Plant Physiol. (1981) 68, 439–442
0032-0889/81/68/0439/04$00.50/0

Effects of Environmental pH on the Internal pH of *Chlorella pyrenoidosa*, *Scenedesmus quadricauda*, and *Euglena mutabilis*[1]

Received for publication October 29, 1980 and in revised form March 9, 1981

ANN E. LANE AND JOHN E. BURRIS
Department of Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

ABSTRACT

The effect of external pH on two laboratory-cultured acid-intolerant species (*Chlorella pyrenoidosa* Chick and *Scenedesmus quadricauda* Turp. Bréb.) and one acid-tolerant species from a natural population (*Euglena mutabilis* Schmitz) was examined by measuring internal pH. These measurements were made with the weak acid ^{14}C-dimethyloxazolidine-2,4-dione after cells had been incubated for 2 and 6 hours at external pH levels from 3.0 to 8.0. Photosynthetic and respiration rates of the three species were also measured over the range of external pH levels.

All three species regulated their internal pH levels over the 6-hour incubation time. *C. pyrenoidosa* and *S. quadricauda* had internal pH levels around 7.0, regardless of external pH. *E. mutabilis* had a wider internal pH range, from 5.0 at low external pH to 8.0 at high external pH. External pH had no effect on either photosynthetic or respiration rates. Statistical comparisons showed that there was a significant difference between the acid-intolerant and acid-tolerant species with regard to the level of internal pH maintained and the response of internal pH to external pH.

Many streams in Pennsylvania and other parts of Appalachia have become polluted with H_2SO_4 from coal mines in the region. As a consequence, the pH levels in these streams are very low, in the range of 1.4 to 4.5 (6). The low environmental pH, along with other factors such as high acidity and high iron concentrations, strongly affects the biota of these streams (3). Algal species diversity is quite low (3, 24) and decreases as the pH level decreases. Nevertheless, certain species of algae flourish in these streams (3, 10, 24).

Studies, such as those cited, have characterized a number of aspects of natural populations of these algae. However, little about the physiology at low external pH of acid-tolerant species is known.

The measurement of internal (cytoplasmic) pH with [^{14}C]DMO[2] provides one method by which the effect of external pH on a cell can be investigated (see Ref. 15 for a review of internal pH and methods of its measurement). None of the algae that have been studied with this method have been acid-tolerant species. Furthermore, internal pH levels have not been measured when external pH levels are as low as those encountered by acid-tolerant species.

In this study, [^{14}C]DMO was used to measure the internal pH of an acid-tolerant species collected from a mine-polluted stream. Measurements were made at external pH levels which varied from 3.0 to 8.0. For comparative purposes, the internal pH levels of two laboratory-cultured acid-intolerant species were also measured over the same external pH range. The objective of the study was to determine whether or not the three species maintained constant internal pH levels regardless of external pH and whether there were differences between the internal pH levels of the acid-tolerant species and the acid-intolerant species.

Results of the internal pH measurements indicate that differences do exist in both the actual values of internal pH that the acid-tolerant and acid-intolerant species have and in the way in which the internal pH of each species responds as external pH changes.

MATERIALS AND METHODS

Organisms. The two acid-intolerant species used in the study were *Chlorella pyrenoidosa* Chick and *Scenedesmus quadricauda* Turp. Bréb., obtained from the Algal Culture Collection of the University of Texas. Cultures were grown in the *Chlorella* medium, as described in Starr (16), and were bubbled with 1% CO_2 at 30 C with constant illumination of 180 μE $m^{-2}s^{-1}$. In order to obtain reliable results from the technique used for internal pH measurements, thick cell suspensions were needed. Suspensions with 0.5 mg Chl *a*/liter were sufficiently thick for experimental use. This concentration of cells was obtained by centrifugation of cells from cultures that were 4 to 7 days old.

The acid-tolerant species was collected from an acidic stream draining the site of a strip mine located approximately 5 miles north of Kylertown, PA. The pH of the stream at the collection point averaged 2.78 ± 0.52. The alga was identified as *Euglena mutabilis* Schmitz, a common inhabitant of acid-polluted streams (5). Microscopic examination of collected material showed it to be unialgal. Any experiments which used *E. mutabilis* were done with algae collected on the day of the experiment to minimize stress to the cells.

Internal pH Determination. To adjust external pH levels, cell suspensions from either the cultures or collected material were centrifuged, and the pellets were resuspended in phosphate-buffered distilled H_2O. In most instances, the cells were washed in this solution by centrifuging and then resuspended for a second time in a volume of the same solution. The buffers were made according to Umbreit *et al.* (18) for pH 3.0, 4.5, 6.2, 7.0, and 8.0. The final buffer concentration was 40 mM.

Internal pH measurements were made using the filtering centrifugation method. The technique, as it appears in this study, is based on the methods described in Werdan *et al.* (25), Gaensslen and McCarty (9), Badger *et al.* (1), and Hiipakka (12). The three layers in the 0.40-ml microfuge tubes were, from bottom to top, 20 μl of a killing solution made up of 1 ml Triton X-100 diluted to 100 ml with a 20% sucrose solution (w/v) (12), 100 μl of a silicone fluid mixture (Versilube F-50:SF 96-20/4:1; General Electric Co., Waterford, NY), and 200 μl of cell suspension. The cell suspension consisted of either buffered or nonbuffered culture or

[1] Contribution Number 200 of the Department of Biology, The Pennsylvania State University.

[2] Abbreviation: [^{14}C]DMO, ^{14}C-labeled 5,5-dimethyloxazolidine-2,4-dione.

collected material prepared as described above.

For each internal pH determination, three labeled compounds were used: [^{14}C]DMO (46.63 mCi/mmol); [^{14}C]ethylene glycol (4.0 mCi/mmol); and [^{14}C]dextran (2.76 mCi/g) (New England Nuclear). [^{14}C]DMO, a weak acid, acts as a pH probe; [^{14}C]ethylene glycol is used to measure cell volume; and the proportion of the labeled compounds that adheres to the exterior of the cells during filtering centrifugation is determined with [^{14}C]dextran. A 12-min incubation period under illumination was sufficient to allow equilibration of the compounds. The tubes were centrifuged for 30 s in a Beckman 152 microcentrifuge and then immediately frozen in liquid N_2. The frozen tips containing the cell pellets were cut off and placed in scintillation vials with scintillation fluid, and radioactivity in each was determined.

Calculations of internal pH proceeded as outlined in Heldt *et al.* (11), using a pK of 6.28 for DMO at 27.5 C (4).

Internal pH determinations were performed on all three species. Cells were placed in distilled buffered H_2O with a replicate of each pH level. The internal pH values for one set of replicates of these cell suspensions were determined after a 2-h incubation; for the other set, pH values were determined after 6 h. Thus, these experiments yielded internal pH data for cells from the same culture or collection after 2- and 6-h exposure to various external pH levels. Internal pH determinations were also made in unbuffered culture medium for *C. pyrenoidosa* and *S. quadricauda* and unbuffered stream water for *E. mutabilis.*

The effect of light on the internal pH of *C. pyrenoidosa* was measured. In this experiment, two groups of cells from the same culture were incubated at all pH levels for 2 h. Internal pH was then determined for one group by the usual procedure. When internal pH was determined for the second group, the procedure remained the same except that the cells were incubated with the labeled compounds in the dark.

In control experiments, to make certain that buffered distilled H_2O did not affect internal pH, *C. pyrenoidosa* cells were incubated for 6 h in both buffered distilled H_2O and buffered fresh growth medium at all pH levels prior to internal pH determination.

For all experiments, the order in which the external pH levels for internal pH determinations were done was randomized.

Photosynthetic and Respiration Rates. Photosynthetic and dark respiration rates were measured for all three species after 2-h incubations in buffered distilled H_2O, in acid stream water (*E. mutabilis*), and either in culture medium in which the cells had been grown or in fresh culture medium (*S. quadricauda* and *C. pyrenoidosa*). The main purpose of these measurements was to determine whether or not the cells were metabolically active in the buffered distilled H_2O.

The rates were measured with a Rank Brothers O_2 electrode (Bottisham, Cambridge, England) at 29 to 30 C with a light level of 1,000 to 1,200 μE $m^{-2}s^{-1}$. The pH of the cell suspensions was recorded before and after the rate measurements.

The Chl *a* in each sample was extracted, using the methods of Strickland and Parsons (17), and the amount of Chl *a* present was measured with a Turner Fluorometer, Model 111 (Turner Associates, Palo Alto, CA).

RESULTS

Internal pH Determinations. A statistical comparison of internal pH values obtained for cells in buffered distilled H_2O and in buffered culture medium showed no significant difference between the two groups (achieved $F = 0.65$ based on 2, 18 degrees of freedom for a $P > 0.50$). The comparison was made by obtaining regression lines for each group of data and then testing these lines for pooling (14). This analysis indicates that cells in distilled H_2O have the same internal pH level as those in the culture medium, and, as a result, the internal pH values obtained for cells in distilled H_2O represent the normal pH of the cell.

An analysis by Wilcoxon's test (23) of the single experiment on the effect of darkness on internal pH showed that the internal pH in the dark was significantly lower than it was in the light ($P = 0.05$).

Inspection of Figures 1 and 2 shows that both *C. pyrenoidosa* and *S. quadricauda* have fairly constant internal pH levels over the range of external pH levels tested. The internal pH for both species varies between approximately 6.5 and 7.15, with some values reaching 8.0 at high external pH.

The internal pH data for *E. mutabilis* are shown in Figure 3. This species apparently has a wider internal pH range in contrast with the narrower range of the other two species. The internal pH of *E. mutabilis* varies from as low as 5.0 to as high as 8.0. This increase in internal pH occurs with increasing external pH.

For each species, separate regression equations were obtained for the 2-h and 6-h incubation data. Two types of models were used: linear ($Y = \beta_0 + \beta_1 x$) and quadratic ($Y = \beta_0 + \beta_1 x + \beta_2 x^2$). During the course of the regression analysis, three outliers were

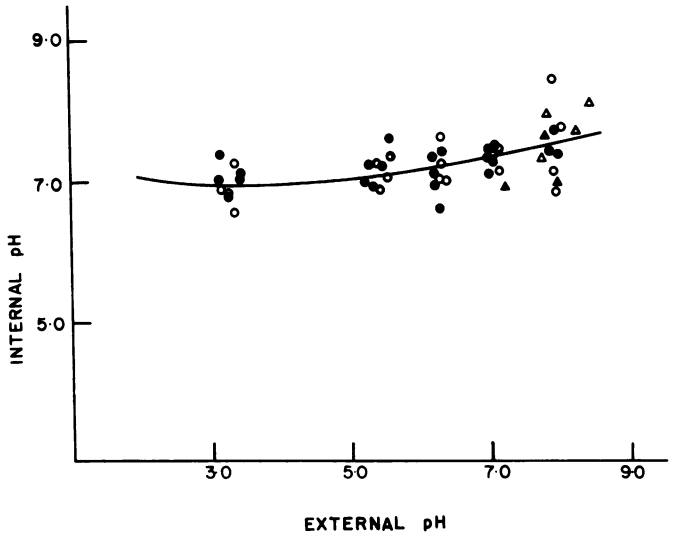

FIG. 1. Internal pH values from 2- and 6-h incubations for *C. pyrenoidosa.* The regression equation is $Y = 7.25 - 0.165x + 0.0234x^2$. (●, ▲), 2-h data; (○, △), 6-h data. (●), buffered distilled H_2O; (▲), culture medium.

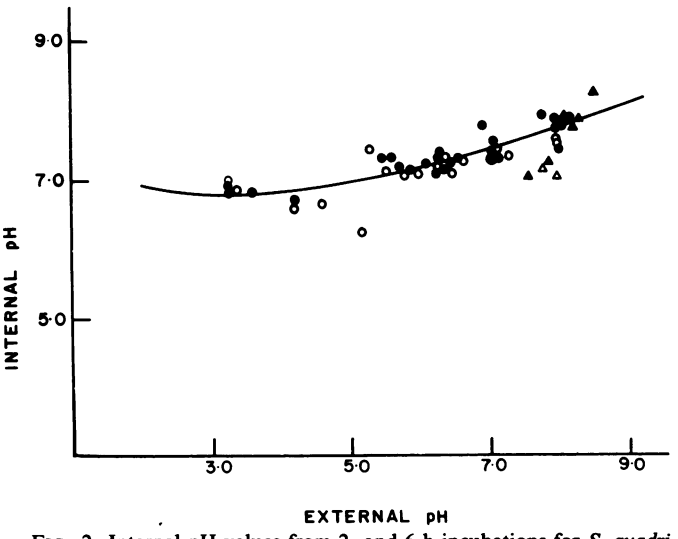

FIG. 2. Internal pH values from 2- and 6-h incubations for *S. quadricauda.* The regression equation is $Y = 7.21 - 0.239x + 0.0372x^2$. (●, ▲), 2-h data; (○, △), 6-h data. (●), buffered distilled H_2O; (▲), culture medium.

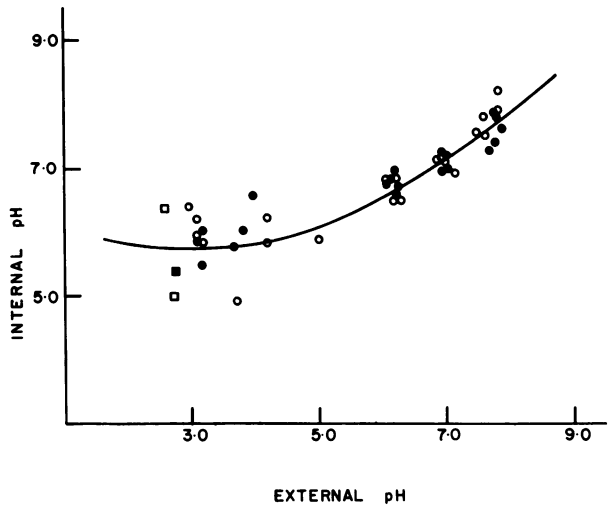

FIG. 3. Internal pH values from 2- and 6-h incubations for *E. mutabilis*. The regression equation is $Y = 6.40 - 0.447x + 0.0792x^2$. (●, ■), 2-h data; (○, □), 6-h data. (●), buffered distilled H_2O; (■), filtered stream water.

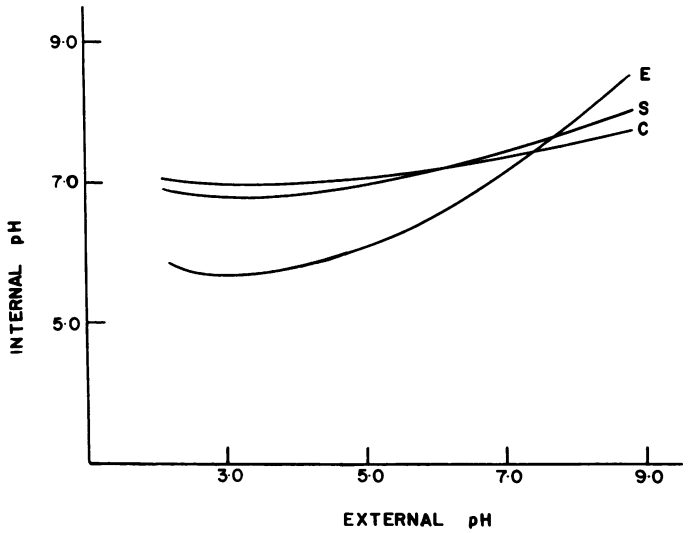

FIG. 4. Quadratic models for the pooled data of *C. pyrenoidosa* (C), *S. quadricauda* (S), and *E. mutabilis* (E). $C = 7.25 - 0.165x + 0.0234x^2$; $S = 7.21 - 0.239x + 0.0372x^2$; and $E = 6.40 - 0.447x + 0.0792x^2$.

removed, two from the *S. quadricauda* data and one from the *E. mutabilis* data. These 2-h and 6-h regressions for each species were compared by the test for pooling (14). By this method, it was determined that there was no significant difference between the 2-h and the 6-h data for any of the species, regardless of the model used ($\alpha = 0.05$). This being the case, all subsequent statistical analysis was done for each species on the combined 2-h and 6-h data.

In order that further statistical comparisons could be made among the three species, it was necessary to decide which of the two regression models, linear or quadratic, was best fitted to all three groups of data. When the linear model was used for the *E. mutabilis* data, plots of the residuals showed a strongly curved trend which disappeared when the quadratic model was used. Also, the contribution to the fit of the quadratic model by β_2 was significant (achieved $t = 3.66$ with 42 degrees of freedom). The contribution of β_2 was significant for the *S. quadricauda* data (achieved $t = 2.84$ with 56 degrees of freedom) as well as for the *C. pyrenoidosa* data (achieved $t = 1.77$ with 49 degrees of freedom). Therefore, based on the significance of β_2, the quadratic coefficient, the quadratic model was determined to be the better of the two in terms of fitting all three groups of data.

When the quadratic regressions of the three species are considered (Fig. 4), it seems that those of *C. pyrenoidosa* and *S. quadricauda* are similar while that of *E. mutabilis* is different from the other two. A comparison of the curves by the pooling method (14) found that the *E. mutabilis* curve was significantly different from the *S. quadricauda* and *C. pyrenoidosa* curves (achieved $F = 33.33$ with 3, 99 degrees of freedom and 50.47 with 3, 130 degrees of freedom, respectively). However, the *S. quadricauda* and *C. pyrenoidosa* curves were also significantly different from each other when $\alpha = 0.05$ (achieved $F = 3.70$ with 3, 145 degrees of freedom) but not when $\alpha = 0.01$. Thus, although the *S. quadricauda* and *C. pyrenoidosa* curves may be similar, they are statistically distinct.

When regression curves are different from one another, the difference can usually be traced to the coefficients of the equations that describe the curves. The coefficients β_0, β_1, and β_2 were tested for differences (14). No significant differences were found between β_0 and β_1 for any of the species or between the β_2 of *C. pyrenoidosa* and that of *S. quadricauda* ($\alpha = 0.10$). However, the β_2 of *E. mutabilis* was significantly different from those of *C. pyrenoidosa* and *S. quadricauda* ($\alpha = 0.005$).

The results from the comparison of the regression lines and the comparison of the coefficients substantiates the observation that *E. mutabilis* has a response to external pH which is different from that of the other two species. The results from the same two tests are less clear for *C. pyrenoidosa* and *S. quadricauda*. No differences were found among the coefficients, even though the lines themselves were found to be significantly different.

Photosynthesis and Respiration Rates. The photosynthetic rates of *C. pyrenoidosa* and *E. mutabilis* were unaffected by external pH and by buffered distilled H_2O. An analysis of variance showed that there was no significant effect of either factor on the photosynthetic rates ($P = 0.78$ for *C. pyrenoidosa* and $P = 0.40$ for *E. mutabilis*). On an average, *C. pyrenoidosa* and *E. mutabilis* maintained photosynthetic rates at 60 to 160 μmol $O_2 \cdot h^{-1} \cdot$ mg Chl a^{-1} and 30 to 90 μmol $O_2 \cdot h^{-1} \cdot$ mg Chl a^{-1}, respectively. For *S. quadricauda*, while no effect of the buffered distilled H_2O was observed, there was a significant effect of external pH on photosynthetic rate ($P = 0.033$). Further analysis of the *S. quadricauda* data by Duncan's multiple range test ($\alpha = 0.05$) (23) showed that photosynthetic rates were maintained around 100 μmol $O_2 \cdot h^{-1} \cdot$ mg Chl a^{-1} when external pH was below 5.5 and around 250 μmol $O_2 \cdot h^{-1} \cdot$ mg Chl a^{-1} when external pH was greater than 5.5. The higher rates occurring when external pH was above 5.5 may have been due to the use of bicarbonate, in addition to CO_2, as a carbon source in photosynthesis (19), or they may have been due to a direct inhibitory effect of external pH below 5.5.

For all three species, no significant effect of external pH on respiration was found by the analysis of variance procedure. For *C. pyrenoidosa*, *S. quadricauda*, and *E. mutabilis*, the *P* values were 0.92, 0.76, and 0.15, respectively. The rates were similar for all three species and were maintained, on an average, between −12 and −72 μmol $O_2 \cdot h^{-1} \cdot$ mg Chl a^{-1}.

DISCUSSION

Two major conclusions can be drawn from the results. First, all three species show maintenance and regulation of their internal pH levels. Second, there are differences both in the actual values of the internal pH levels maintained and in the response of internal pH to external pH dependent upon whether a species is acid-tolerant or acid-intolerant.

All three species were able to maintain a certain internal pH level even when incubated for 6 h at any given external pH. This is indicated by the finding of no difference between the 2- and the 6-h internal pH data for any of the species. Because the internal pH maintenance is relatively longterm, the process may be energy-

requiring. Evidence for this possibility is the observed significant drop in internal pH that occurred in the dark. This effect has been noted by other researchers as well (7, 22).

While maintenance of internal pH is common to all three species, the levels at which internal pH is regulated differ. The two acid-intolerant species, *C. pyrenoidosa* and *S. quadricauda*, maintain their internal pH levels somewhere near neutrality (Figs. 1 and 2). In the literature, there are examples of other species which also maintain a nearly-neutral pH (2, 8, 22). In contrast, the acid-tolerant *E. mutabilis* maintains its internal pH level below 6.0 for much of the external pH range (Fig. 3). Although values of the internal pH of *E. mutabilis* have not been previously measured, the internal pH of *E. gracilis* has been found to be as low as pH 5.89 (21). This value is in good agreement with the data presented here, which suggests that relatively low internal pH levels may be characteristic of the genus *Euglena*.

There is also a difference between acid-tolerant and acid-intolerant species with regard to the response of internal pH as external pH increases. This conclusion is supported by the statistical comparisons of the β_2 coefficients in which the β_2 of *E. mutabilis* was significantly different from those of *C. pyrenoidosa* and *S. quadricauda*. The internal pH of *E. mutabilis* increases relatively rapidly when external pH is above 6.0 so that internal pH is very nearly equal to external. However, the internal pH levels of *C. pyrenoidosa* and *S. quadricauda* increase only slightly in the same external pH range.

Based on the data presented here, it appears that, in the low external pH range (2.0 to 6.0), *E. mutabilis* has adapted to its acid stream environment by maintaining a lower internal pH. The two acid-intolerant species, in contrast, maintain relatively higher internal pH levels. If the pH-regulatory process is energy-requiring, *E. mutabilis* then has an energy advantage over the other two species at low external pH. Energy equivalent to that expended by *C. pyrenoidosa* and *S. quadricauda* in maintaining internal pH can be used by *E. mutabilis* in other processes such as growth and replication. While no growth rate measurements for the three species were done in this study, there is evidence that the growth of *S. quadricauda* at low pH is strongly inhibited (13). This supports the idea that there may be an energy deficit in the acid-intolerant species at low external pH.

At external pH levels above 6.0, acid-adapted *E. mutabilis* appears to lose its ability to regulate internal pH while the two acid-intolerant species continue to maintain their internal pH levels near neutrality. The extent of the loss of the ability to regulate by *E. mutabilis* is not known, however. The photosynthetic and respiration rates of *E. mutabilis* were unaffected after a 2-h exposure to high pH levels, and Van Dach (20) found that *E. mutabilis* exhibited maximal growth when culture pH ranged from 2.1 to 7.7. Thus, since *E. mutabilis* seems able to live at high external pH, perhaps it does not lose its ability to regulate but simply tolerates higher internal pH levels.

It cannot be determined conclusively from our results whether or not *E. mutabilis* has the ability to regulate its internal pH levels when external pH levels are high. However, it has been determined that, at external pH levels below 6.0, all three species have the ability to regulate internal pH at characteristic levels. The lower internal pH level that *E. mutabilis* maintains may help it to survive in the acid environment in which it lives.

Acknowledgments—The authors would like to thank Dr. Jane Gibson for technical advice on the measurement of internal pH, Dave Umbach for assistance in the statistical analyses of the data, and Dr. Robert Hamilton for suggestions regarding the manuscript.

LITERATURE CITED

1. BADGER MR, A KAPLAN, JA BERRY 1977 The internal CO_2 pool of *Chlamydomonas reinhardtii*: Response to external CO_2. Carnegie Inst Wash Year Book 362–366
2. BADGER ME, A KAPLAN, JA BERRY 1978 A mechanism for concentrating CO_2 in *Chlamydomonas reinhardtii* and *Anabaena variabilis* and its role in photosynthetic CO_2 fixation. Carnegie Inst Wash Year Book 77: 251–261
3. BENNETT HD 1968 Algae in relation to mine water. Castanea 34: 306–338
4. BORON WF, A ROOS 1976 Comparison of microelectrode, DMO, and methylamine methods for measuring intracellular pH. Am J Physiol 231: 799–809
5. BUETOW DE 1968. The Biology of *Euglena*, Vol. 1. Academic Press, New York
6. BUTLER RL, EL COOPER, JK CRAWFORD, DC HALES, WG KIMMEL, CC WAGNER 1973 Fish and food organisms in acid mine waters of Pennsylvania. U S Environmental Protection Agency Ecological Research Series EPA-R3-73-032. U S Govt Printing Office, Washington
7. DAVIS RF 1974 Photoinduced changes in electrical potentials and H^+ activities of the chloroplast, cytoplasm, and vacuole of *Phaeoceros laevis*. *In* U Zimmermann, J Dainty, eds, Membrane Transport in Plants. Springer-Verlag, Berlin, pp 197–201
8. FALKNER G, F HORNER, K WERDAN, HW HELDT 1976 pH changes in the cytoplasm of the blue-green alga *Anacystis nidulans* caused by light-dependent proton flux into the thylakoid space. Plant Physiol 58: 717–718
9. GAENSSLEN RE, RE MCCARTY 1972 Determination of solute accumulation in chloroplasts by rapid centrifugal transfer through silicone fluid layers. Anal Biochem 58: 504–514
10. HARGREAVES JW, EJ LOYD, BA WHITTON 1975 Chemistry and vegetation of highly acidic streams. Freshw Biol 5: 563–576
11. HELDT HW, K WERDAN, M MILOVANCEV, G GELLER 1973 Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space. Biochim Biophys Acta 314: 224–241
12. HIIPAKKA RA 1978 Transport and phosphorylation of 2-deoxyglucose by bull and ram spermatozoa. PhD Thesis. The Pennsylvania State University, University Park
13. MOSS A 1973 The influence of environmental factors on the distribution of freshwater algae: An experimental study. II. The role of pH and the CO_2 bicarbonate system. J Ecol 61: 157–177
14. NETER J, W WASSERMAN 1974 Applied Linear and Statistical Models: Regression, Analysis of Variance, and Experimental Designs. Richard D. Irwin, Inc, Homewood
15. SMITH FA, JA RAVEN 1979 Intracellular pH and its regulation. Annu Rev Plant Physiol 30: 289–311
16. STARR RC 1971 The culture collection of algae at Indiana University—Additions to the collection July 1966-July 1971. J Phycol 7: 350–362
17. STRICKLAND JDH, TR PARSONS 1972 A practical handbook of seawater analysis. Fish Res Bd Can Bull 167 (2nd ed)
18. UMBREIT WW, RH BURRIS, JF STAUFFER 1964 Manometric Techniques: A Manual Describing Methods Applicable to the Study of Tissue Metabolism, 4th ed. Burgess Publishing Co, Minneapolis
19. VAN TK, WT HALLER, G BOWES 1976 Comparison of the photosynthetic characteristics of three submersed aquatic plants. Plant Physiol 58:761–768
20. VAN DACH K 1943 The effect of pH on pure cultures of *Euglena mutabilis*. Ohio J Sci 43: 47–48
21. VOTTA JJ, TL JAHN, RH LEVENDAHL 1971 The mechanism of onset of the stationary phase in *Euglena gracilis* grown with 10 mM succinate: Intracellular pH values. J Protozool 18: 166–169
22. WALKER NA, FA SMITH 1975 Intracellular pH in *Chara corallina* measured by DMO distribution. Plant Sci Lett 4: 125–132
23. WALPOLE RE, RH MYERS 1978 Probability and Statistics for Engineers and Scientists, 2nd ed. MacMillan, New York
24. WARNER RW 1971 Distribution of biota in a stream polluted by acid mine drainage. Ohio J Sci 71: 302–316
25. WERDAN K, HW HELDT, G GELLER 1972 Accumulation of bicarbonate in intact chloroplasts following a pH gradient. Biochim Biophys Acta 283: 430–441