To the Graduate Council:

I am submitting herewith a thesis written by Henry-York Steiner II entitled "The Effects of pH on Ammonium Uptake in Plant Cell Culture." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

Donald K. Dougall, Major Professor

We have read this thesis
and recommend its acceptance:

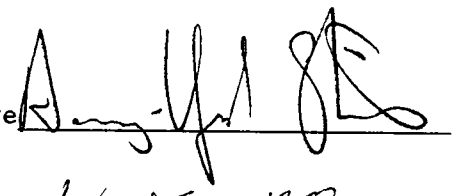
Accepted for the Council:

Vice Provost
and Dean of The Graduate School

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirments for a Master's degree at The University of Tennessee, Knoxville, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of the source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Head of Interlibrary Services when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature 

Date November 25 1987

THE EFFECTS OF pH ON AMMONIUM UPTAKE IN PLANT CELL CULTURE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Henry-York Steiner II

December 1987

ACKNOWLEDGMENTS

I would like to express my sincere thanks to several people who have helped me during the course of my Master's degree. First, I extend my deepest gratitude to Dr. Dougall for his wisdom, guidance, and unwaivering patience throughout this study. I would also like to thank the gentlemen on my thesis committee, Dr. G. Frazier, Dr. R. Henke, Dr. W. Smith, and Dr. G. Stacey, for their very helpful comments and suggestions. Special thanks go to Gayle Darugh for her assistance in preparation of the thesis. Finally, to my parents, Mary and David, this work is dedicated. Without their love and continued support, none of it would have been possible.

ABSTRACT

Plant cell suspension cultures were used to investigate the effects of pH on ammonium uptake in wild carrot cells. Cultures were grown at three pH's (4.5, 5.5, and 6.5) in a media containing ammonium as the sole nitrogen source. Cells were cultured initially semi-continuously followed by batch culture and growth, pH, and ammonium uptake followed in both. Both nitrogen limited and phosphate limited conditions were tested. The results indicated that pH effected ammonium uptake rate in carrot cell cultures. Nitrogen limited pH 4.5 semi-continuous cultures showed a higher uptake rate than of pH 5.5 or 6.5 cultures. In phosphate limited cultures, pH 5.5 showed the highest uptake rate. Little difference in ammonium uptake rate occured in batch culture at three pH's of semi-continuously grown cells. Large uptake differences did exist between cells previously cultured semi-continuously at different pH's. Cell morphology was also effected by pH. Greater than 75% of an individual culture grown at pH 5.5 or 6.5 was composed of cell aggregates 310 microns or greater. At pH 4.5, 71% of an individual culture was composed of cell aggregates 310 microns or less. From the results it was hypothesized that pH effects ammonium uptake rate through the aggregation or disaggregation of cells.

TABLE OF CONTENTS

SECTION		PAGE
INTRODUCTION		1
I.	LITERATURE REVIEW	3
	NH_4-NO_3 Mixtures and pH	3
	Nitrogen Ratios, Nitrogen Feeding Regimes And Medium pH	4
	Effect of Controlled pH Levels	4
	Effect of Buffer on NH_4 Uptake	5
	Growth, Culture pH and Ammonium as a Limiting Nutrient	6
	Summary	7
II.	MATERIALS AND METHODS	8
	Plant Tissue	8
	Culture Medium	8
	Semi-continuous Culture	8
	Ammonium Uptake Experiments	9
	Batch Culture	9
	Dry Weight Determinations	10
	pH Determinations	10
	Ammonium Ion Determinations	10
	Cell Screenings	11
III.	RESULTS	12
	Preliminary Experiments	12
	Semi-continuous Culture of Ammonium Limited Carrot Cells	12
	Attainment of steady state culture density	12
	Stability of pH	13
	Ammonium Uptake Rates of Ammonium Limited, Semi-continuous Cultures	16
	Batch Culture of Ammonium Limited, Semi-continuous Cultures	16
	Phosphate Limited, Semi-continuous Culture	29
	Ammonium uptake experiments	29
	Batch Culture of Phosphate Limited, Semi-continuous Cultures	32
	Aggregate Size Distribution Experiments	36
IV.	DISCUSSION	44
V.	CONCLUSION	50

SECTION PAGE

LIST OF REFERENCES 52

APPENDICES 55
APPENDIX A. WCM-1 MEDIUM 56
APPENDIX B. WCM-4 MEDIUM 57
APPENDIX C. DRY WEIGHT DETERMINATION 58
APPENDIX D. AMMONIUM ION DETERMINATION 60

VITA 62

LIST OF TABLES

TABLE		PAGE
I.	Ammonium Uptake Rates of Nitrogen Limited, Semi-continuous Cultures at pH 4.5 and 5.5	18
II.	Ammonium Uptake and Growth Rates in Batch Culture and Ratio of These Two for Nitrogen and Phosphate Limited Cells at Three pH's . .	24
III.	Ammonium Uptake Rates of Phosphate Limited, Semi-continuous Cultures at pH 4.5, 5.5, and 6.5	33
IV.	Summary of Ammonium Uptake Rates in Semi-continuous and Batch Culture at All pH's . .	45

LIST OF FIGURES

FIGURE		PAGE
1.	Culture Density of Ammonium Limited, Semi-continuous Cultures at pH 4.5 and 5.5	14
2.	Culture Density and Ammonium Concentration of Ammonium Limited, Semi-continuous Cultures at pH 4.5 and 6.5	15
3.	Ammonium Uptake Rates of Ammonium Limited, Semi-continuous Cultures at pH 4.5, 5.5, and 6.5 . .	17
4.	Growth of Batch Cultures at pH 4.5, 5,5, and 6.5 Innoculated with Tissue from Ammonium Limited, Semi-continuous Cultures	20
5.	Ammonium Uptake of Batch Cultures at pH 4.5, 5.5, and 6.5 Innoculated with Tissue from Ammonium Limited, Semi-continuous Cultures	21
6.	Growth and Ammonium Uptake of Batch Culture at pH 4.5, 5.5, and 6.5 Innoculated with Tissue from Ammonium Limited, Semi-continuous Cultures	22
7.	Semi-log Plot of Dry Weight of Figure 4 Data . .	25
8.	Semi-log Plot of Ammonium Uptake of Figure 5 Data	26
9.	Semi-log Plot of Dry Weight of Figure 6B Data . .	27
10.	Semi-log Plot of Ammonium Uptake Data of Figure 6B	28
11.	Culture Density of Phosphate Limited, Semi-continuous Cultures at pH 4.5 and 5.5	30
12.	Culture Density of Phosphate Limited, Semi-continuous Cultures at pH 4.5 and 6.5	31
13.	Dry Weight and Ammonium Uptake in Batch Culture at pH 4.5 and 5.5 Innoculated with Tissue from Phosphate Limited, Semi-continuous Cultures	34

FIGURE PAGE

14. Dry Weight and Ammonium Uptake in Batch Cultures at pH 4.5, 5.5, and 6.5 Innoculated with Tissue from Phosphate Limited, Semi-continuous Cultures 35

15. Semi-log Plots of Dry Weight and Ammonium Uptake of Figure 13 Data 37

16. Semi-log Plots of Dry Weight and Ammonium Uptake of Figure 14 Data 38

17. Aggregate Size Distribution of a Stock Culture . 40

18. Aggregate Size Distribution of a Nitrogen Limited, Semi-continuous Culture at pH 4.5 and 5.5 41

19. Aggregate Size Distributions of Nitrogen Limited, Semi-continuous Cultures at pH 4.5, 5.5, and 6.5 42

INTRODUCTION

The continued growth of plant cells in suspension culture depends upon the organic and inorganic nutrients supplied by the medium. Physical factors, such as pH, light, and temperature, also effect growth. Knowledge of whether any or all of these factors operate singly or together to effect growth is important in understanding the complexities behind such a living system.

Previous observations have indicated that both pH and nitrogen source effect growth of plant cells in culture. Carrot cells grown under nitrogen limited conditions exhibited different growth and anthocyanin yield constants when cultured at different pH's (Dougall et al. 1983). Medium pH also effects nitrogen uptake. Ipomoea cells grown under pH control, batch culture conditions, showed decreasing ammonium uptake rates as pH decreased (Martin and Rose 1975). Other observations of large pH fluctuations during culture can also be found in the literature. From these observations pH is a common factor and as such suggests an approach by which a relationship between pH, nitrogen uptake, and growth may be discovered.

Unfortunately, Martin and Rose's experiments supplied cells with two nitrogen sources simultaneously and the possible interaction of both plus pH on nitrogen uptake was not clarified. The general applicability of their results also remained untested. Dougall et al.'s results presents a complicating factor as semi-

continuous culture methodology may have a different effect upon nutrient uptake than batch culture. Dougall and Weyrauch (1980b), using semi-continuous carrot suspension cultures, showed that in a range of concentration of a limiting nutrient (phosphate) in the input medium and at a specific growth rate, steady state culture density was proportional to the input nutrient concentration.

The objective of this study was to investigate the relationship between pH and nitrogen uptake by simplifying the system being studied. To accomplish this, one nitrogen source, ammonium, was supplied to carrot cell cultures at various pH's. The cells were cultured initially semi-continuously followed by batch culture and the uptake of ammonium followed in both. The methodology of semi-continuous culture provided cells growing exponentially for an extended period of time, which significantly increased reproducibility of experiments and the frequency with which experiments could be made. By utilizing both culture methodologies, the investigation was extended by providing a means to examine the effects of culture methodology on ammonium uptake rates.

I. LITERATURE REVIEW

To date, only one review has been written on nutrition and metabolism in plant tissue culture (Dougall, 1980). In examining ammonium as a sole nitrogen source and effects of nitrate and ammonium on medium pH during growth, two general conclusions arose. One, plant cell cultures would grow on NH_4 provided that the pH of the medium was controlled. This could be accomplished through the addition of di- and tri-carboxylic acids, some of which were Kreb's cycle intermediates. The specific function of the acids in allowing growth on NH_4, however, remained unclear. Two, the effects of NH_4 and NO_3 on culture pH plus the effects of culture pH on assimilation of these nutrients suggested a mechanism by which cells could control the medium pH within tolerable limits. Although the investigation of this area is by no means extensive, a number of reports examine, both directly and indirectly, the relationship(s) between nitrogen source and pH.

NH_4-NO_3 Mixtures And pH.

Sheat et al. (1959) noticed a pH drift following nitrogen uptake in nitrogen-depleted tomato roots and concluded that pH drifts were caused by assimilation of NH_4-NO_3 mixtures. Drifts in pH during uptake of NH_4-NO_3 mixtures were also recorded by Bayley et al. (1972) where pH fell from 5.9 to 4.8 and then rose to 7.0 in soybean root cell suspension cultures. No account for the pH change was given. Nash and Davies (1972) have also reported pH shifts from

pH 5.9 to 6.6 to 5.5 to 5.8 during the course of a batch culture without pH control.

Nitrogen Ratios, Nitrogen Feeding Regimes And Medium pH.

pH fluctuations in culture media during 10 day experiments with different ratios of NH_4 and NO_3 in carrot cell cultures prompted Veliky and Rose (1973) to propose that medium pH changes, along with ammonium and nitrate utilization, might influence biomass production. In 200 hour experiments with Ipomoea cell cultures, NH_4-citrate was added periodically to maintain constant NH_4 concentration (Rose and Martin, 1975). During the course of culture, medium pH rose from 5.1 to 6.2 by 25 hours, dropped to 4.4 by 90 hours, and then leveled off at 4.8. In cultures where NH_4-citrate was added continuously for the first 150 hours, pH initially rose sharply from 5.3 to 6.2 by 25 hours, fell to 4.8 by 50 hours followed by a steady rise to 5.5. In cultures grown without supplementary nutrients for 137 hours, with supplementary ammonium citrate and sucrose between 137 and 194 hours, and no supplementary nutrients from 194 hours on, pH fluctuated throughout the experiment. By 137 hours pH had climbed to 7.4 corresponding to depletion of NH_4. During and after the feeding period, pH steadily dropped from 7.4 to 4.0., and ammonium was depleted by hour 250.

Effect Of Controlled pH Levels.

In rose suspension cultures whose pH was controlled with MES buffer, pH effected maximum fresh weight yields (Nesius and

Fletcher, 1973). When the pH of the cultures was increased from about 5.5 to 6.0, the fresh weight of the cultures also increased.

Ipomoea cells grown at controlled pH levels indicated that pH had a definite effect on NH_4 and NO_3 uptake and total N-accumulation (Martin and Rose 1975). At pH 7.1, 6.3, and 5.6, ammonium was depleted by 90 hours at all three pHs whereas NO_3 uptake increased as pH decreased. The initial rate of NH_4 uptake was slow to none at pH 5.6 and 4.8 followed by a rapid increase in uptake. In the pH 5.6 culture, NH_4 depletion marked the begining of rapid NO_3 uptake. Cultures at pH 4.8 performed in a similar manner but at 90 hours the cells excreted NH_4 into the medium. However, NO_3 uptake was rapid from the start.

The presence of more than one nitrogen source in both of Martin and Roses' experiments complicates the interpretation of the results. Though the rates of both ammonium and nitrate uptake are clearly affected by pH and vice versa, the effects of one nitrogen source upon the other and the contribution to these effects by pH is difficult to separate.

Effect of Buffer on NH_4 Uptake.

In cultures grown on ammonium as the sole nitrogen source, rapid acidification of the medium occurs killing the cells. Control of the hydrogen ion concentration was first achieved by the addition of buffers to the medium (Gamborg and Shyluk, 1970).

In examining the effect of an organic acid buffer on NH_4

uptake, Behrend and Mateles (1976) observed that the rate of ammonium uptake in tobacco cells was not influenced by the presence of the buffer (succinate) in the medium. They concluded that organic acids were not connected with NH_4 transport but with NH_4 metabolism the reason being that (in this case) NH_4 and succinate were consumed in parallel and at a fixed ratio. pH affects were not taken into account as the pH of the cultures was initially between 5.0-6.5 and subsequently dropped to 4.3-4.7 during the course of the experiment.

Dougall and Weyrauch (1980a) observed an increase in pH in the presence of succinate as a buffer while NH_4 was assimilated, but contrary to Behrend and Mateles, concluded that the function of the dicarboxylic acids added to medium of plant cells grown on NH_4 was to limit pH change. They suggested that the organic acid was modifying the physiology of the cells to produce fewer H^+'s or the cells were taking up unionized forms of the organic acid.

Growth, Culture pH and Ammonium as a Limiting Nutrient.

In semicontinuous chemostat cultures where ammonium was the limiting nutrient, dilution rate and pH affected growth yield and anthocyanin accumulation in wild carrot cells (Dougall et al. 1983). Changing the pH from 4.5 to 5.5 or 4.2 reduced the yield constant for growth by up to 20%.

Summary

Nitrogen type, nitrogen ratio, nitrogen feeding regime, medium pH, medium buffer, and limiting nutrient all appear to be interrelated in some way with respect to nitrogen uptake, medium pH fluctuation, and growth. The possible effect of all these factors on NH4 uptake rate cannot be investigated here. However, simplifing the system under study, as described earlier, may provide an answer to at least one of these relationships, that being the effect of pH on ammonium uptake rate.

II. MATERIALS AND METHODS

Plant Tissue.

Cell suspensions were a wild carrot (Daucus carrota L.) cell line WC-63 initiated from a petiole section in 1963 (Wetherell and Dougall 1976). Stock cultures of this tissue were maintained by serial passage (a volume of culture equal to 5% of the fresh medium) at two week intervals in liquid culture medium WCM-1 (Wetherell, 1969) (see Appendix A). Cultures were grown in the dark at 25°C in 250 ml Erlenmeyer flasks plugged with cotton and agitated on a rotary shaker at 120 rpm.

Culture Medium.

All experimental cultures were grown in a medium known as WCM-4 (Dougall and Weyrauch, 1980a) (see Appendix B), an improved media for anthocyanin production, buffered with potassium succinate. All media were additionally buffered with MES (N-morpholino-ethane sulfonic acid). All media were filter sterilized using either Gelman 0.2 um pleated cartridges or Millipore filters equiped with 0.2 um Gelman filter membranes.

Semi-continuous Culture.

Ninety-five mls of WCM-4 at pH 4.5, 5.5, or 6.5 was added to 500 ml Erlenmeyer flasks equiped with silicon sponge enclosures. Five mls of inoculum was added to each flask for a final 1:20 density. The inoculum was 7 day old WC-63 suspension. After a 3-4

day adaptation period, the daily cycles of media turnover began. In a laminar flow hood, each flask was agitated and 20 mls of culture was removed, followed by the addition of an equal amount of fresh media. Replacement of the culture medium occured at the same time every 24 hours and sterile distilled water was added to compensate for evaporation. Every fourth or fifth day, the cultures were transferred to new flasks to check culture volume and minimize heterogeneity due to cells adhering to the walls of the flask.

Ammonium Uptake Experiments.

Uptake experiments were performed at the begining of a turnover period after the addition of fresh medium and lasted a full 24 hours. 1 or 2 ml samples were taken at intervals, usually in the first 12 hours. A total of 20 mls of culture was available for samples. Two 10 ml samples were taken at the end of the previous 24 hour period and pH, ammonium concentration, and dry weight were determined. The dry weight value, corrected for dilution, served as the culture density used to calculate ammonium uptake rates on a dry weight basis.

Batch Culture.

Batch cultures were initiated with semi-continuously grown cells. At the end of a semi-continuous culture experiment, 100 mls of WCM-4 (6 mM NH_4Cl) at each of three pH's (4.5, 5.5, 6.5), was inoculated with 20 or 25 mls of semi-continuous culture suspension. Growth was allowed to proceed for 7 days while NH_4 disappearance,

pH, and dry weight were followed. No sample was taken the first 24 hours to allow growth to be initiated. Two 10 ml samples were taken the second and third day, and two 5 ml samples every day thereafter.

Dry Weight Determinations.

Culture samples were centrifuged at 650xg for 9 minutes in a Beckman Table Top centrifuge. The supernatant was decanted and saved for pH determination and ammonium analysis. The pellet was resuspended in an equal volume of deionized water and filtered onto a preweighed 24 mm glass fiber disk (Whatman GF/D) and washed with approximately 30 mls of deionized water. The disks were dried at 65^{o}C for at least 12 hours. Samples were then weighed on a Mettler H51AR Analytical balance to the nearest 0.01 mg. This methodology is further detailed in Appendix C.

pH Determinations.

pH was measured using an Orion Research model 601A digital Ionalyzer equiped with a research grade combination pH probe.

Ammonium Ion Determinations.

Ammonium disappearance from the medium was measured colorimetrically. The assay was a sodium nitroprusside, phenol and sodium hydroxide reaction (Berthelot's reagent, see Appendix D) (Balis, 1971). The optical density of samples was measured in a Gilford Stasar II spectrophotometer at 640nm.

Cell Screenings.

Samples were washed through a stainless steel wire screen with distilled water. Samples were first washed through a 580 micron screen and the residue collected for dry weight measurement. The remaining cells were then washed through a 310 micron screen and the residue collected, dried and weighed. A 105 micron screen was used to filter the remaining cells and both the filtrate and the cells remaining on the screen were collected on glass fiber disks for dry weight measurement.

III. RESULTS

Preliminary Experiments.

Initial experiments determined proper dilution rate, limiting nutrient concentration, sample intervals and minimum time to reach steady state.

Semi-continuous Culture of Ammonium Limited Carrot Cells.

Attainment of Steady State Culture Density. To define a steady state culture density, dry weight values should be randomly distributed about a horizontal line. Any deviation of this line from zero slope indicates that the culture is either gaining or losing biomass with time. The semi-continuous culture methodology provides a means to achieve steady state.

Nearly all of the semi-continuous culture density data indicated that steady state had not been achieved in any of the experiments. Although some portions of the growth curves showed random distribution about a horizontal axis (see 1a days 35-42 and 1b days 19-25 on page 14, days 20-26 on page 15, days 10-16 pH 4.5 on page 30), linear regressions of the entire culture period showed positive or negative deviations from zero slope.

Since, in most experiments, some of the errant dry weight values were in concert between flasks, an environmental factor may have influenced the culture density (see page 15). Another possibility is that sampling for ammonium uptake experiments may have brought the culture density out of steady state if samples were

not accurate. Despite these fluctuations, most of the cultures were very close to steady state.

Cells were grown in media at pH 4.5 and 5.5 in which the initial ammonium chloride concentration was 0.8 mM. Both cultures neared steady state after 21 days or the equivalent of four hydraulic volumes of fresh media. Figure 1 shows the culture densities at pH 4.5 and 5.5 at a daily replacement rate of 0.2 for one of three separate experiments. The average culture density of the 4.5 culture was 1.26 ± 0.08 mg/ml and for pH 5.5 cultures was 1.10 ± 0.16 mg/ml. Standard deviations for each pH are 8 duplicate samples from three flasks. No ammonium was detectable in the culture medium for pH 4.5 and 5.5 cultures at the end of the 24 hour replacement cycle. The initial NH_4Cl concentration was reduced to 0.6 mM for pH 6.5 cultures due to their inability to utilize all the ammonium and thus become limited. Even so, 0.1 to 0.2 mM NH_4Cl remained in the pH 6.5 medium after 24 hours for all but the last few days of semi-continuous culture (Figure 2). The dry weight of the culture increased by about 30% and then decreased from day 26 to 31.

Stability of pH. With both potassium succinate and MES as buffers in the culture medium, the pH of each culture remained acceptably constant. The pH for all runs of 4.5, 5.5, and 6.5 cultures was 4.52 ± 0.07, 5.39 ± 0.01, and 6.28 ± 0.02 respectively. Standard deviations are 8 or more duplicate samples from 1 to 3

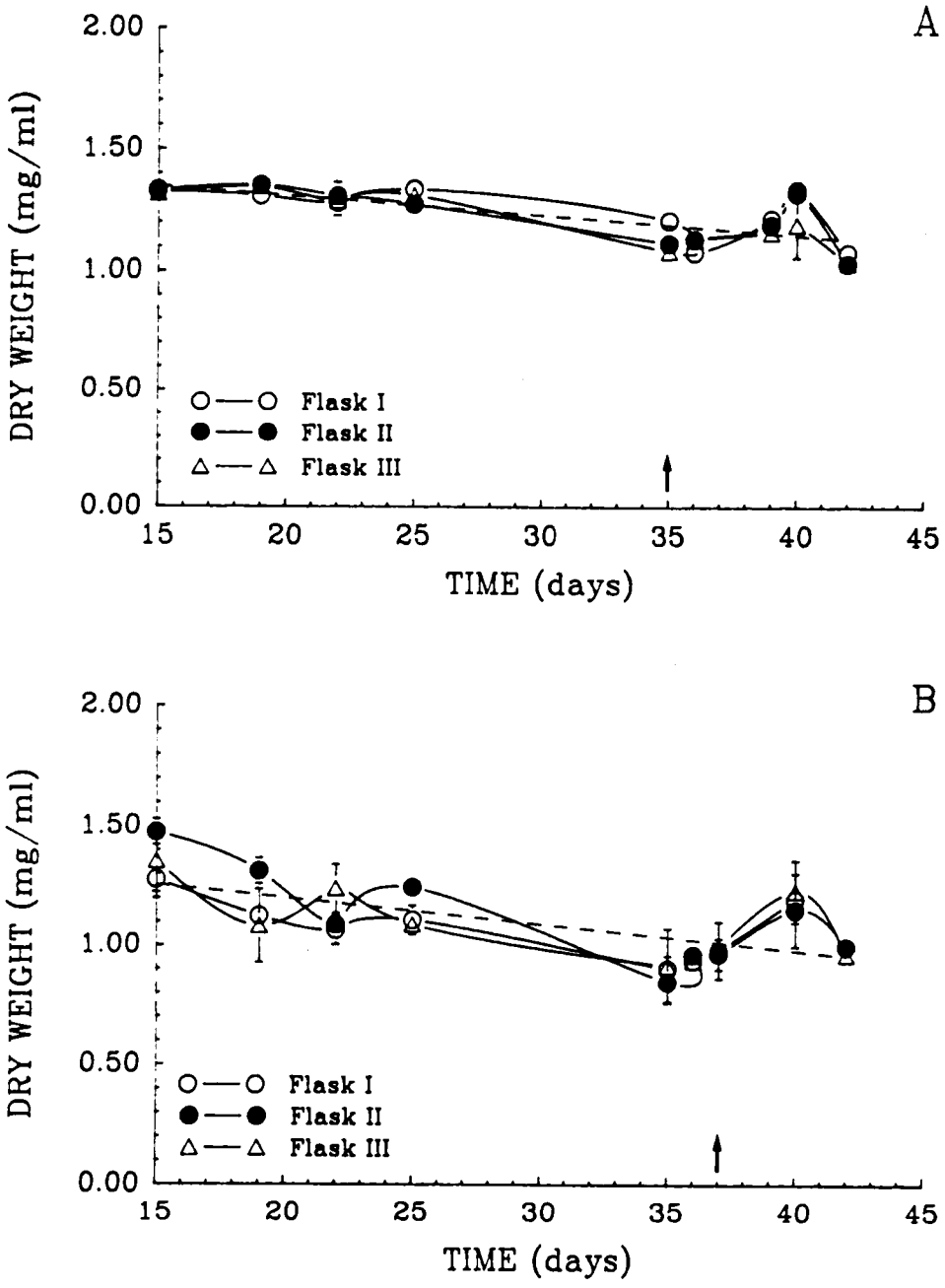


Figure 1. Culture density of ammonium limited, semi-continuous cultures at pH 4.5 and 5.5. The dashed line is the linear regression of all the data points. A--pH 4.5, B--pH 5.5. Arrow indicates time of ammonium uptake measurement.

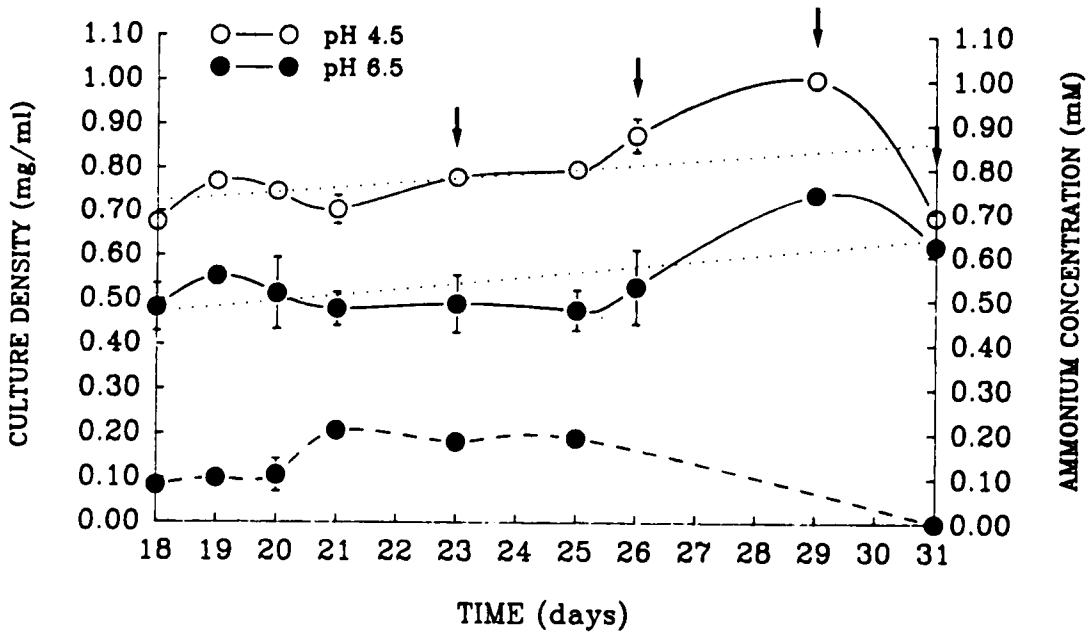


Figure 2. Culture density and ammonium concentration of ammonium limited, semi-continuous cultures at pH 4.5 and 6.5. Linear regression (dotted lines); ammonium concentration (dashed line). Arrow indicates time of ammonium uptake measurement.

cultures showed no detrimental effects on growth or ammonium uptake in batch cultures up to 40 mM, the highest concentration tested (data not shown).

Ammonium Uptake Rates of Ammonium Limited, Semi-continuous Cultures.

Figure 3 shows results from uptake experiments at pH 4.5 and 5.5 and in a separate experiment at pH 4.5 and 6.5. At all three pH's ammonium uptake appeared linear with time. The average uptake rate for pH 4.5 and 5.5 cells was 0.066±0.017 mM/hr and 0.047±0.015 mM/hr respectively. At pH 6.5, the average NH_4 uptake rate was 0.033±0.006 mM/hr versus 0.062±0.006 mM/hr for pH 4.5. When corrected for differences in culture density, the differences in uptake rate remained. Table I compares the NH_4 uptake rates for all ammonium limited experiments at pH 4.5 and 5.5. The average uptake rate for pH 4.5 and 5.5 experiments was 0.055±0.004 and 0.046±0.003 umoles/mg/hr respectively. For pH 4.5 and 6.5 experiments, the average NH_4 uptake rates were 0.075±0.017 and 0.057±0.016 umoles/mg/hr respectively (four replicates each). Although some variability existed between experiments, the difference between pH's within each experiment was repeatable. Clearly, the rate is significantly higher for pH 4.5 cells than of pH 5.5 or 6.5 cells with the difference between pH 5.5 and 6.5 cultures less appearent.

Batch Culture of Ammonium Limited, Semi-continuous Cultures.

To examine the effect of an excess of ammonium and different

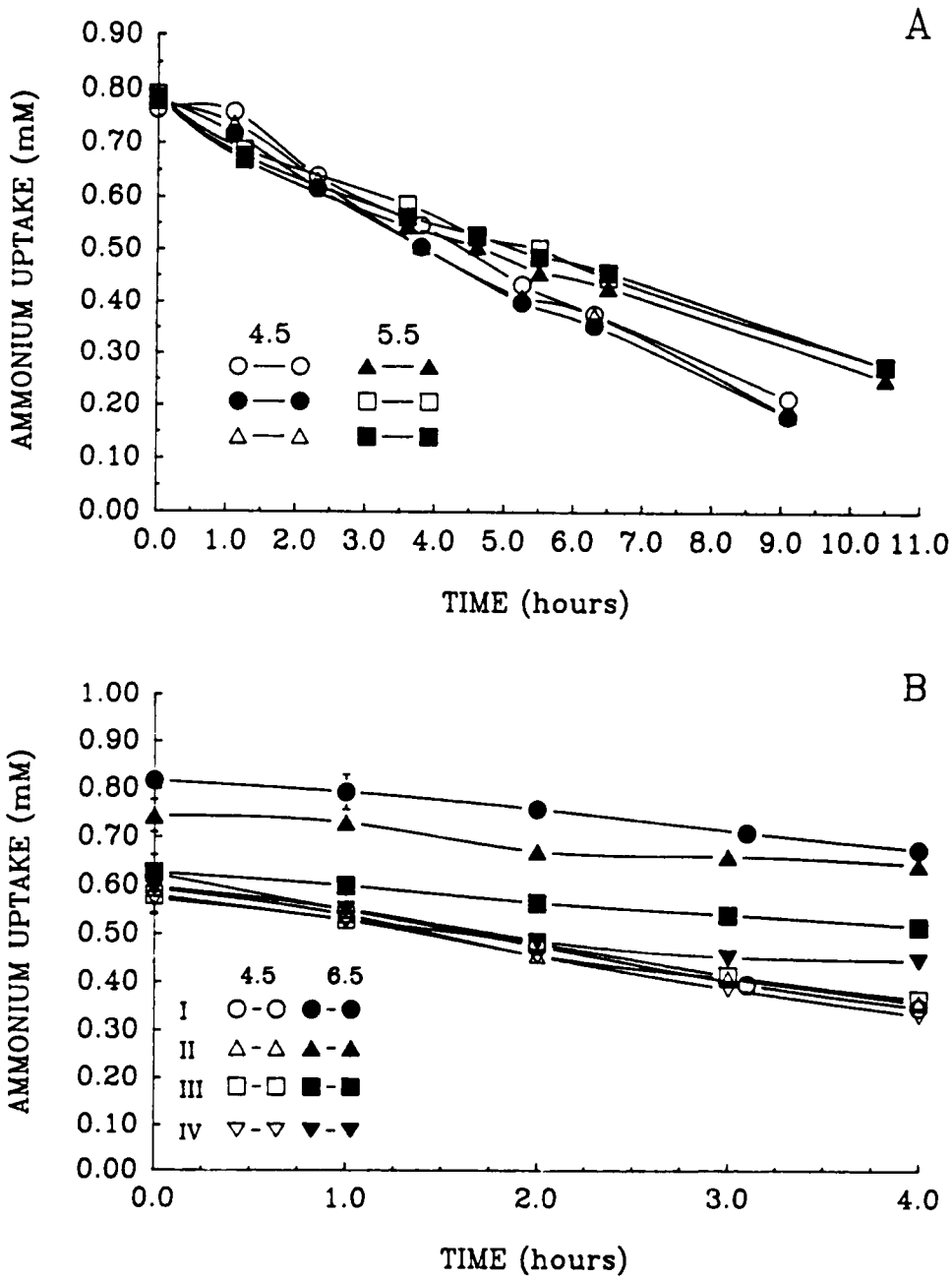


Figure 3. Ammonium uptake rates of ammonium limited, semi-continuous cultures at pH 4.5, 5.5, and 6.5. A--pH 4.5 and 5.5, B--pH 4.5 and 6.5.

Table I.
Ammonium Uptake Rates of Nitrogen Limited Semi-continuous Cultures at pH 4.5 and 5.5.

AMMONIUM UPTAKE MEASUREMENT[1]	REPLICATE FLASKS[1]	AMMONIUM UPTAKE RATE (μmoles/mg/hr)									
		SET I[1]		SET II		SET III		SET IV		SET V	
		4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5
I	1	.043	.040	.049	.050	.055	.047	.053	.050	.058	----
	2	----	----	.050	.047	.055	.044	.060	.049	.061	----
	3	----	----	----	----	----	----	.062	.046	.062	.051
II	1	.065	.056	.055	.053	.052	.045	----	----	----	----
	2	.066	.046	.049	.043	.049	.041	----	----	----	----
III	1	.053	.036	----	----	.057	.047	----	----	----	----
	2	.049	.032	----	----	.059	.042	----	----	----	----
	$\bar{x}$	.055	.042	.051	.048	.054	.044	.058	.048	.060	.051
Std. Dev.		.010	.009	.003	.004	.003	.002	.004	.002	.002	----

Summary:

	4.5	5.5
$\bar{x}$	.055*	.046*
Std. Dev.	.004	.003

*significantly different at the 1.0% level.
[1]Each Set is a separate semi-continuous culture experiment. At different times during the course of culture, 1 to 3 ammonium uptake measurements were performed with the indicated number of replicate flasks.

culture methodologies on the ammonium uptake rate, cells from semi-continuous culture were grown in batch culture. Due to the difference in size class distribution of the cells during semi-continuous culture at the three pH's, the range of deviation from the mean of dry weight samples varied considerably. For cells previously grown at pH 4.5, the range of deviation was less than 10% of the mean. Cells previously grown at pH 5.5 or 6.5 showed larger fluctuations (up to 50% of mean) in the range of deviation of dry weights. For ammonium measurements, the range of deviation of replicate samples was less than 5% of the mean at all pH's.

The growth and NH_4 uptake from two separate experiments are shown in Figures 4-6. Cells which had previously grown semi-continuously in pH 4.5 medium were substantially different in growth and NH_4 uptake rates from pH 5.5 and 6.5 cells despite growing in batch culture in pH 4.5, 5.5, or 6.5 medium. Within a cell type (i.e., cells which had previously been cultured semi-continuously at one of the three pH's) little difference in growth and ammonium uptake rate occured between the three different pH's in which the cells were grown in batch culture. Because the growth and uptake curves appeared to be logrithmic with time, each was plotted semi-logrithmically. The calculated slopes from these plots yielded a more accurate average growth and uptake rate, according to the correlation coefficients which were 0.997 ± 0.003 for logrithmic plots and 0.966 ± 0.034 for linear plots (16 determinations each). From the equation, $\ln \cdot W_t/W_o = kt$ (W_t and W_o are dry weight or ammonium

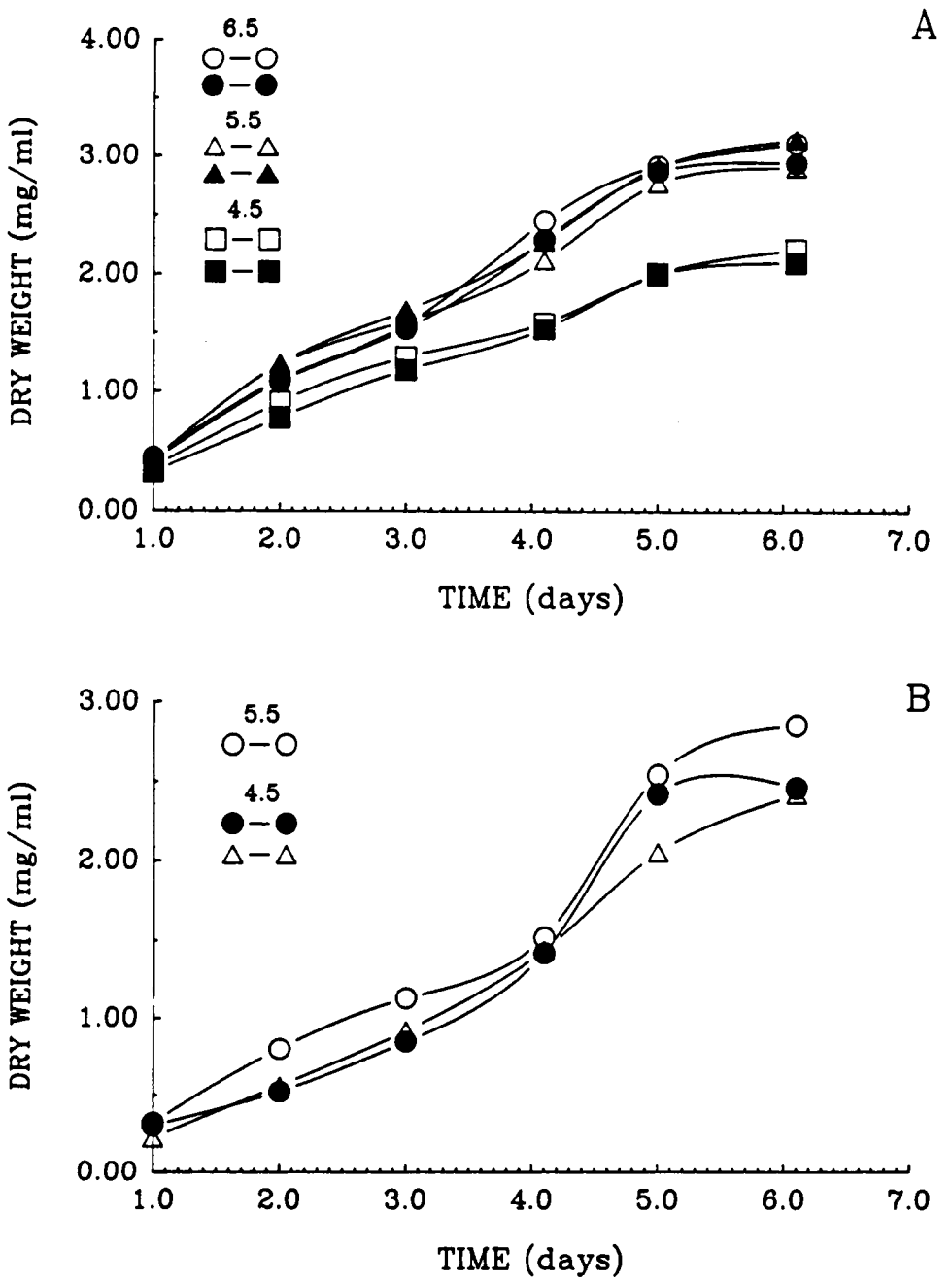


Figure 4. Growth of batch cultures at pH 4.5, 5.5, and 6.5 innoculated with tissue from ammonium limited, semi-continuous cultures. A--tissue from semi-continuous cultures at pH 4.5. B--tissue from semi-continuous cultures at pH 5.5.

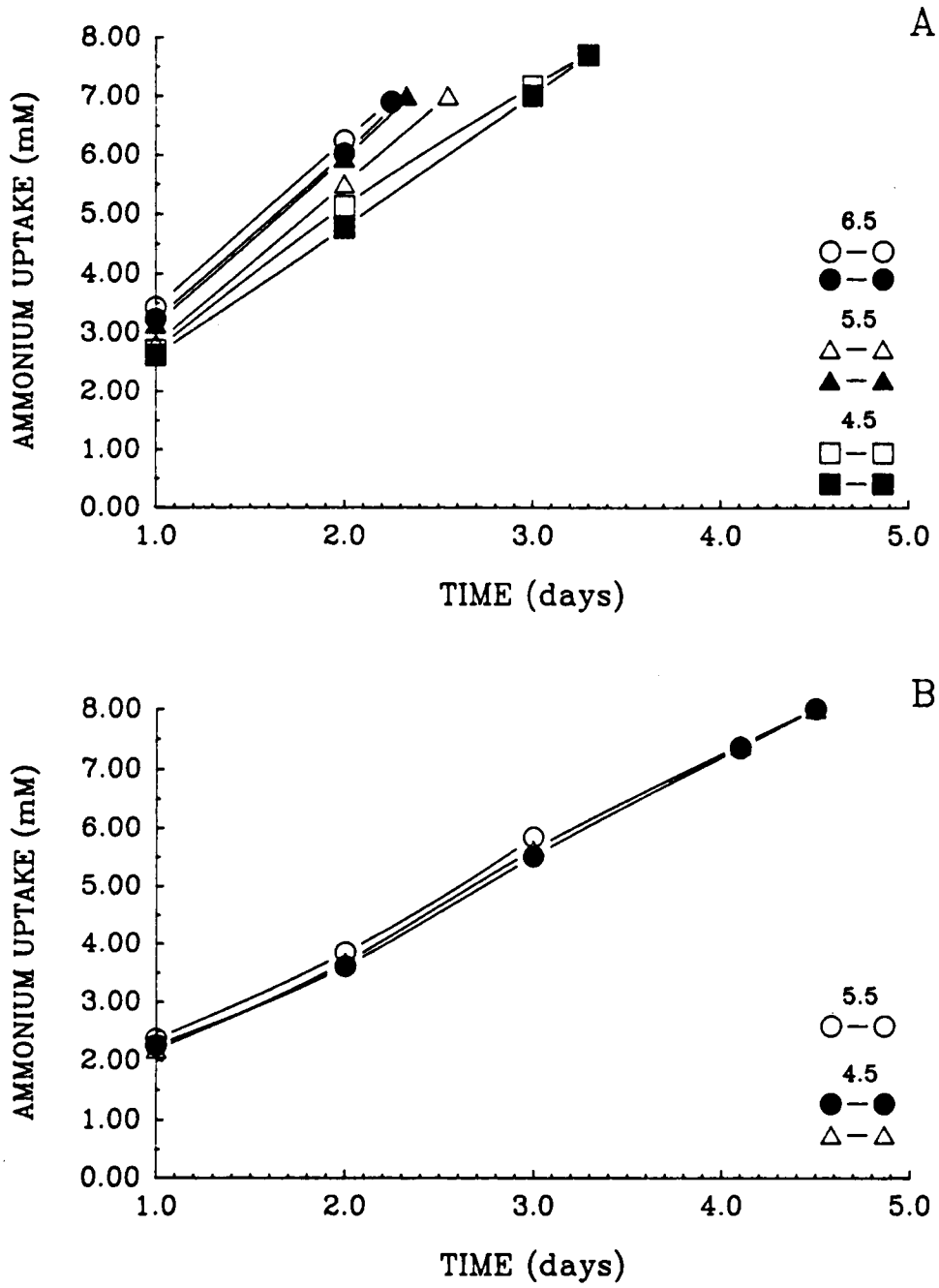


Figure 5. Ammonium uptake of batch cultures at pH 4.5, 5.5, and 6.5 innoculated with tissue from ammonium limited, semi-continuous cultures. A--tissue from semi-continuous cultures at pH 4.5. B--tissue from semi-continuous cultures at pH 5.5.

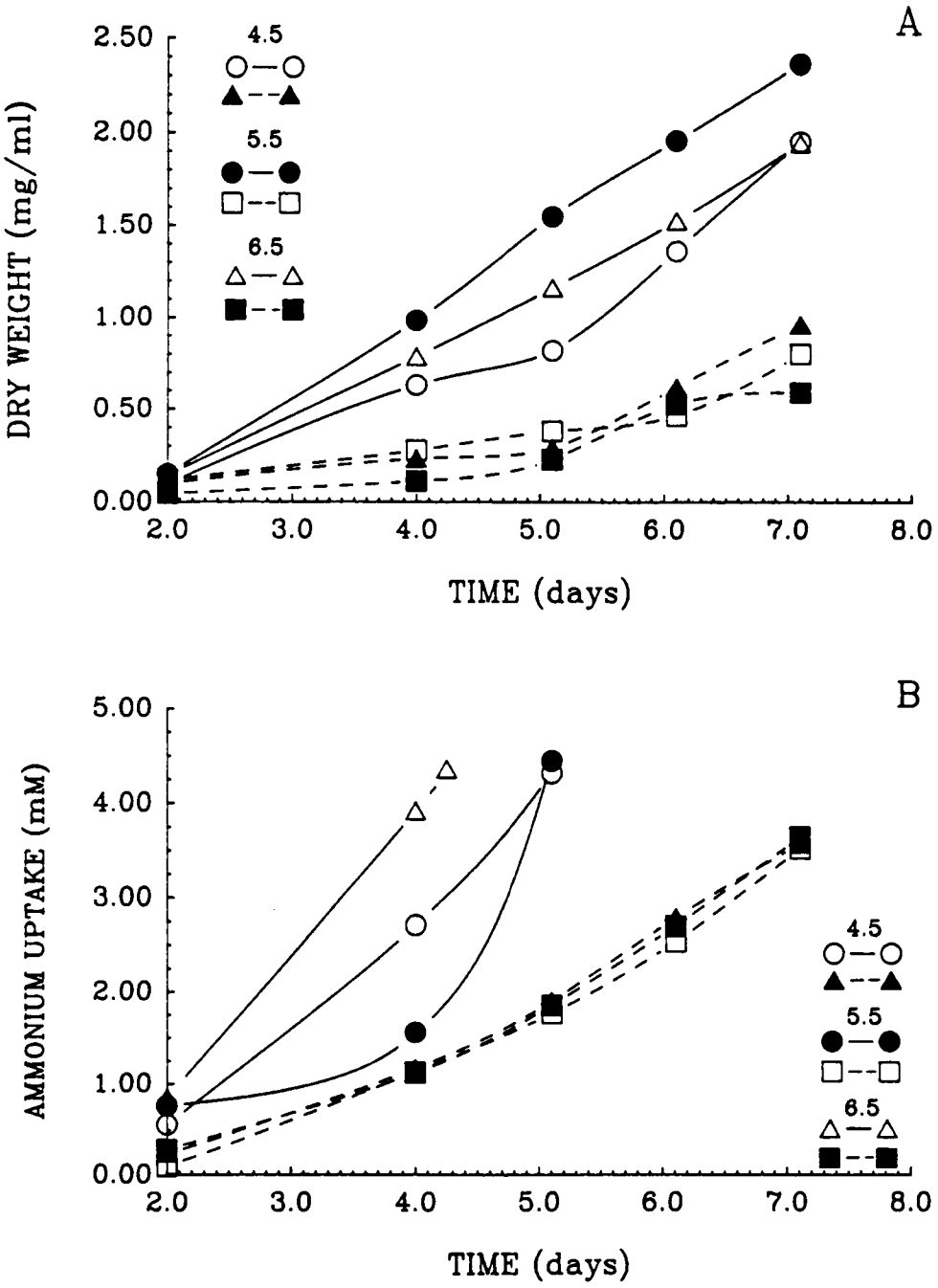


Figure 6. Growth and ammonium uptake of batch culture at pH 4.5, 5.5, and 6.5 innoculated with tissue from ammonium limited, semi-continuous cultures. Solid line--tissue from semi-continuous cultures at pH 4.5, dashed line--tissue from semi-continuous cultures at pH 6.5.

concentration at time t and time zero repectively, k is a constant or the specific growth rate, and t is a point in time during culture), a rate, with units day^{-1}, was calculated for both growth or ammonium uptake. When NH_4 uptake rate was divided by growth rate, the unitless ratio describing the logrithmic ammonium uptake rate on a dry weight basis for batch culture was obtained. In addition, this ratio was indicative of the composition of the cells during batch culture. Those cells which had a ratio greater than 1 were taking up ammonium proportionately faster than they were growing in biomass. Likewise, those cells whose ratio was less than 1 were growing proportionately faster than they were taking up ammonium. Table II provides a summary of ammonium uptake rates and growth rates in batch culture.

For pH 4.5 and 5.5 cells grown at three pH's (Figures 7 and 8), uptake rate/dry weight growth rate for NH_4 was 1.67 ± 0.16 (6 determinations) and 0.96 ± 0.11 (3 determinations) respectively. For pH 4.5 and 6.5 cells grown at 3 pH's (Figures 9 and 10), the uptake rate/growth rates were 1.21 ± 0.22 and 0.93 ± 0.11 (3 determinations at each pH).

Though little effect of medium pH in batch culture on growth and uptake rate could be seen, a large effect of previous culture pH occures. From the above data, the uptake rate of pH 4.5 cells was 74% higher than for pH 5.5 cells. Between pH 4.5 and 6.5 cells, a 30% difference occured. Thus, previous culture history has a profound effect on subsequent culture behavior.

TABLE II.

Ammonium Uptake and Growth Rates in Batch Culture and Ratio of These Two for Nitrogen and Phosphate Limited Cells at Three pH's.

pH In Semi-continuous Culture	pH In Batch Culture: 4.5 NH4 Uptake Rate $days^{-1}$	4.5 Growth Rate $days^{-1}$	4.5 Ratio	5.5 NH4 Uptake Rate $days^{-1}$	5.5 Growth Rate $days^{-1}$	5.5 Ratio	6.5 NH4 Uptake Rate $days^{-1}$	6.5 Growth Rate $days^{-1}$	6.5 Ratio	$\bar{X}\pm SD$[4]
NITROGEN LIMITED										
4.5	0.67	0.57	1.17	0.67	0.35	1.91	0.60	0.36	1.67	
	0.49	0.32	1.53	0.64	0.36	1.73	0.62	0.36	1.72	$1.52\pm0.29^{2,3}$
	0.49	0.34	1.44	0.54	0.54	1.00	0.74	0.51	1.45	
5.5	0.40	0.41	0.97	0.45	0.41	1.10				
	0.41	0.46	0.89							$0.99\pm0.11^{1,2}$
6.5	0.36	0.44	0.82	0.38	0.36	1.05	0.50	0.54	0.92	$0.93\pm0.12^{1,3}$
PHOSPHATE LIMITED										
4.5	0.67	0.72	0.93	0.67	0.75	0.89	0.65	0.63	1.03	
	0.85	0.53	1.60	0.91	0.60	1.52				1.23 ± 0.32^{2}
	0.90	0.62	1.45							
5.5	0.63	0.68	0.92							0.92
6.5	0.65	0.70	0.93	0.46	0.72	0.64	0.48	0.70	0.68	$0.75\pm0.18^{1,2}$
$\bar{X}\pm SD$	0.59 ± 0.18	0.52 ± 0.14	1.15 ± 0.30	0.59 ± 0.17	0.51 ± 0.16	1.23 ± 0.45	0.60 ± 0.10	0.52 ± 0.14	1.24 ± 0.43	

[1] Range of deviation from the mean.
[2] Significantly different at the 2.0% level.
[3] Significantly different at the 1.0% level.
[4] $\bar{X}\pm SD$ of Ratios only.

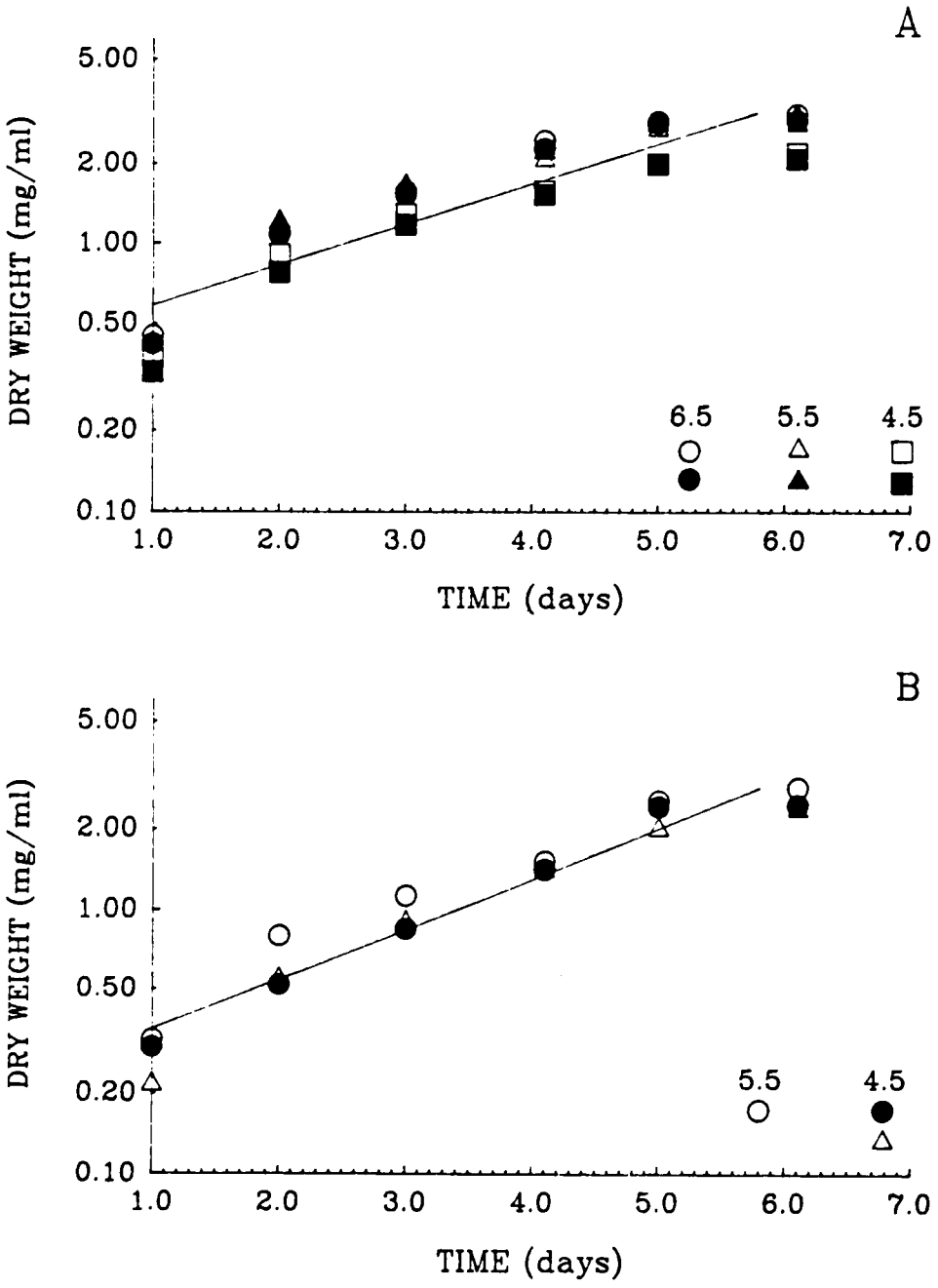


Figure 7. Semi-log plot of dry weight of figure 4 data. A--cells previously grown at pH 4.5. B--cells previously grown at pH 5.5.

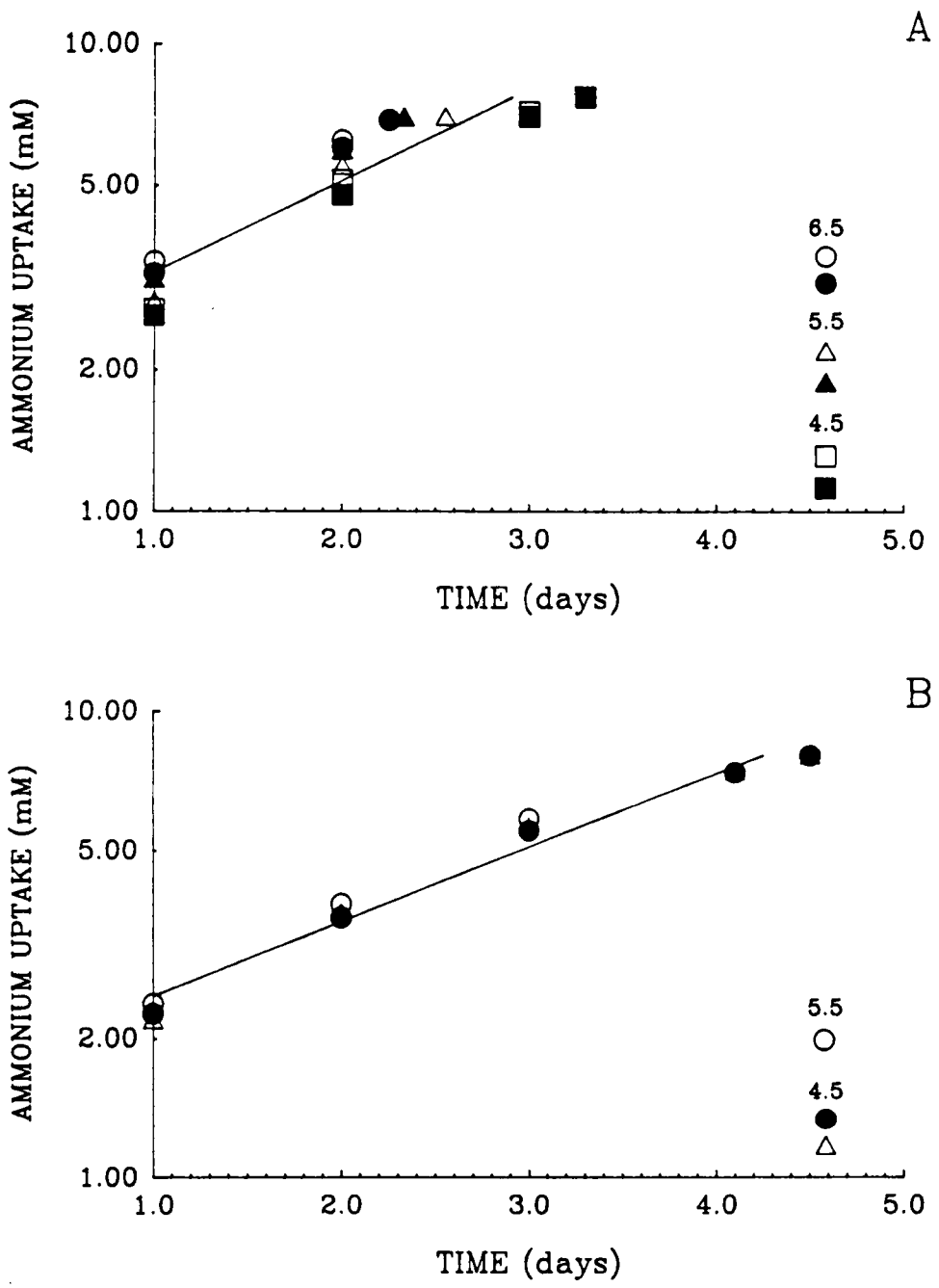


Figure 8. Semi-log plot of ammonium uptake of figure 5 data. A--cells previously grown at pH 4.5. B--cells previously grown at pH 5.5.

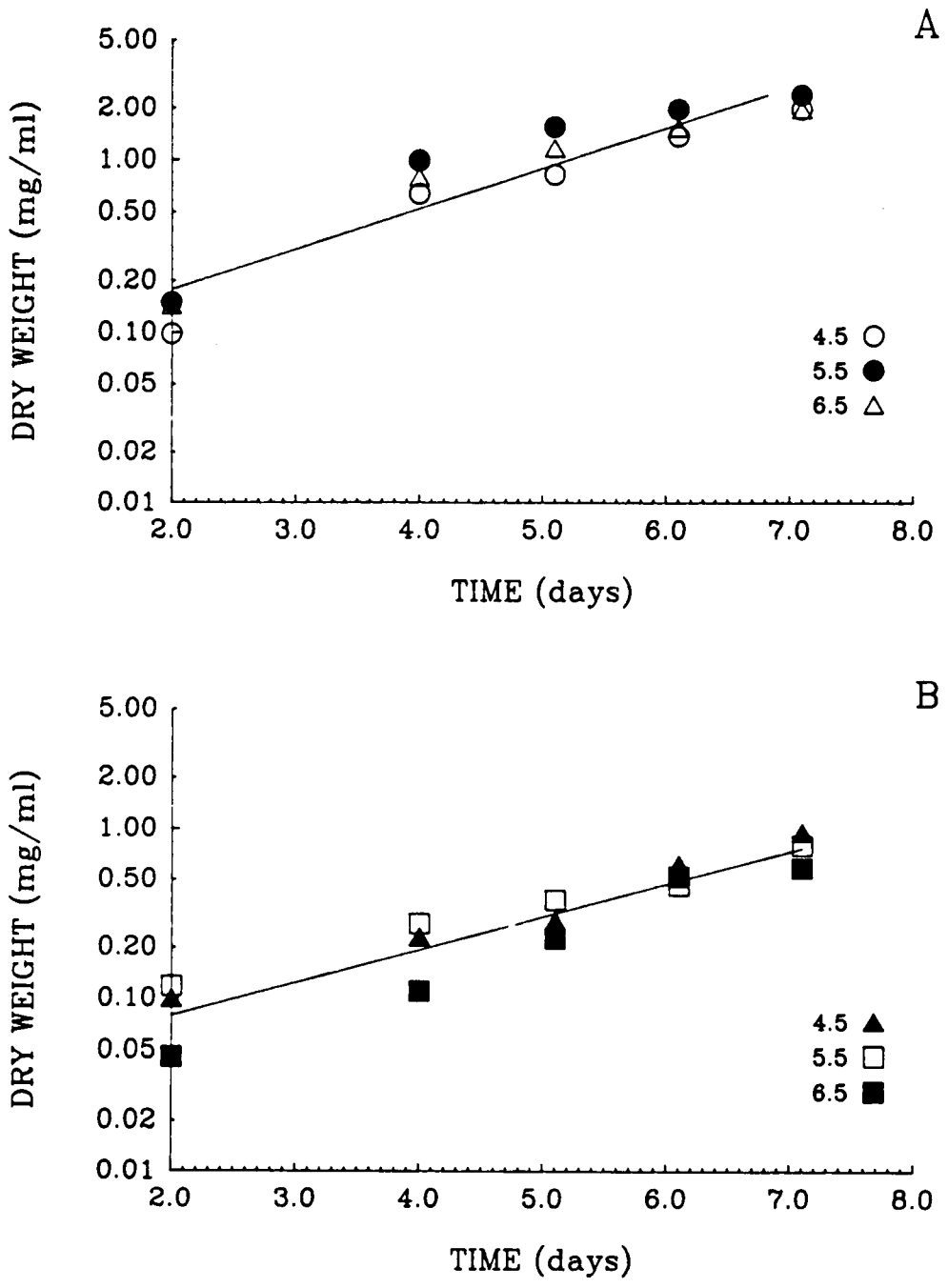


Figure 9. Semi-log plot of dry weight of figure 6B data. A--cells previously grown at pH 4.5. B--cells previously grown at pH 6.5.

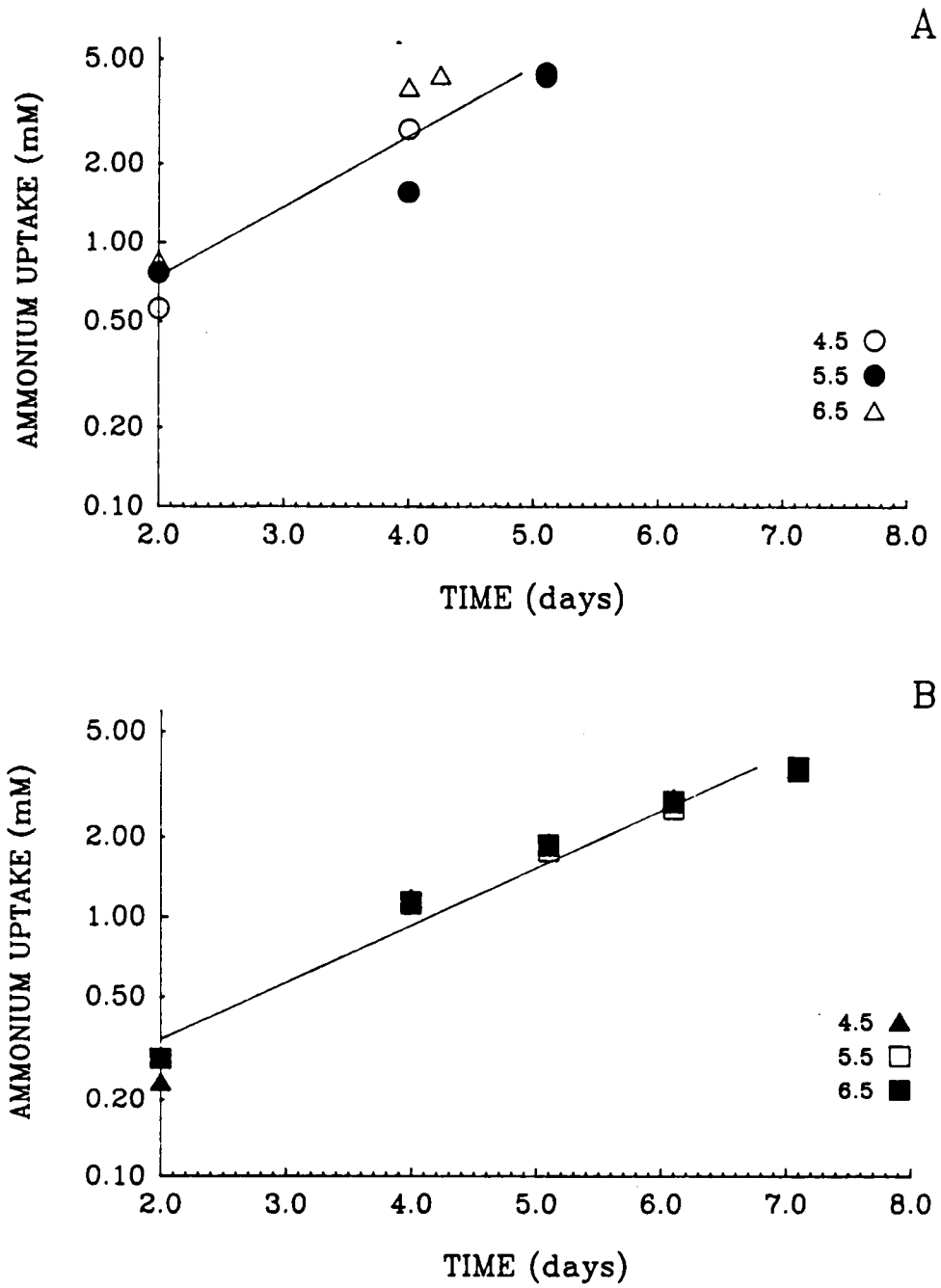


Figure 10. Semi-log plot of ammonium uptake data of figure 6B. A--cells previously grown at pH 4.5. B--cells previously grown at pH 6.5.

Phosphate Limited, Semi-continuous Culture.

To test whether or not the condition of nitrogen limitation had any effect on the ammonium uptake rates observed in the nitrogen limited experiments, cells were grown semi-continuously under phosphate limited conditions. The normal concentration of phosphate in WCM-4 (0.5 mM) was lowered to 0.2 mM. This concentration had previously been shown to be limiting in semi-continuous carrot cell cultures (Dougall et al. 1983).

Two experiments were run, one at pH 4.5 and 5.5, and the other at pH 4.5 and 6.5. In the first experiment, the total run time was 22 days with the culture nearing steady state about day 14 or 15 (Figure 11). The average culture density was 0.857±0.09 mg/ml for pH 4.5 (average of duplicate samples from each of three replicates) and 0.968±0.16 mg/ml for pH 5.5 (duplicate samples from a single flask). In the second experiment, total run time was 29 days with culture nearing steady state for pH 6.5 about day 15 (Figure 12). The average culture density was 0.517±0.065 mg/ml for pH 6.5 (duplicate samples from each of two replicate flasks) and 0.665±0.17 mg/ml for pH 4.5 (duplicate samples from a single flask). The pH 4.5 culture appeared not to be at steady state.

Ammonium Uptake Experiments. The ammonium uptake experiments were performed as described earlier in nitrogen limited semi-continuous culture.

pH 5.5 cells took up ammonium faster than pH 4.5 cells by as

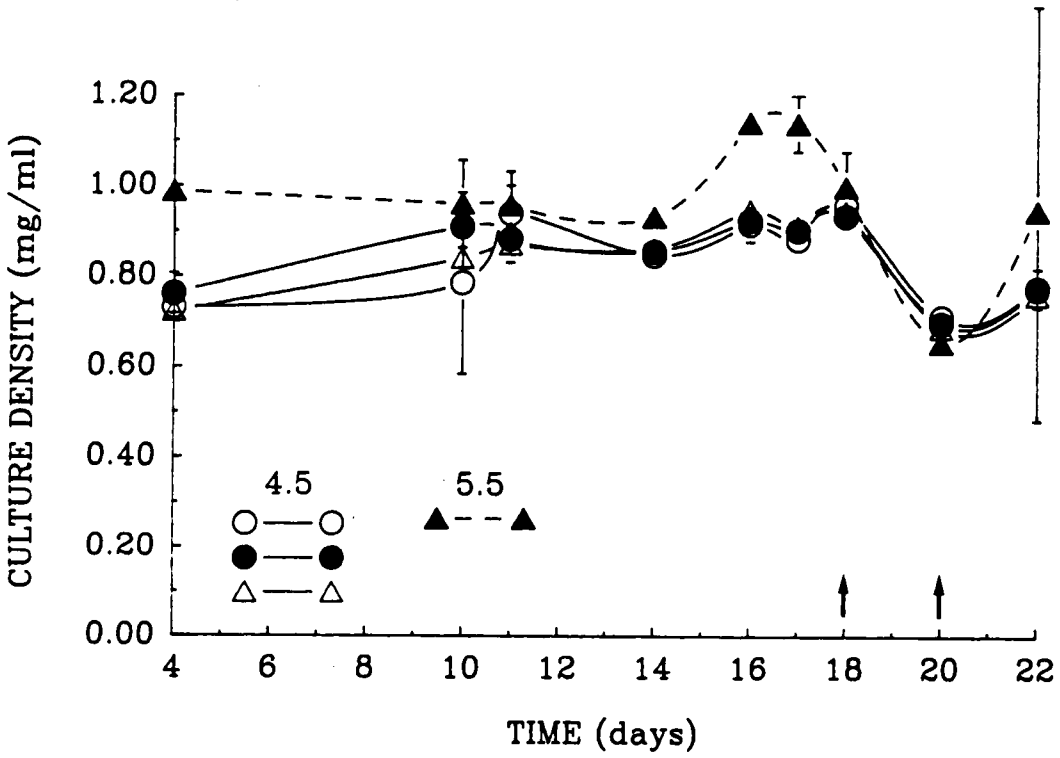


Figure 11. Culture density of phosphate limited, semi-continuous cultures at pH 4.5 and 5.5. Each data point represents duplicate samples. Arrows indicate time of ammonium uptake measurement.

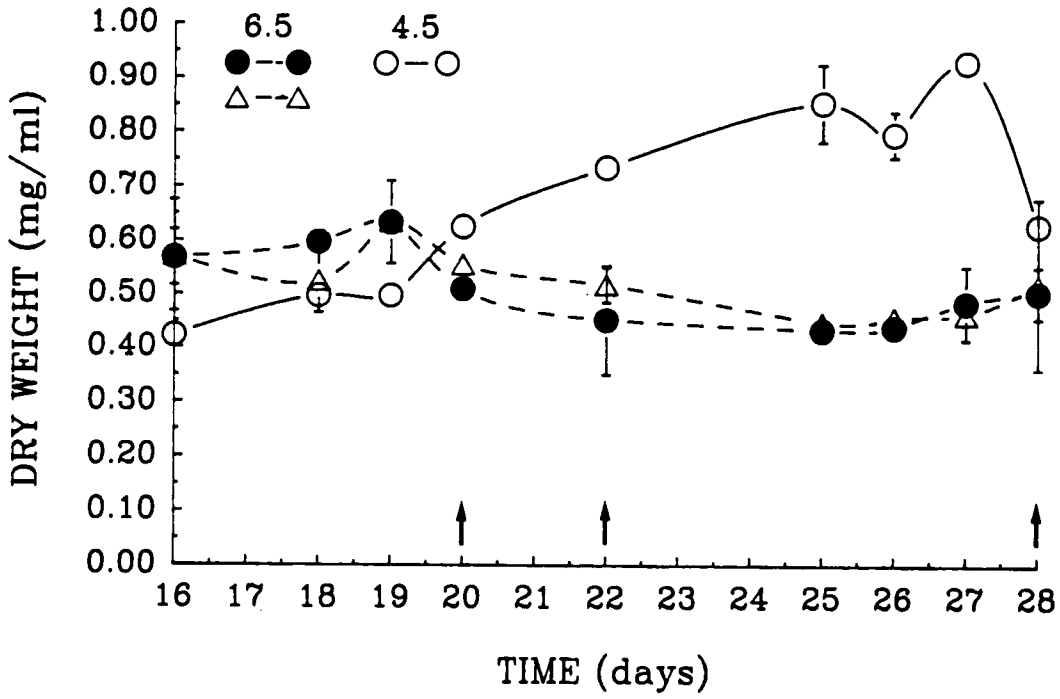


Figure 12. Culture density of phosphate limited, semi-continuous cultures at pH 4.5 and 6.5. Each data point represents duplicate samples. Arrows indicate time of ammonium uptake measurement.

much as 27%, while pH 4.5 cells showed a 45% greater uptake rate than pH 6.5 cells (Table III). Although only one culture was available for pH 5.5 experiments, the difference in uptake rate from pH 4.5 remained. In pH 6.5 cultures, the rate of uptake of ammonium was considerably less than that of pH 4.5. The marked difference between uptake rates at pH 6.5 from the four separate experiments is difficult to explain. Perhaps, the cellular aggregation exhibited at the higher pH's caused dry weight samples to fluctuate so the calculated ammonium uptake rates likewise fluctuated. Some of the raw data suggests this may be true. In any case, the uptake rate for NH_4 in phosphate limited cultures was highest at pH 5.5, approximately 20% less at pH 4.5 and lower still at pH 6.5.

In nitrogen limited cultures, pH 4.5 showed the greatest ammonium uptake rate. In phosphate limited cultures, pH 5.5 showed the highest rate. The results suggest a different effect of pH on the ammonium uptake rate with NH_4 or PO_4 as the limiting nutrient.

Batch Culture of Phosphate Limited, Semi-continuous Cultures.

After semi-continuous, phosphate limited growth, cells in batch culture showed the same phenomena as did the nitrogen limited cultures (see Figures 13 and 14). When curves for growth and ammonium uptake were plotted semi-logrithmically, the uptake rate/dry weight growth rates for cells previously grown at pH 4.5 and pH 5.5 were 1.52 ± 0.07 (duplicate samples from three replicates) and 0.92 ± 0.0 (duplicate samples from a single flask) respectively

Table III.
Ammonium Uptake Rates of Phosphate Limited, Semi-continuous Cultures at pH 4.5, 5.5, and 6.5.

Experiments	Replicate flasks	AMMONIUM UPTAKE RATE (μmoles/mg/hr)			
		4.5 vs. 5.5		4.5 vs. 6.5	
I	1	.035	.042	.053	.006
	2	.038	----	----	.034
	3	.036	----	----	----
II	1	.043	.078	.054	.016
	2	.058	----	----	.041
	3	.054	----	----	----
	$\bar{x}$	.044	.060	.053	.024
Std. Dev.		.009	.018[1]	.001[1]	.016

[1] range of deviation from the mean.

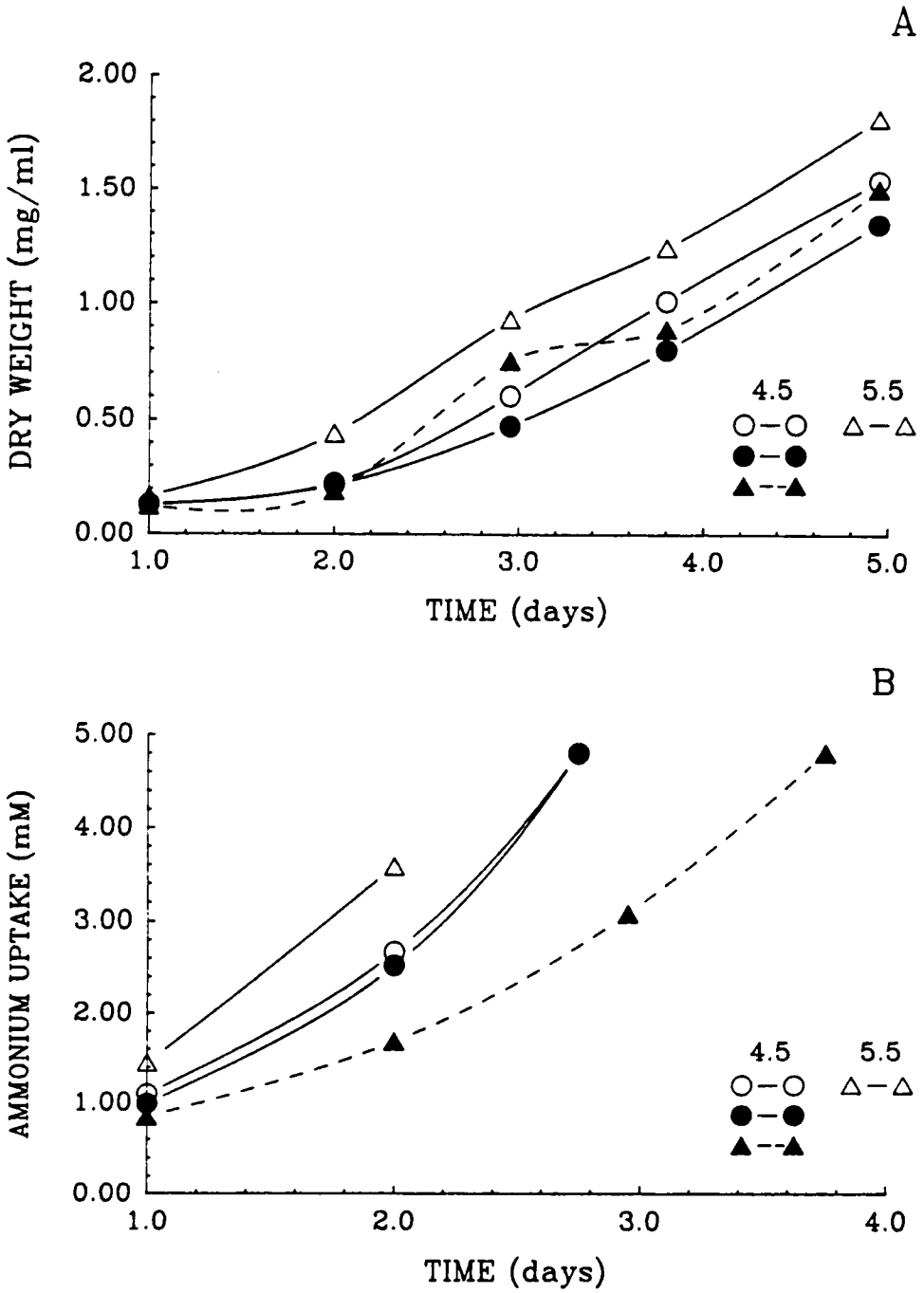


Figure 13. Dry weight and ammonium uptake in batch culture at pH 4.5 and 5.5 innoculated with tissue from phosphate limited, semi-continuous cultures. Solid lines--tissue from semi-continuous cultures at pH 4.5. Dashed lines--tissue from semi-continuous cultures at pH 5.5.

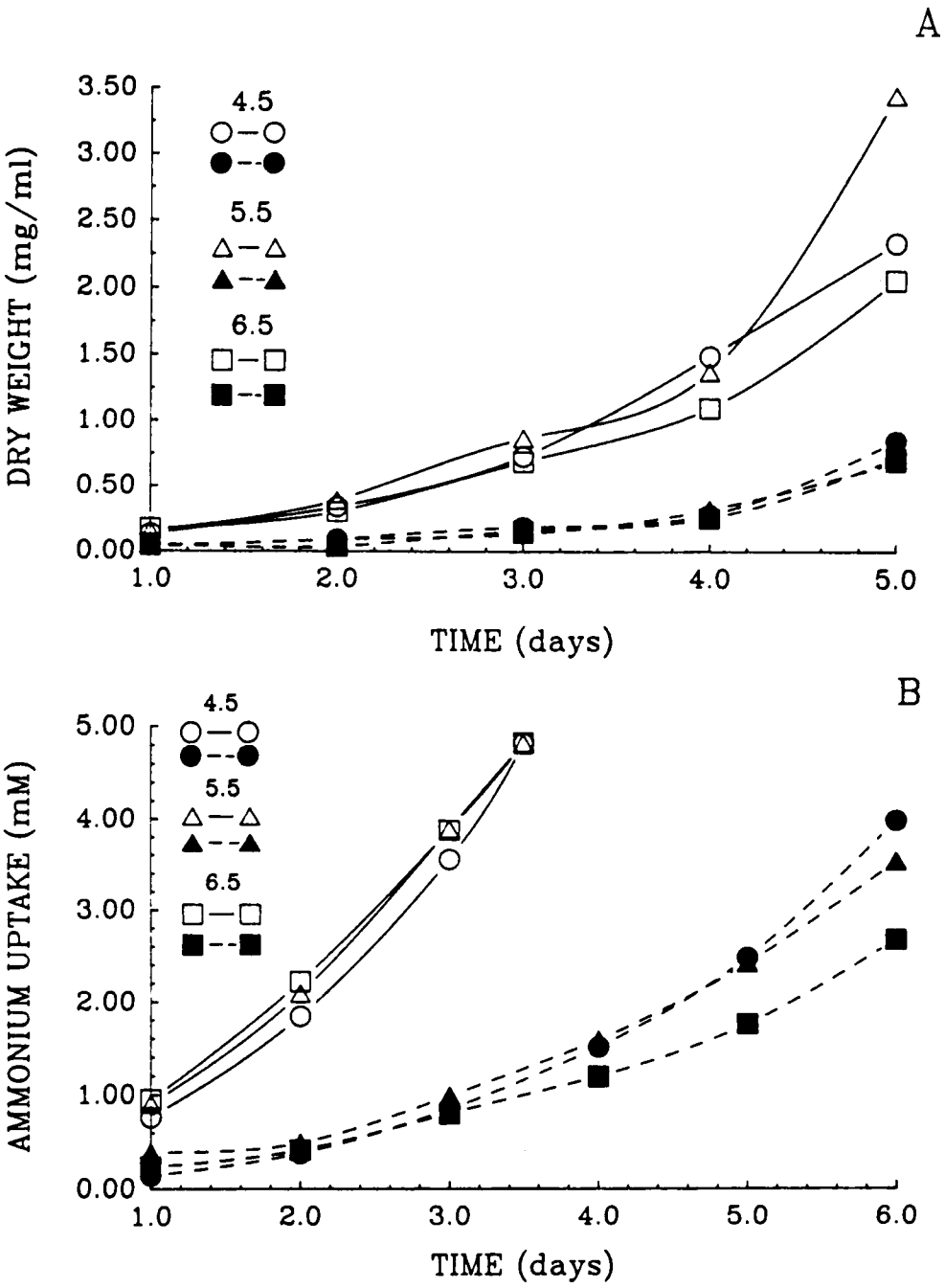


Figure 14. Dry weight and ammonium uptake in batch cultures at pH 4.5, 5.5, and 6.5 innoculated with tissue from phosphate limited, semi-continuous cultures. Solid lines--tissue from semi-continuous cultures at pH 4.5. Dashed lines--tissue from semi-continuous cultures at pH 6.5.

(Figure 15). In a separate experiment, uptake rate/dry weight growth rate was 0.95±0.07 and 0.75±0.15 for cells previously cultured in pH 4.5 and 6.5 medium respectively (Figure 16). Again, these numbers are unitless but indicate that pH 4.5 cells were utilizing ammonium faster on a dry weight basis than pH 5.5 or pH 6.5 cells regardless of what pH at which they were currently growing. Thus, in batch culture, ammonium uptake rate was higher with cells previously grown at pH 4.5 than at pH 5.5 or pH 6.5 while the relationship between pH 5.5 and 6.5 remained unclear.

In phosphate limited semi-continuous culture, the ammonium uptake rate was higher at pH 5.5 than pH 4.5. In subsequent batch culture this trend reversed. The reversal in ammonium uptake rates verses pH in phosphate limited cultures is difficult to explain in relation to nitrogen limited cultures where the higher rates occur at the lower pH in both semi-continuous and in subsequent batch cultures.

Aggregate Size Distribution Experiments.

During the course of nitrogen limited, semi-continuous culture the morphology of the aggregrates changed depending on pH at which the cells were growing. Though no screening experiments were conducted, aggregate distribution in phosphate limited cultures appeared to be similar to nitrogen limited cultures. At pH 4.5 the cell suspension was fine and dispersed. At pH 5.5 or 6.5 the cells increased in aggregrate size. To measure the extent of this

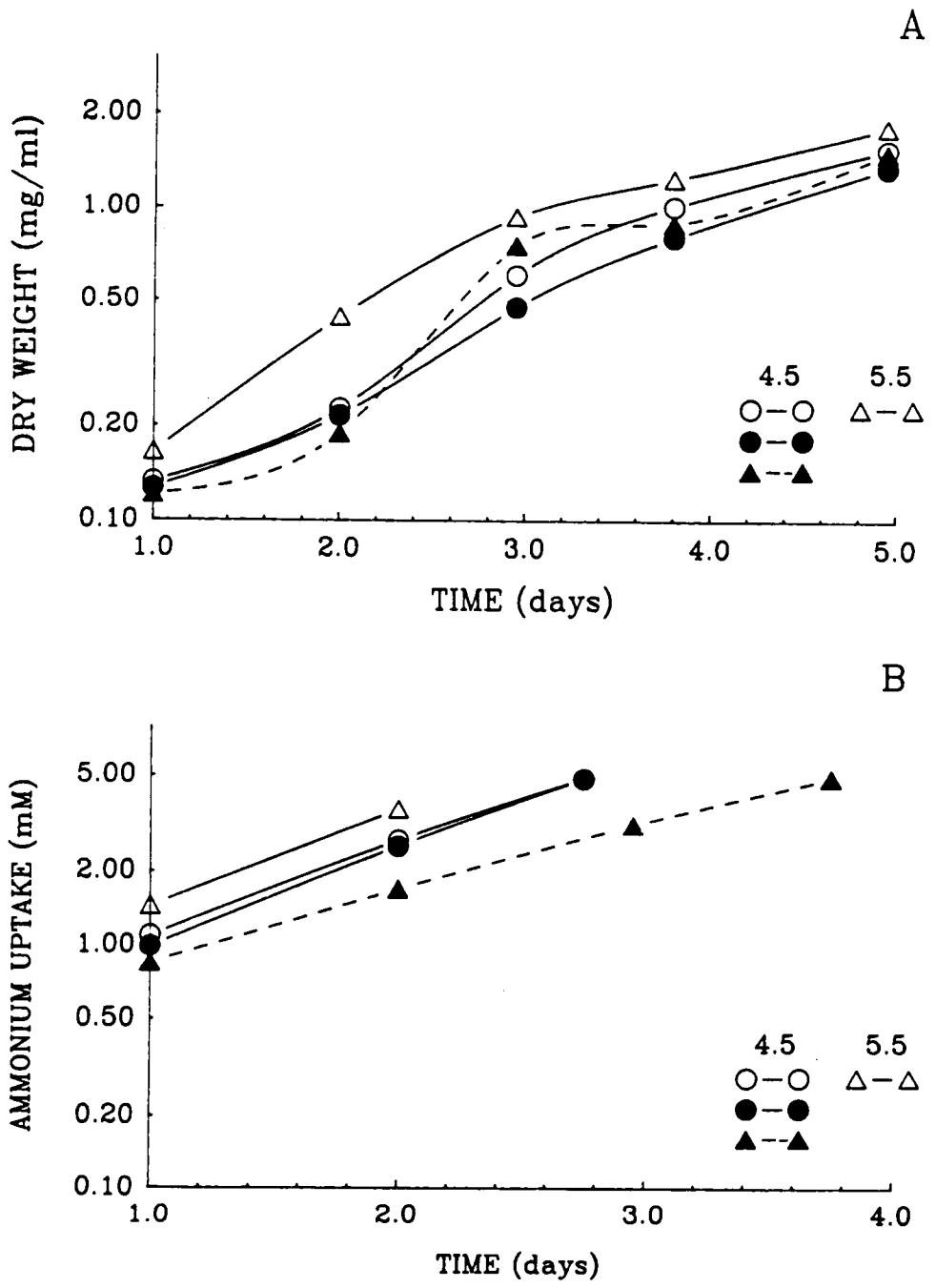


Figure 15. Semi-log plots of dry weight and ammonium uptake of figure 13 data. Solid and dashed lines same as Figure 14.

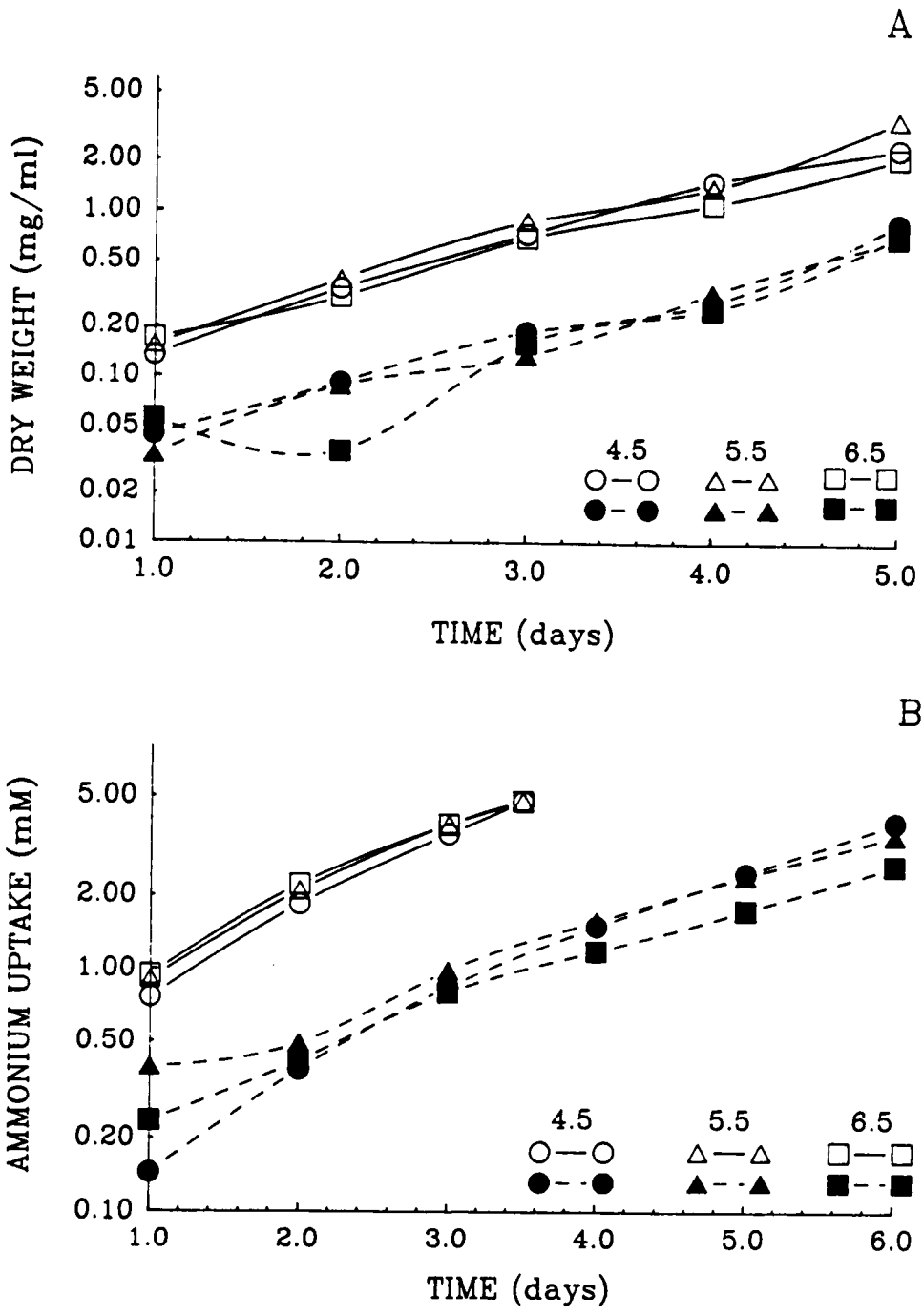


Figure 16. Semi-log plots of dry weight and ammonium uptake of figure 14 data. Solid and dashed lines same as Figure 14.

increase, samples from cultures were screened into various size classes and the percentage of each size class in relation to total dry weight of the sample was calculated. Three such experiments were done. Figure 17 shows the aggregate size distribution of tissue typically used to inoculate the semi-continuous cultures. 70.4±5.0% of the culture was composed of aggregates 210 microns or greater, the remainder, 210 microns or less. Figure 18 shows a screening of cells grown semi-continuously at pH 4.5 and 5.5 under nitrogen limited conditions for 40 days. Of the percent total dry weight, pH 4.5 showed 32.0±5.0% 310 microns or greater, 53.0±4.0% 310 to 105 microns, and 15.0±2.0% less than 105 microns. At pH 5.5, 61.0±2.1% were 310 microns or greater, 22.0±1.5% 310 to 105 microns, and 16.5±0.06% less than 105 microns. In the last experiment (Figure 19) 7-10 day old semi-continuous cultures were screened. At pH 4.5, 27.8±5.2% of the aggregates were greater than 310 microns, 49.6±2.9% were between 310 and 105, and 22.6±2.9% were less than 105 microns. At pH 5.5, 89.3±0.8% of the culture comprised aggregates greater than 310 microns, 8.8±0.5% between 310 and 105 microns, and 2.0±0.3% less than 105 microns. Finally, 74.6±8.5% of the culture at pH 6.5 was larger than 310 microns, 18.5±6.6% between 310 and 105 microns, and 6.9±1.9% below 105 microns.

Clearly, as the cultures progress, the cells aggregate or disaggregate according to the pH. Starting with an inoculum, whose compostion was roughly 75% clumps greater than 310 microns, the percentage of aggregates greater than 310 microns would either

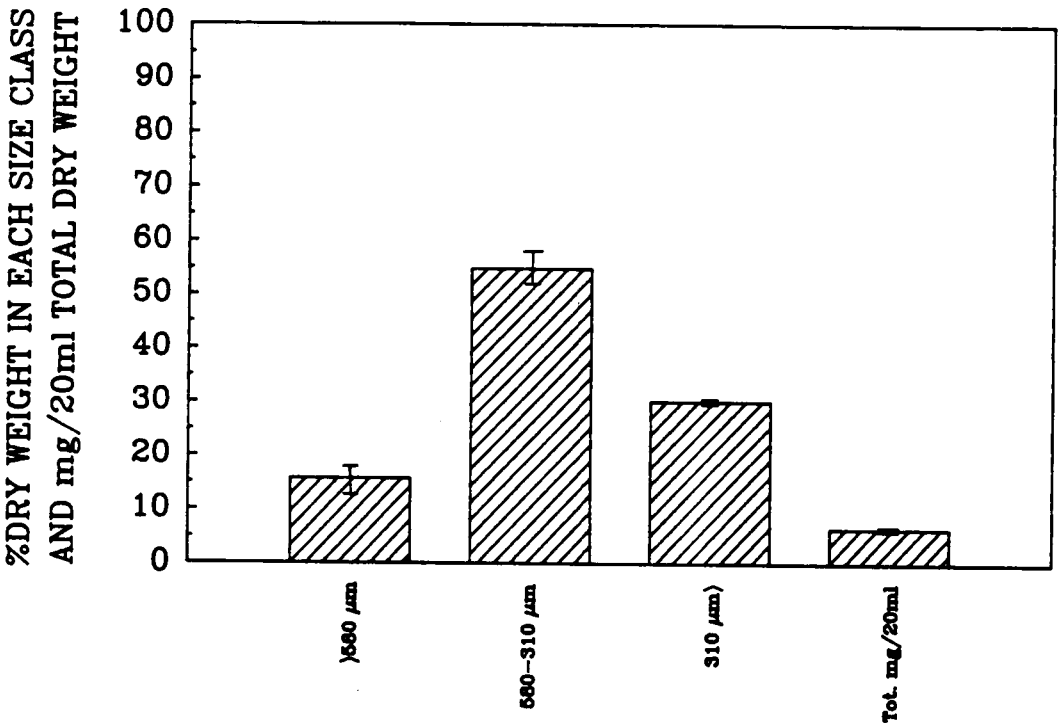


Figure 17. Aggregate size distribution of a stock culture. Data given as mean and standard deviation of samples from three flasks.

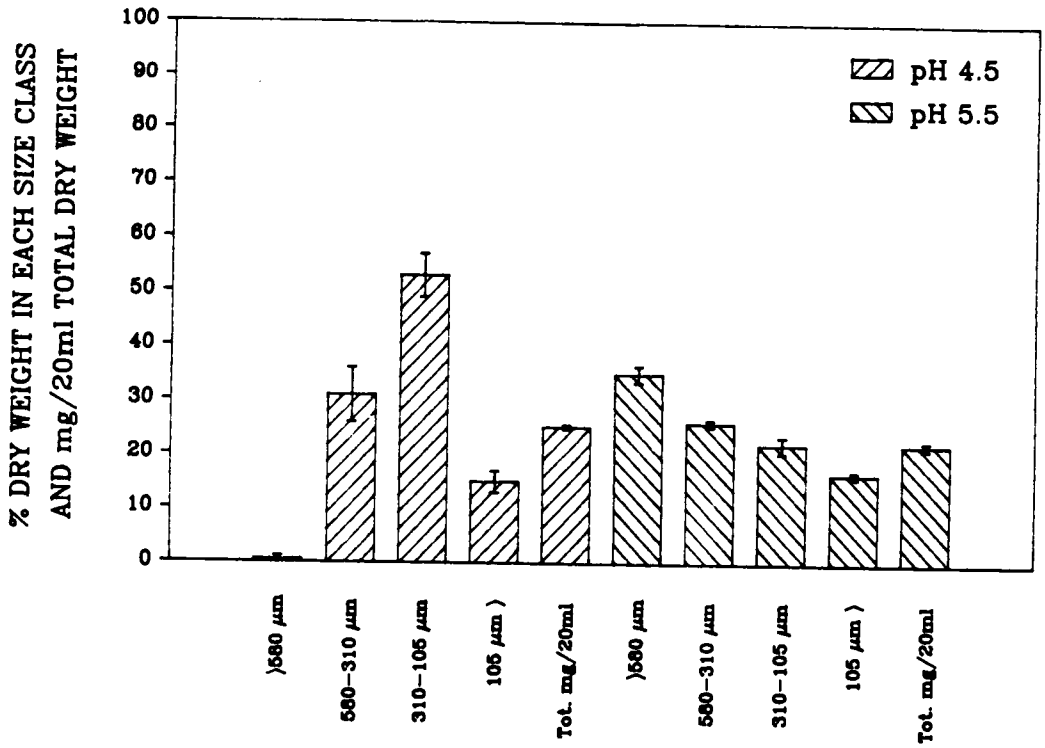


Figure 18. Aggregate size distribution of a nitrogen limited, semi-continuous culture at pH 4.5 and 5.5. Data given as mean and standard deviation of samples of 2 flasks.

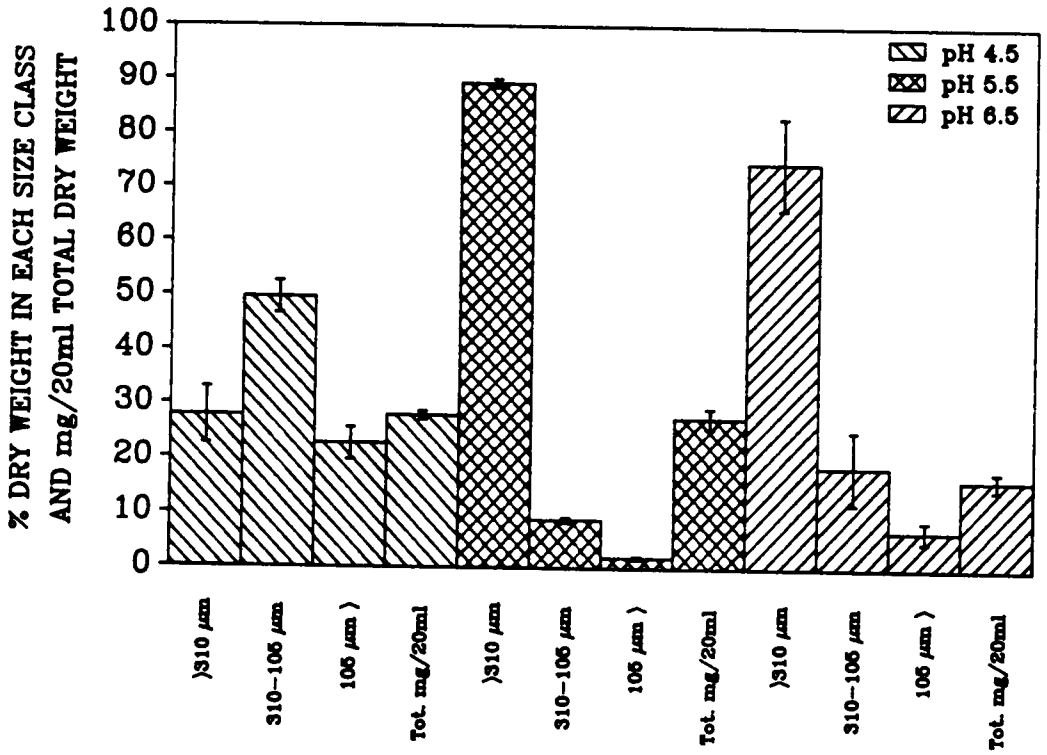


Figure 19. Aggregate size distributions of nitrogen limited, semi-continuous culture at pH 4.5, 5.5, and 6.5. Data given as mean and standard deviation of samples from three flasks except for pH 6.5 where the mean and the range of samples from duplicate flasks is given.

increase at pH 5.5 and 6.5 or decrease dramatically until 71.0% of the aggregates were less than 310 microns at pH 4.5. These data suggest a significant effect of pH on carrot cell culture morphology.

IV. DISCUSSION

From semi-continuous and batch culture experiments, it is clear that pH effected ammonium uptake in carrot suspension cultures. Nitrogen limited pH 4.5 semi-continuous cultures showed a higher uptake rate than of pH 5.5 or 6.5 cultures. In phosphate limited cultures, pH 5.5 showed the highest uptake rate, although this result may be questionable. Table IV summarizes the ammonium uptake rates in both semi-continuous and batch culture. Batch culture at three pH's of semi-continuously grown cells showed little difference in ammonium uptake. However, large uptake differences existed between cells previously cultured at different pH's. pH also effected cell morphology. Cells grown in pH 5.5 or 6.5 medium aggregated while cells grown in pH 4.5 medium were dispersed.

Some, but not all, of the results confirm experiments with Ipomoea suspension cultures at controlled pH levels (Martin and Rose, 1976). Their results also showed that pH had an effect on ammonium uptake. However, the trend of ammonium uptake verses pH was different. With Ipomoea cultures, as pH decreased, the uptake rate decreased, precisely opposite of the results shown here. In their pH 4.8 and 5.6 cultures, the initial NH_4 uptake rates were zero and highest at hour 49 and 52 in the culture period respectively. At hour 125 the pH 4.5 culture excreted ammonium into the culture medium. None of these results were seen in any of the experiments discussed here.

TABLE IV.

Summary of Ammonium Uptake Rates in Semi-continuous and Batch Culture at All pH's.

	Ammonium Uptake Rate					
	pH of Nitrogen Limited Cells[1]			pH of Phosphate Limited Cells[1]		
	4.5	5.5	6.5	4.5	5.5	6.5
Semi-continuous Culture Uptake Rates, μM/mg/hr	0.055 ± 0.004	0.046 ± 0.003	———	0.044 ± 0.009	0.060 ± 0.018	———
	0.075 ± 0.017	———	0.057 ± 0.016	0.053 ± 0.001	———	0.024 ± 0.016
Batch Culture Uptake Rates, ratio[2]	1.67 ± 0.16	0.96 ± 0.11	———	1.52 ± 0.07	0.92[3]	———
	1.21 ± 0.22	———	0.93 ± 0.11	0.95 ± 0.07	———	0.75 ± 0.15

[1] pH of the medium in which the cells were cultured semi-continuously.
[2] NH_4 uptake rate/growth rate.
[3] Single determination.

Several possible explanations for these differences exist. One of the more obvious explanations is that cells from a different species of plant were used in the two studies. Second, the physical size of the cultures were different. Martin and Rose used 7.5-liter stirred jar fermentors while all the cultures in these experiments were done in shake flasks with no more than 125 ml total volume. Another more plausible explaination is that two nitrogen sources were available to the Ipomoea cells at the same time, while only one nitrogen compound was supplied to the carrot cells. Though Martin and Rose, in their discussion, conclude that nitrogen utilization was controlled by pH and not by some suppressive or competitive effect between ammonium and nitrate, a relationship, undetected by their experiments, may exist between the uptake of ammonium and nitrate and pH.

Finally, the culture methodology itself may lead to differing results. The use of the semi-continuous culture methodology to provide as homogenous as possible a culture at each pH, may have been a significant factor. Two major differences arise. One, the cells that served as inoculum for the batch cultures in Martin and Roses' experiments may not have undergone repeated, frequent cycles of nutrient limitation as did the cells in the semi-continuous cultures. Two, the pH of the Ipomoea cells before batch culture may have been fluctuating as evidenced by a batch culture experiment without pH control where the pH underwent an initial rapid rise from pH 5.0 to pH 6.5 during inoculation, followed by a fall to pH 5.0

and then a steady rise to pH 7.0 over 9 days. The pH of semi-continuously cultured cells was controlled and showed little deviation from the set pH's. The uptake rates at each pH between ammonium and phosphate limited semi-continuous culture suggest that nutrient limitation may play a role in ammonium uptake. Further experiments are needed to clarify this point. Some very preliminary experiments involving culturing carrot cells at different ammonium concentrations suggest that differing initial levels of ammonium do not effect ammonium uptake.

The fact that very dissimilar results were obtained between Martin and Roses' studies and those reported here, indicates that previous culture history may play a large part in the subsequent behavior of the cells. It is quite plausible that had Martin and Rose extended their pH controlled batch cultures over several passages, that the ammonium uptake curves and calculated rates world have been much different from those they reported. Furthermore, their results may be more indicative of a combined effect of previous culture history and an effect of pH. An experiment that might clarify the degree of influence of semi-continuous culture on ammonium uptake rates is to culture cells semi-continuously for different periods (i.e. 5, 10, 15, 20 days) followed by batch culture at different pH's to determine when the change from pH responsiveness to non-responsiveness in batch culture occurs.

During the course of their experiments, Martin and Rose made no

mention of changing culture morphology. In this study, the morphology of the semi-continuous cells did change and did so in response to pH and in as short a time as 10 days (see Figure 19, page 42). The importance of this may lie in altered diffusion of nutrients into the cells. A dispersed culture consisting of single cells or very small clumps would have a greater amount of surface area exposed to the media, plus a shorter distance for nutrients to diffuse within the aggregates. Therefore, single cells and small aggregates may have a higher uptake rate than clumps orders of magnitude greater in size. Large differences in growth rate and ammonium uptake rate were seen between cells that had aggregated and those that had not. This suggested that an effect of aggregation on ammonium uptake rate was present.

The effect of pH on ammonium uptake is complex. Is it pH which effects aggregation which effects ammonium uptake which then effects growth? Or is pH effecting ammonium uptake which effects both aggregation and subsequent growth? The results with batch culture experiments indicate that pH does not have a direct effect on NH_4 uptake rate. The semi-continuous culture experiments are more difficult to analyze. During these periods, the aggregation process is ongoing and how much this contributes to the characteristic ammonium uptake rate and how much pH is effecting the rate alone is uncertain. It is well documented that pH effects cell wall mechanical properties, cell wall extension and various other growth parameters (Cleland, 1971; Taiz, 1984), but these phenomena have not

been correlated with cell aggregation or nutrient uptake. Clearly, further experiments are necessary to first qualify and then quantify the interrelationships between all these processes.

V. CONCLUSION

The results presented here show that ammonium uptake rate in carrot cell cultures was higher at pH 4.5 than at pH 5.5 or 6.5 contrary to the results of Martin and Rose. The type of limiting nutrient and culture methodology may also effect ammonium uptake rate though the investigation of the relationship between these factors is incomplete. Clearly, the immediate pH of batch culture does not effect the uptake rate of ammonium. The uptake rates between pH 4.5, 5.5, and 6.5 in batch culture were not significantly different (see Table II, page 24). The previous pH history of the cultures appears to influence ammonium uptake rate. Cells previously cultured semi-continuously at pH 4.5 showed significantly higher ammonium uptake rates compared to cells grown at pH 5.5 or 6.5 in either nitrogen or phosphate limited semi-continuous cultures (see Table II, page 24).

Medium pH also effected culture morphology. On average, $70\pm2.1\%$ of cultures grown at pH 4.5 were composed of aggregates less than 310 microns in diameter compared to 25% for cultures grown at pH 5.5 or 6.5. When these data were compared to data on ammonium uptake rates, a pattern began to emerge. pH 4.5 cultures, which showed the highest ammonium uptake rate, were principly composed of single cells or very small clumps of cells. pH 5.5 or 6.5 cultures, whose composition was 75% large aggregates, showed the lower uptake rate. Furthermore, cultures that had dispersed or aggregated were

not effected by medium pH and retained their characteristic uptake rates.

From this data a working hypothesis could be formulated. pH effects ammonium uptake rate not directly but through the process of aggregation or disaggregation of cells. The smaller clumps of cells growing at low pH will take up ammonium at a faster rate than large clumps of cells growing at a higher pH.

Although these results are preliminary in nature, they do provide a solid foundation for future experiments. Some of those experiments include how fast or slow is the aggregation/disaggregation process and precisely at what pH does the aggregation/disaggregation process begin. Does the ammonium uptake rate change proportionately with degree of aggregation? How long does a culture have to be maintained before the culture conditions set the pattern of nutrient uptake? If pH causes cell aggregation, how? These are but a few of the many interesting questions that remain to be answered about this fascinating plant culture system.

LIST OF REFERENCES

Balis, M.E. 1971 Determination of glutamine and aspartic acids and their amides. p. 123. In Methods of Biochemical Analysis, Vol. 20. Ed. M. Earl Dolis.

Bayley, J.M., King, J., Gamborg, O.L. 1972 The effect of the source of inorganic nitrogen on growth and enzymes of nitrogen assimilation in soybean and wheat cells in suspension cultures. Planta. 105:15-24.

Behrend, J., Mateles, R.I. 1976 Nitrogen metabolism in plant cell suspension cultures. II. Role of organic acids during growth on ammonia. Plant Physiol. 58:510-512.

Cleland, R.E. 1971 Cell wall extension. Ann. Rev. Plant Physiol. 22:197-222.

Dougall, D.K. 1980 Nutrition and metabolism. In Plant Tissue Culture As A Source Of Biochemicals. Ed. E. John Staba. pp. 21-58.

Dougall, D.K., Weyrauch, K.W. 1980a Abilities of organic acids to support growth and anthocyanin accumulation by suspension cultures of wild carrot cells using ammonium as the sole nitrogen source. In Vitro. 16(11):969-975.

Dougall, D.K., Weyrauch, K.W. 1980b Growth and anthocyanin production by carrot suspension cultures grown under chemostat conditions with phosphate as the limiting nutrient. Biotech. Bioeng. 22:337-352.

Dougall, D.K., LaBrake, S., Whitten, G.H. 1983 The effects of limiting nutrients, dilution rate, culture pH, and temperature on the yield constant and anthocyanin accumulation of carrot cells grown in semicontinuous chemostat cultures. Biotech. Bioeng. 25:569-579.

Gamborg, O.L., Shyluk, J.P. 1970 The culture of plant cells with ammonium salts as the sole nitrogen source. Plant Physiol. 4.5:598-600.

Martin, S.M., Rose, D. 1975 Growth of plant cell (Ipomoea) suspension cultures at controlled pH levels. Can. J. Bot. 54:1264-1270.

Nash, D.T., Davies, M.E. 1972 Some aspects of growth and metabolism of Paul's Scarlet Rose cell suspensions. J. Exp. Bot. 23:75-91.

Nesius, K.K., Fletcher, J.S. 1973 Carbon dioxide and pH requirements of non-photosynthetic tissue culture cells. Physiol. Plant. 28:259-263.

Rose, D., Martin, S.M. 1975 Effect of ammonium on growth of plant cells (Ipomoea sp.) in suspension cultures. Can. J. Bot. 53:1942-1949.

Sheat, D.E.G., Fletcher, B.H., Street, H.E. 1959 Studies on the growth of excised roots. VIII. The growth of excised tomato roots supplied with various inorganic sources of nitrogen. New Phytol. 58:128-141.

Taiz, L. 1984 Plant cell expansion: regulation of cell wall mechanical properties. Ann. Rev. Plant Physiol. 35:585-657.

Veliky, I.A., Rose, D. 1973. Nitrate and ammonium as nitrogen nutrients for plant cell cultures. Can. J. Bot. 51:1837-1844.

Wetherell, D.F. 1969 Phytochrome in cultured wild carrot tissue. I. Synthesis. Plant Physiol. 44:1734-1737.

Wetherell, D.F., Dougall, D.K. 1976 Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. Physiol. Plant. 37:97-103.

APPENDICES

APPENDIX A. WCM-1 MEDIUM[1]

Component	mg/l	mM
KNO_3	4000	39.59
NH_4Cl	540	10.10
$MgSO_4$-$7H_2O$	185	0.75
$CaCl_2$	166	1.50
KH_2PO_4	68	0.50
Na_2EDTA	18.6	0.05
$FeSO_4$-$7H_2O$	13.6	0.05
$MnSO_4$-H_2O	7.0	0.04
$ZnSO_4$-$7H_2O$	4.0	0.01
H_3BO_3	2.4	0.04
$(NH_4)_6Mo_7O_{24}$-$4H_2O$	0.01	$8x10^{-6}$
KI	0.38	$2x10^{-3}$
$CuSO_4$-$5H_2O$	0.015	$6x10^{-5}$
THIAMINE-HCl	3.0	$9x10^{-3}$
SUCROSE	20,000	58.43
2,4-D	0.5	$2x10^{-3}$

pH 5.5

[1]Wetherell, 1969

APPENDIX B. WCM-4 MEDIUM[1]

Component	mg/l	mM
NH_4Cl	320.94	6.00
$MgSO_4$-$7H_2O$	185	0.75
$CaCl_2$	166	1.50
K_2HPO_4	87.1	0.50
Na_2EDTA	18.6	0.05
$FeSO_4$-$7H_2O$	13.6	0.05
$MnSO_4$-H_2O	7.0	0.04
$ZnSO_4$-$7H_2O$	4.0	0.01
H_3BO_3	2.4	0.04
$(NH_4)_6Mo_7O_{24}$-$4H_2O$	0.01	$8x10^{-6}$
KI	0.38	$2x10^{-3}$
$CuSO_4$-$5H_2O$	0.015	$6x10^{-5}$
THIAMINE-HCl	3.0	$9x10^{-3}$
2,4-D	2.5	$1x10^{-2}$
SUCROSE	20,000	58.43
K_2 SUCCINATE	3885.8	20
MES	5856	30

pH 4.5

[1]Dougall and Weyrauch, 1980a

APPENDIX C. DRY WEIGHT DETERMINATION[1]

1. Number sequentially 2.4 cm disks of Whatman #40 filter paper. The number of disks required is either one per sample and 5 blanks for less than 40 samples; or one blank for each 8 samples when the number of samples is in excess of 40.
2. Arrange the disks in sequence in 100 mm Petri dishes containing filter paper.
3. With the lid of the Petri dish underneath the bottom, dry the circles for at least 3 hours at 70°C.
4. Cover the Petri dishes with their lids and cool for at least one hour in a dessicator.
6. Weigh the papers on the analytical balance and record the weights. Take the following precautions.
   a. Turn on the balance 15 minutes prior to use.
   b. Zero the balance before weighing papers from each Petri dish.
   c. One disk at a time is to be removed from the Petri dish and kept closed at all other times.
   d. The papers must be handled with forceps.
   e. The balance should not be arrested between weighings unless the paper and tissue exceeds 100 mgs, i.e., the papers must be added and removed from the free swinging pan gently and with care.
   f. If the balance swings excessively, arrest and release it.
   g. Re-zero the balance every time it has been arrested except where the paper and tissue weight exceeds 100 mg.
   h. Read the weight of the paper as soon as possible because the paper on the pan will increase in weight due to water absorption.
7. With a filter paper circle in the filter holder, add enough distilled water to wet the paper and then pour in the sample of tissue. Wash the tissue remaining in the culture vessel into the filter holder and wash the tissue on the filter holder with a total of 25 mls of distilled water.
8. Record the sample number or designation and the paper number.
9. Blank papers are to be distributed evenly throughout the samples and are to be washed with 25 mls of distilled water as in (7).
10. For each sample, check the upper part of the filter holder for adhering tissue after washing. Remove any tissue adhering to the filter holder and transfer to filter dish using a spatula. Wash the upper part of the filter holder with distilled water.
11. Slip the blade of the spatula between the filter paper disk and the top of the filter holder to break the vacuum and to move the disk off-center on the holder so that the vacuum remains broken.

12. Remove the disk plus tissue to a Petri dish, arrange in sequence so that the disks do not over-lap.
13. When the Petri dish has the required number of disks in it, dry it at 70°C with the lid underneath for at least 24 hours.
14. Repeat Steps 4-6.
15. Calculations
    a. Subtract the weight of the disks from the weight of the disks plus tissue and subtract initial from final weights of the blank disks. It is important that the sign of the difference be recorded.
    b. For the blanks, average the values obtained in (a) by adding all the plus weights and separately all the minus weights. Subtract the total for the minuses from the total for the plus values and record the sign. Divide this difference by the total number of blank disks to give the average differnce for blanks.
    c. If the average difference for blanks is plus, subtract this value from <u>each</u> of the values of (disks plus tissue) - (disk). If the average difference for blanks is minus, add this value to <u>each</u> of the values of (disk plus tissue) - (disk).
    d. Average the values from (c) for each treatment.

[1]Laboratory Procedures from the Alton W. Jones Cell Science Center, Lake Placid, New York.

APPENDIX D. AMMONIUM ION DETERMINATION[1]

STANDARD SOLUTION: NH_4Cl, 12.5 mM, from this make a series of dilutions to get 2.5, 5.0, 7.5, 10.0 mM to be used in the standard curve.

REAGENTS:

Reagent A: 10.0 g Phenol
50.0 mg Sodium nitroferricyanide
(sodium nitroprusside)
Vol. to 1.0 l with D.D. H_2O

Reagent B: 5.0 g NaOH
5.0 ml NaOCl 2N (Chlorox Bleach)
Vol. to 1.0 l with D.D. H_2O

Solutions are stored a 4°C, the bottles are covered with aluminum foil to prevent light exposure.
Solutions are stable up to two months.
Allow reagents to warm up to room temperature before using.

PROCEDURE:

Standard Curve:

mM NH_4 (in tube)	NH_4Cl	Std. mM	D.D. H_2O
0	40 ul	0	960 ul
0.1	40 ul	2.5	960 ul
0.2	40 ul	5.0	960 ul
0.3	40 ul	7.5	960 ul
0.4	40 ul	10.0	960 ul
0.5	40 ul	12.5	960 ul

Unknown:	sample size	D.D. H_2O	
	40 ul	960 ul	(1/25 dilution)

For both used and unused medium not being concerned with the fact that it is the limiting nutrient or not.

To all tubes:

Add 2.5 ml Reagent A, then add 2.5 ml Reagent B.
Mix with vortex.
Let stand for 30 minutes to allow for full color development.
Read O.D. at 640 nm on a spectraphotometer.
Zero with D.D. H_2O.

[1]Laboratory Procedures from the Alton W. Jones Cell Science Center, Lake Placid, New York.

VITA

Henry-York Steiner II was born in Amarillo, Texas on November 3, 1961. As his father was in the United States Armed Forces, he attended numerous elementary, middle and high schools around the U.S. and the world. He received his high school diploma from Vacaville High School, CA in June 1980. The following August he entered Grinnell College in Iowa and in May 1984 received a Bachelor of Arts degree in Biology. In September 1984 he accepted a Graduate Teaching Assistantship at the University of Tennessee Botany Department and began study toward a Master's Degree in Botany. This degree was awarded in December 1987.

The author is a member of the American Association for the Advancement of Science, and the Tissue Culture Association. Mr. Steiner will persue the degree of Ph.D. in the Botany Department at the University of Tennessee under the direction of Dr. D.K. Dougall.