iron

EXPERIMENT FIVE

To study the effect of iron on the assimilation of form of nitrogen by Spirodela polyrhiza, and to test the hypothesis that the addition of an organic acid, such as citric acid, will keep sufficient iron in solution available for plant growth, three experiments were performed. The forms of nitrogen used were ammonium and nitrate at a concentration of 9 mg. nitrogen per liter. This concentration of nitrogen was selected because the ammonium and nitrate series plants grew approximately equally well at this concentration, as was found in Experiment Four. In the first experiment, a concentration of 2 p.p.m. of iron as $FeCl_3 \cdot 6H_2O$ was used and amounts of citric acid were selected so that molar ratios of citric acid to iron were 1:1, 2:1, 4:1, and 8:1.

Quantitative colorimetric test (57) showed there was less than one part iron per 100,000,000 as an impurity in the nutrient solutions as prepared. It was also established by use of the nitrate solutions at concentrations of 92 mg. and 46 mg. nitrogen per liter at pH 6.0 without addition of iron that the rate of growth of Spirodela

was retarded. The plants became chlorotic in less than a week, indicating that sufficient iron for plant growth was not present as an impurity in the reagent chemicals used to prepare the nutrient solutions.

The results in Table VI show that the 4:1 citric acid: iron ratio appears to be the best ratio both for the nitrate series and the ammonium series. In the 1:1 series a fine reddish brown sediment was noticed on the bottom of each flask. This sediment was not present in the higher ratios. The ammonium series plants for all the ratios appeared to grow better than the nitrate series. The growth of Spiro-dela at pH 7.0 was very similar in both the ammonium and nitrate series.

TABLE VI

Values for Σ (logN-logN_o) for Twenty-fourth Day for Solutions Containing 9 mg. Nitrogen per Liter, 2 p.p.m. Iron, and Various Molar Ratios of Citric Acid to Iron

pH	Σ Value	Remarks
Nitrate (1:1 Ratio)		
5	1.38	small, yellowish, fine reddish sediment
6	1.22	small, light green, fine reddish sediment
7	1.84	normal size, pale green, fine reddish sediment
8	1.66	normal size, yellowish, fine reddish sediment
Ammonium (1:1 Ratio)		
5	2.16	small, wrinkled fronds, fine reddish sediment
6	1.92	small, yellowish green, fine reddish sediment
7	1.88	normal size, light green, fine reddish sediment
8	1.90	normal size, pale green, fine reddish sediment
Nitrate (2:1 Ratio)		
5	1.44	small, light green
6	1.72	normal size, light green
7	2.12	normal size, light green
8	1.95	normal size, pale green
Ammonium (2:1 Ratio)		
5	2.14	small, wrinkled fronds
6	1.85	normal size, yellowish green
7	2.00	normal size, pale green
8	1.95	normal size, pale green
Nitrate (4:1 Ratio)		
5	1.50	small, light green
6	1.94	normal size, green
7	2.28	normal size, green
8	2.36	normal size, yellowish green
Ammonium (4:1 Ratio)		
5	1.90	small, wrinkled fronds
6	2.30	normal size, pale green
7	2.25	normal size, green
8	2.26	large, light green
Nitrate (8:1 Ratio)		
5	1.98	small, yellowish green
6	1.54	small, pale green
7	2.00	small, light green
8	2.11	normal size, pale green
Ammonium (8:1 Ratio)		
5	2.15	small, wrinkled fronds
6	2.15	small, light green
7	2.15	normal size, pale green
8	2.15	normal size, pale green

EXPERIMENT SIX

Since the optimum iron concentration for the growth of Spirodela was not known, the following experiment was performed. Concentrations of iron of 10, 20, 40, and 80 p.p.m. were selected, using a citric acid:iron ratio of 4:1 and the same nutrient solution formulations that were used in Experiment Five. The results in Table VII show that a concentration of 10 p.p.m. of iron produced the best growth under these conditions. At concentrations of 40 p.p.m. of iron and above, the growth of the Spirodela plants was greatly retarded. At 80 p.p.m. of iron growth was very poor indeed, the plants being tiny, chlorotic, and scorched even at the higher pH's where growth occurred. The best growth at all concentrations took place at pH's 7.0 and 8.0.

The best general appearance of the plants at all pH values, however, took place at a concentration of 2 p.p.m. of iron. This is found by comparing the Σ values for the 4:1 ratios of Tables VI and VII.

TABLE VII

Values for $\Sigma(\log N - \log N_0)$ for Twenty-fourth Day for Solutions Containing 9 mg. Nitrogen per Liter, Various Concentrations of Iron, and a 4:1 Citric Acid to Iron Molar Ratio

pH	Σ Value	Remarks
Nitrate (10 p.p.m. Fe)		
5	.62	small, yellowish
6	.98	normal size, pale green
7	1.90	normal size, light green
8	1.96	normal size, light green
Ammonium (10 p.p.m. Fe)		
5	.60	small, chlorotic
6	1.24	small, pale green
7	1.98	normal size, green
8	1.80	normal size, green
Nitrate (20 p.p.m. Fe)		
5	.04	whitish
6	.23	small, chlorotic
7	.74	small, chlorotic
8	1.88	small, pale green
Ammonium (20 p.p.m. Fe)		
5	-	died
6	.32	small, chlorotic
7	.94	small, pale green
8	2.24	normal size, green
Nitrate (40 p.p.m. Fe)		
5	-	died
6	.31	whitish
7	.74	chlorotic
8	1.88	normal size, green
Ammonium (40 p.p.m. Fe)		
5	.21	whitish
6	.55	small, chlorotic
7	1.76	small, yellowish, speckled brown
8	1.54	small, green
Nitrate (80 p.p.m. Fe)		
5	-	died
6	.05	white, died
7	.42	tiny, chlorotic
8	1.14	small, yellowish, scorched edges
Ammonium (80 p.p.m. Fe)		
5	-	died
6	.14	whitish
7	1.10	tiny, yellowish
8	.42	tiny, scorched

EXPERIMENT SEVEN

As a test of the hypothesis of Hopkins and Wann (36) that increasing the citrate content of the nutrient solution decreases the ionization of the ferric citrate present so that growth is retarded because of a low concentration of Fe^{+++} in solution, the following experiment was performed. A concentration of iron equal to 10 p.p.m. was selected, and citric acid:iron ratios of 16:1 and 32:1 were prepared using the same nutrient solution formulations as in Experiments Five and Six. A concentration of iron of 10 p.p.m. was selected because it was thought that 2 p.p.m. might not furnish adequate iron for plant growth at the high citric acid:iron ratios used.

The results in Table VIII show that at the 16:1 ratio, growth has been retarded by the relatively high concentration of citrate and that the nitrate series plants appear better than the ammonium series. At the 32:1 ratio the growth of the Spirodela plants was very greatly retarded by the high concentration of citrate at all pH values and the fronds were very small, and pale, yellowish, or chlorotic.

TABLE VIII

Values for $\Sigma(\log N - \log N_0)$ for Twenty-fourth Day for Solutions Containing 9 mg. Nitrogen per Liter, 10 p.p.m. of Iron, and Various Molar Ratios of Citric Acid:Iron

pH	Σ Values	Remarks
Nitrate (16:1 Ratio)		
5	1.40	very small, yellowish
6	1.82	small, pale
7	1.72	normal size, pale
8	1.91	normal size, light green
Ammonium (16:1 Ratio)		
5	1.16	very small, chlorotic
6	1.89	small, frond edges scorched
7	1.93	small, pale
8	1.82	small, pale
Nitrate (32:1 Ratio)		
5	1.44	tiny, yellowish, speckled brown
6	1.42	tiny, yellowish, speckled brown
7	.25	small, yellowish
8	.08	small, chlorotic
Ammonium (32:1 Ratio)		
5	.90	small, pale
6	.68	small, pale
7	.20	small, chlorotic
8	.28	small, chlorotic, individuals

DISCUSSION

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The results obtained in Part I seem to indicate that the effectiveness of any form of nitrogen in promoting growth is influenced primarily by concentration. When the reaction of the medium is not extreme (i.e., pH 5 to 8), nitrate nitrogen seems to be almost equally efficient at all concentrations in promoting growth, providing, of course, that it is not present to a toxic excess, or in insufficient amounts. Ammonium nitrogen appears to be toxic for plant growth in solution cultures over the same range of concentrations that nitrate is effective. However, at much lower concentrations, ammonium appears to be a more efficient form of nitrogen than nitrate. It thus seems that plants are able to tolerate and utilize much larger concentrations of nitrate than ammonium at moderate reactions. It should be noted, however, that much lower concentrations of ammonium nitrogen than of nitrate nitrogen are needed to produce excellent growth. This fact may find some practical application in the use of commercial fertilizers.

Urea nitrogen is intermediate in its effects on the growth of *Spirodela* between nitrate and ammonium. Urea

is apparently more efficient at neutral and slightly alkaline reactions. The fact that nitrogen is present as NH_2 in the urea molecule would seem to indicate its easy absorption and assimilation. The ammonium form must first be oxidized to NH_2. The ammonium form itself is toxic above certain low concentrations. This fact may be a clue to the question as to why the Spirodela plant can tolerate higher concentrations of urea than ammonium. The fact that Spirodela can tolerate higher concentrations of nitrate than urea may be explained by the fact that plants absorb and store relatively large amounts of nitrate, reducing only such amounts as they require for assimilation. Urea, having the NH_2 form, is immediately available for assimilation, and in that form the plant can only utilize certain amounts without toxic results.

Guanidine was found to be inefficient as a nitrogen source for the growth of Spirodela at all concentrations used, although growth was promoted at the lowest concentration. Purpling of the fronds increased as the guanidine concentration decreased. This purpling may be an indication of some unbalanced condition in the nitrogen metabolism of the plant. Such purpling of the fronds is often noticed in ponds containing Spirodela polyrhiza, particularly

in the mid-West.

At nitrogen concentrations which were about equally effective for mainting growth, ammonium and nitrate were approximately equally efficient in neutral solutions. However, nitrate was somewhat more effective below neutrality (pH 7) and ammonium above neutrality.

The results in Part II suggest that the addition of an organic acid, such as citric acid, will maintain sufficient iron available in solution for plant growth, even in alkaline solutions. It was found, however, that there must be more than a molar equivalence of citric acid to iron to maintain all the iron in solution. Struthers and Sieling (61) reported that more than one mole of citric acid is required per mole of iron to maintain all the iron in solution. This fact was substantiated in these experiments. However, it was found that a higher mole ratio, viz, 4:1, promoted more effective growth. Subsequent work in this laboratory has shown that in dilute solutions one mole of citric acid will maintain one mole of iron in solution between pH 4 and pH 6, but the citric acid is somewhat less effective at higher and lower pH values.

Lanford and Quinan (42) have found evidence that iron forms a complex with citric acid whose formula may be re-

presented by:

$$Fe(H\ citr)^{+} \rightleftharpoons Fe^{+++} + H\ citr^{--}$$

This formula is of the same type as that for the complex formed by the reaction of ferric ion with phosphoric acid, which has been shown to be $FeHPO_4^{+}$ (41). This suggests that the form in which iron is taken up by plants is ferric organic complex ion instead of Fe^{+++} as has been widely accepted, and that $Fe(H\ citr)^{+}$ is probably the form absorbed when citrates are present. In inorganic media the iron is probably complexed by organic substances present in the epidermis of the roots, and thus enters the plant as ferric organic complex ion. Work in this laboratory has shown that pectic and uronic acids (compounds which are among those present in roots) are capable of forming stable complex ions with iron. This does not mean to infer that Fe^{+++} would not be utilizable by plants, but that there is not enough Fe^{+++} in solution at moderate pH values to supply sufficient iron for plant growth. Since the solubility product of $Fe(OH)_3$ is 1×10^{-36}, the concentration of Fe^{+++} at pH 7 is determined as follows:

$$(Fe^{+++}) \times (OH)^3 = 1 \times 10^{-36}$$

$$(Fe^{+++}) \times (10^{-7})^3 = 1 \times 10^{-36}$$

$$(Fe^{+++}) = 1 \times 10^{-15}\ \text{molar}$$

At pH 5 the concentration of Fe^{+++} is 1×10^{-9} molar.

Such low concentrations of iron cannot account for the toxicity which results from an excess of iron. It is quite probable, however, that apparent toxicity may really be iron deficiency on the assumption that though the plant may absorb a ferric organic complex ion, it utilizes only the Fe^{+++} which it obtains from the complex. Thus, an excess of complexing agent may repress the ionization of the ferric organic complex ion to such an extent that the plant would be unable to obtain sufficient Fe^{+++} from the complex ion.

In comparing the Σ values in Tables VI, VII, and VIII, it would seem that the optimum concentration of iron for the growth of Spirodela is 2 p.p.m., and the optimum ratio of citric acid:iron is 4:1. It is possible, however, that a much lower concentration of iron as $Fe(H\ citr)^{+}$ is adequate for maintaining good growth of Spirodela. The concentration of $Fe(H\ citr)^{+}$ in the vario s nutrient solutions was not determined.

The experimental results indicate that at a concentration of 2 p.p.m. of iron, a molar ratio of citric acid to iron of 1:1 was insufficient to maintain all the iron in solution. When the ratio was increased to 2:1, healthier growth resulted, but the 4:1 ratio produced the best growth.

This would seem to indicate that at the 2:1 ratio, the citric acid kept all the iron in solution, as far as it was possible to observe, but that the chemical conditions within the nutrient solutions were such that the amount of citric acid required to furnish the optimum amount of $Fe(H\ citr)^{+}$ was not present until the 4:1 ratio was used.

Work in this laboratory has shown that the $Fe(H\ citr)^{+}$ complex is most stable between pH values 4:0 and 6:0 since more $(H\ citr)^{--}$ is present. This fact can explain the growth results reported in Tables VI, VII, and VIII. The best growth took place in the more basic solutions, and the poorest growth in the more acid solutions. This suggests that the $Fe(H\ citr)^{+}$ concentration was somewhat excessive in the acid solutions, but that it was present in "safe" amounts in the basic solutions. The addition of (OH) ions seems to reduce the concentration of $Fe(H\ citr)^{+}$ because it changes the $(H\ citr)^{=}$ to $(citr)^{---}$ and reduces the $Fe(H\ citr)^{+}$ form to a "safe" level for plant growth. In the case of the higher ratios either the concentration of citrate or complex ion, or iron itself becomes toxic.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Spirodela polyrhiza plants were grown in sterile culture solutions under conditions of constant temperature and illumination. The composition of the culture solutions was similar in all cases except that the form of nitrogen was different. The uptake of such forms of nitrogen as guanidine, ammonium, urea, and nitrate was studied under various conditions of pH and concentration of nitrogen. The effect of iron and an organic acid (citric acid) on the uptake of ammonium and nitrate under various conditions of pH and various molar ratios of citric acid to iron was also studied.

The experimental results indicate that the effectiveness of any form of nitrogen in promoting growth is influenced primarily by concentration. Nitrate was a more effective source of nitrogen than any other form of nitrogen studied over a greater range of concentrations. Ammonium nitrogen was toxic at the higher concentrations where nitrate was very effective in promoting growth. However, at much lower concentrations, ammonium was more efficient in promoting growth than nitrate.

Urea was intermediate in its effects on plant growth between ammonium and nitrate.

Guanidine was inefficient in promoting growth at all the concentrations used in these experiments, and caused a purpling of the undersides of the fronds which increased as the guanidine concentration decreased in the culture solutions.

A citric acid to iron ratio greater than 1:1 maintained all the iron in solution, even in basic solutions. The optimum ratio for the growth of Spirodela polyrhiza was 4:1. The optimum concentration of iron was 2 p.p.m.. Growth was greatly retarded above 20 p.p.m. of iron and a 16:1 ratio. The best growth took place in the basic solutions, and the poorest growth in the acidic solutions.

Evidence was found to corroborate the postulate that iron enter the plant as ferric organic complex ion, or is transported to the plant root where it is utilized as Fe^{+++} subsequent to its release from the complex ion.

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