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Seventh Semiannual Status Report

National Aeronautics and Space Administration

Research Grant No. NsG-70-60

NASA ↓

NASA CR- 55554

t: A STUDY OF PHYCOPHYSIOLOGY IN CONTROLLED ENVIRONMENTS Semiannual...

No. 7, Apr. 1, 1963 – Oct. 1, 1963

Prepared by the Director

Dr. Robert W. Krauss 1 Oct. 1963 43p refs

Professor of Plant Physiology

Department of Botany

University of Maryland U., College Park

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College Park

October 1, 1963

Introduction

The investigations reported here were under an extension of Research Grant NsG-70-60 made to the University of Maryland for the period 1 April, 1963 to 31 March, 1965. The initial grant was for a three year period beginning 1 April, 1960 to 31 March, 1963. This report covers the period from 1 April, 1963 to 1 October, 1963. The outline of proposed research is given in the proposal for continuation submitted on 15 February, 1963. This report has been proposed in accordance with the instructions of the NASA Office of Grants and Contracts and is designed to provide the requested information with regard to financial details as well as general progress of research. The details of certain parts of the completed research can be found in the appendix where manuscripts and reprints are included.

The studies being supported by this grant are designed to provide basic information about photosynthetic organisms which may serve as components of closed, or partially closed, ecological systems developed for long range or long term exploration of space. It is the mission of the research team associated with this project to search for as much basic information about the physiology, growth, and biochemistry of algae and other photosynthetic microorganisms as is possible with the personnel and equipment available. These investigations are not developmental in nature but are designed to increase the store of knowledge available for those who are engaged in the development of operational systems. Any engineering based on a biological organism becomes

enormously complex even for such a thoroughly studied creature as man. The number of variables associated with the growth and oxygen evolution of the algae are just becoming well documented. As additional information is accumulated the point may be reached where computer analysis of the variables will make possible a prediction of performance under any given set of conditions. It may well be that when this is indeed possible the engineering design for a gas exchanger will become obvious.

The brief summaries of research reported here are designed to give only a review of the areas under study. The papers and manuscripts in the appendices of each report give the detailed results of each completed stage of research.

Sterols

A study to determine the quantity and identity of sterols present in different species of Chlorella is nearing completion. Ergosterol is the major sterol in most Chlorella species with a smaller amount of another sterol which has a longer retention time on gas chromatography but a shorter retention time on column chromatography. This suggests that it may be a C-29 sterol. Chlorella vulgaris has been found to contain a rare C-29 sterol, chondrillasterol, which has not been found in other species of the genus. Chlorella protothecoides contains at least five sterols, three of which are present in minor quantities. These sterols may be precursors to the major sterol of the organism, and should be a fine starting point for a study on the

biosynthesis of sterols in algae. The identification of these sterols is not as yet final.

A new drug (triparanol) which inhibits the biosynthesis of sterols has been obtained. Use of triparanol may show whether Chlorella can survive and grow in the presence of triparanol and valuable information concerning the biosynthesis of algal sterols may be obtained by analyzing the cells for intermediates which accumulate due to the triparanol inhibition.

Recent work has shown that earlier estimates on the amount of 3β-hydroxy sterol present have been low. Acid hydrolysis of the cells which have previously been extracted in acetone yields an additional 50% to the sterol yield if the second extraction is with absolute ethanol. This may be due to release of sterol which is bound in the cell, possibly to protein.

Recent reports of the presence of steroids, possibly of hormonal significance to humans, are under study. At this point we have been unable to verify the presence of any hormonal steroid in the species of Chlorella in which they have been reported. The resolution of this problem is a significant part of our program for the future.

Special Effects of Light

Any cell capable of photosynthesis depends on light for its increase in mass and its total metabolism. However in recent years light has been shown to have profound effects on the cell quite exclusive of the energy conversion reactions of photosynthesis. Two of these effects are under study at this time. The first of these is the effect of light in permitting the growth of Chlorella vulgaris in

the dark, and the second is the inhibitory effect of light on the process of cell division. This work can be summarized as follows:

The mechanism of acceleration of dark growth by small amounts of light:

Certain plants, including Chlorella vulgaris Beyerinck (Emerson's strain), fail to grow in the dark even when sugars are provided. This phenomenon was clearly demonstrated in the alga, C. vulgaris, for which the growth rate in darkness on a glucose medium remained constant for two days and then declined approaching zero. Pigment concentrations also declined in darkness. Changes in flow rate of 1% CO_2-in-air from zero to 7 ml/min caused a progressive increase in the dark growth rate over a five day period but did not maintain growth in the dark. Rates above 7 ml/min produced no changes in growth rate.

White light intensities below the compensation point of the alga maintained heterotrophic growth. The saturation value for this response was 0.8 $\mu w/cm^2$. White light also initiated growth in non-growing cultures transferred from darkness to light.

The action spectrum for heterotrophic growth indicated a porphyrin as the active pigment. Blue light in the 425 mμ region was four times as effective as white light in stimulating heterotrophic growth. A secondary peak of growth stimulation occurred in the 575 mμ region.

The respiration of glucose by the alga was stimulated by low intensities of white light. This response was not immediate, but was clearly present after the third day of incubation.

Sodium malonate and KCN were inhibitory to growth of C. vulgaris on inorganic medium or glucose medium under 300 f-c of white light. These data suggested that the succinic dehydrogenase and cytochrome oxidase systems were present.

Substances inhibitory to growth were excreted into the medium under dark growth conditions. Two substances identified were formic and acetic acids.

The evidence suggested that respiration of glucose cannot proceed for an extended period of time in darkness. The reason for this is postulated to be the lack of a cytochrome or a cytochrome precursor.

The inhibition of cell growth by light:

A major obstacle in making a quantitative study of the inhibitory effects of light in photosynthesizing organisms has been the presence of the competing process of photosynthesis which is a growth-promoting light phenomena. In order to better isolate and quantitatively study the inhibitional effect of light a non-photosynthetic organism closely related to Chlorella should prove an effective tool. Prototheca zopfii, a colorless member of the order Chlorococcales, was chosen as the organism for study. This organism is essentially a Chlorella without chlorophyll. Preliminary studies with Prototheca definitely established that light had an inhibitional effect independent of absorption by chlorophyll. A series of experiments were performed to quantitatively measure this phenomena at various light intensities in Prototheca as well as in Saccharomyces and Tetrahymena. Growth rates

were determined for cultures grown in an organic media at the following light intensities; 100, 150, 200, 400, 600, 800, 1000, and 1200 foot candles. It was found that an inhibition coefficient could be defined which is constant for cells grown at 200 f-c or below, accelerates between 200 f-c and 400 f-c and then becomes a constant again between 400 f-c and 1200 f-c. It has thus become possible to give an exact mathematical formula for growth rate as a function of light intensity.

Studies are currently underway to determine whether the observed inhibition arises from a delay in cell division independent of increases in mass and volume, or whether it is due to a decrease in cellular synthesis. For this study the cultures are being synchronized and a Coulter counter is being utilized so that volume changes as well as times of division can be exactly determined.

Shifts in Chlorophyll a and b ratios:

Studies completed during the last report period indicate shifts in the chlorophyll a/chlorophyll b ratios in cells growing in the recyclostat at different light intensities and different population densities. Therefore a detailed study has been initiated to follow the changes in chlorophyll a and chlorophyll b in Chlorella pyrenoidosa (van Niel's strain) as a function of growth phase, optical density, and light intensity. The in vitro method of Comar and Zscheile is employed for such determinations; however, since this method requires approximately four hours and sacrificing cells, an in vivo method is desirable.

It was decided to begin the investigation with log phase cells at various light intensities. To date, cells grown at 500 and 1000 f-c have been studied. Attempts are being made to correlate *in vitro* and *in vivo* spectra.

Employment of the opal glass device with circular cuvettes has been very encouraging. It enables a very close matching of the *in vitro* $OD_{660\ m\mu}$ with the corresponding *in vivo* $OD_{680\ m\mu}$. Both the *in vitro* OD_{660} and the *in vivo* OD_{680} are maximum absorption peaks. There is trouble correlating the *in vitro* $OD_{642.5\ m\mu}$ to the corresponding *in vivo* $OD_{650\ m\mu}$. These last two points are taken on a sharp slope which probably accounts for much of the difficulty. It is to be hoped that the development of an *in vivo* method will permit continuous monitoring of total chlorophyll as well as chlorophylls *a* and *b* in culture in which these values are progressively changing.

Responses and efficiencies of cells of different developmental stages:

Studies of photosynthesis were conducted on cells of non-synchronized populations of *Chlorella* 7-11-05 separated into age groups by fractional centrifugation. The basis for these studies was an assumption that since two fractions of cells separated by centrifugation-one consisting mostly of small cells and another predominantly of large cells-before being separated into fractions were subjected to exactly the same environmental conditions, their performance after separation is due to nothing else than the difference in their size (and, therefore, age) composition. Thus, doubts concerning the effects of synchronizing

agents could be evaluated and the universal decline in photosynthetic activity with the age of cells was hoped to be ascertained.

Special attention was given to the technique of centrifugation. Resolution of the centrifugation technique is rather poor. One of the conditions for the successful separation of cells into size fractions is a larger possible diversity of cell sizes in the suspension to be subjected to centrifugation. A larger difference in sizes of young daughter cells and of old mother cells is assured by having an organism dividing into many cells. In high-temperature Chlorella 7-11-05, a cell can be observed to divide into a number--16, 32, 64, and even more--of cells. The actual number of daughter cells emerging from one mother cell depends on environmental conditions. Thus, a second factor for having a suspension of algal cells suitable for successive fractional separation is optimal conditions for growing such a suspension. The actual size of cells in nonsynchronized suspensions used in our experiments ranged from 1-2 μ for daughter cells to 11-12 μ for well developed mother cells. Another condition for reasonable separation of cells into size fractions is repeated centrifugation and elimination of cells of intermediate sizes. It has been proved necessary to subject cells to 3 or 4 low-speed, short-time successive centrifugations and to discard, as intermediate cells, as much as 9/10 to 19/20 of the original cell mass.

All efforts for possible rigorous separation of cells by fractional centrifugation may fail if large cells are allowed to divide in the process of centrifugation. The large cell fraction will then

actually consist of a mixture of small and large cells. Our studies indicated that cell division can be arrested by suspension of cells in phosphate buffer at pH 4.5.

As has been shown in previous investigations, cells of different age differ not only and even not so much in their photosynthetic activity, as determined for short intervals of time, as in the direction in which the activity changes in prolonged observations. Photosynthetic activity of small and large cell fractions may differ little during a short period after the beginning of measurements or, actually, the activity of larger cells may be even somewhat higher than the activity of small cell fraction. But inevitably the activity of the large cell fraction declines with time at a higher rate than the activity of the small cell fraction.

Actual measurements of photosynthetic gas exchange in the small and large cell fractions were made in phosphate buffer at pH 4.5, in bicarbonate buffer at pH 6.8, and in Warburg buffer No. 9 at pH 9.3. The choice of buffers was affected by our previous studies of photosynthetic activity in synchronized cells conducted in the same buffers. Studies were done under various temperature and illuminance conditions and the superior photosynthetic activity of smaller (younger) cells as compared to larger (older) cells was invariably ascertained, provided a good separation into size fractions was achieved and the large cells were prevented from dividing in the process of separation.

Three groups of processes can be identified as responsible for changes which take place in batches of nonsynchronized cells under nongrowing conditions: (1) the depletion of reserve material in the

process of respiration; (2) change in age composition of cells under observation; and (3) progressive degradation of metabolic mechanisms due to absence of growth. The variability in the activity of photosynthetic mechanisms observed by many investigators can be attributed to changes bound with cell development and metabolic turnover affecting both the enzymatic and the photochemical part of the photosynthetic apparatus.

Studies on buffering capacity of cells and on its relation to cell division were also continued. Von Slyke's β-values for cell secretions were obtained for a variety of the suspending fluids and the time course of the process was traced. This line of research will be actively pursued in the immediate future.

Papers

Krauss, R. W. Algal Physiology and Biochemistry. Invitational address, Harvard University. May 6, 1963.

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Sorokin, C. The Efficiency of Photosynthesis in Algae. Invitational Paper, Symposium on Photosynthetic Mechanisms in Green Plants. Warrenton, Virginia. October 18, 1963.

Publications

Sorokin, C. The capacity for organic synthesis in cells of successive developmental stages. Archiv für Mikrobiologie 46: 29-43. 1963.

Sorokin, C. Injury and recovery of photosynthesis in cells of successive developmental stages: temperature effects. Microalgae and Photosynthetic Bacteria, Special Issue of Plant and Cell Physiology, Japanese Society of Plant Physiologists, eds. p. 99-109. 1963.

Plans for the Future

During the next report period the work on the special accelerating effect of light will be completed and in manuscript ready for publication. Also during this period a paper on the sterols of Chlorella will be completed. The monograph on Chlorella which is now complete except for the steel engraving of the color plates is also due for release. In order to prepare adequate color engravings it was necessary to rephotograph from standard cultures all of the species and strains described in the monograph. It was decided that this considerable delay was preferable to photomicrographs that were poorly reproduced.

During the next period all other research that is in progress will be continued. Dr. Soeder from the University of Freiburg will aid in studies on pure culture techniques, CO_2 toxicity, and temperature effects on growth. In essence the research program in concept, in budget, and in personnel is proceeding precisely as outlined in the proposal.

Presented at the Symposium on Photosynthetic Mechanisms of Green Plants,

Airlie House, Warrenton, Virginia (Va.)
14-18 October, 1963

1485994 Maryland U., College Park

ON THE VARIABILITY IN THE ACTIVITY OF THE PHOTOSYNTHETIC MECHANISMS

Constantine Sorokin

In its ... (p refs Presented at the Symp. on Photosyn. Mech. of Green Plants, ...

Photosynthetic activity was observed to vary from one observation to another and in the course of one experiment. For example, in measurements of the enhancement effect, the photosynthetic rate and enhancement were changing over a considerable period of time estimated to last for the red alga Porphyridium for about 4 hours (1). In Chlorella, the dark current was observed to drift during the first 12 hours after placing cells upon the electrode (2), and stabilization of photosynthetic oxygen production required up to 2 or 3 days. Poor reproducibility and inconsistency of the activity with time, even for the same batch of cells, implied that some factor or factors were possibly overlooked.

Studies on photosynthetic activity in the course of the development of algal cells indicated that the activity level does not remain constant during the life cycle of cells. As a cell proceeds in its development, the activity first rises during the initial portion of the life cycle, attains a maximum in cells of intermediate age, and then declines toward the time of cell division (Fig. 1).

One group of investigators observed the rising part of the curve to coincide with the initial portion of the illumination period and the declining part of the curve with the later portion of the illumination period (3-7). Under conditions of synchronization used by another group of researchers; the recovery of the rate was accomplished before the beginning of illumination; thus, the decline in the photosynthetic rate was usually

observed as extending over the whole light period (8, 9).

A decline in photosynthetic activity beginning at some earlier or later developmental stage is a common feature characteristic of the life cycle of cells. Old cells are sluggish (6), have lower quantum efficiency (10), and are more sensitive to adverse environmental conditions, particularly to the effects of strong light (6).

In prolonged measurements, the rate of photosynthetic gas exchange declines in old cells from the very beginning of observations (Fig. 2). In younger cells, photosynthetic rate may greatly increase in the course of observations, even if cells are suspended in a nitrogen-free medium (11-14).

Thus, the size and/or activity of photosynthetic apparatus may increase in younger cells even under conditions generally considered unfavorable for photosynthesis. Under the same and more favorable conditions, the activity and/or the size of photosynthetic machinery in old cells inevitably declines.

The decline in the active part of photosynthetic apparatus with aging of cells has been attributed to changes in the rate of metabolic turnover: the increase in catabolic activity and the decline in intensity of anabolic processes characteristic of the process of cell development. An intense anabolic activity is coupled in younger cells with higher capacity to use metabolic intermediates for rebuilding photosynthetic machinery (16-17).

Respiration activity (18) and the capacity of cells for growth (19) have been also reported to decline toward the end of the life cycle. Available information on nitrogen accumulation (20), nucleic acid content (21), ash constituents (22), phosphorylation activity (23), and contents of several vitamins (24) also indicates that all these variables depend on the developmental status of cells.

Surprisingly, observations on the dependence of metabolic activity on the developmental status of cells have made small impact on studies of metabolism,

and particularly on studies of photosynthesis. A plausible explanation for the little consideration given to the developmental aspect of photosynthesis lies, perhaps, with the fact that most of the above observations were made on specially synchronized algal cells.

There has been wide-spread concern (25-28) that the observed variations in metabolic activity may be due to synchronization technique; that they are not characteristic of the normal life cycle of cells but are caused by the synchronizing agent. The effect of a synchronizing agent on the subsequent activity of cells cannot be denied any more than can the effect of any other environmental condition. However, a decline in metabolic activity with the age of cells was observed also for the naturally synchronized green colonial alga Hydrodictyon (29) and for individual cells of Amoeba (30) and Tetrahymena (31).

Another technique used in studies of the relative photosynthetic activity in young and old algal cells consists of separation of cells of an originally nonsynchronized suspension into fractions by centrifugation. After centrifugation, the small cell fraction presumably consists mostly of young cells and the large cell fraction of predominantly old cells. Before being centrifuged from a nonsynchronized suspension, these two groups of cells are subjected to the same environmental conditions and the difference in their performance must, therefore, be attributed only to the difference in the age composition of these groups.

Using this technique, the late Dr. R. Emerson studied quantum efficiency of small and large cells and made two comments. In one report in 1954 (32), he stated that large cells have higher quantum requirement per molecule of photosynthetic oxygen production. In 1957 (33), he reversed his opinion, stating that difference in quantum efficiency of large and small cells is

transient and therefore illusory. Neither report was published in detail, and no description of the technique used by Dr. Emerson is available. Dr. Emerson's observations, and particularly his denial of the age changes in photosynthetic activity, probably contributed to the delay in the acceptance of the concept of aging of cells as a universal biological reality.

Centrifugation technique may lead to false interpretations unless some basic conditions are observed. These conditions include:

1. A wide diversification in cell sizes of nonsynchronized suspension to be subjected to centrifugation;

2. A careful, repeated centrifugation and rejection of the largest portion of the intermediate cells;

3. A prevention of large cells from dividing during the process of centrifugation;

4. Prolonged observations on the activity of small and large cell fractions.

In our investigations under various temperature and illuminance conditions and in such diverse suspending fluids as phosphate buffer at pH 4.5, bicarbonate buffer at neutral pH, and carbonate-bicarbonate buffer at pH above 9, the fraction of larger cells consistently displayed an inferior photosynthetic activity provided the separation of cells into fractions by centrifugation was reasonably good (Fig. 3).

In studies on nonsynchronized algal populations a gradual, slow change in age composition of the batch of cells and an accumulation of small cells occur. Actual progress in cell division in a batch of cells under observation depends on the hereditary constitution of the organism, the external conditions, and the physiological state of the cells, the last one largely dependent on their prehistory. An interaction of these factors affects individual cells

against their developmental background. Thus, the chance of a particular cell to enter the division stage, the degree of completion of cell division, and the time course of the division process depend on how far the cell progressed in its development by the time it was taken for observation. The complexity of the situation makes it unreasonable to expect a ready standardization of age composition of cells used in a series of experiments.

Eventually all cells capable of division divide and the age composition of the batch of cells stabilizes. These cells have been described as "active dark" cells (2). However, for a number of reasons, cells maintained on the electrode surface hardly can be called "active dark" cells. For one reason, under unfavorable conditions, not all the cells divide and some cells do not attain the status of a small (dark) cell. For another reason, the "active dark" cells, in the sense used by Tamiya group (8), are recognized to be actually starved cells (34, 35). Thirdly, physical conditions on the electrode surface are even less favorable for cell division and maintenance of cells than those stipulated by Tamiya's synchronization technique.

Growth under the described conditions (2) is negligible or absent. The importance of maintaining growth conditions has been generally disregarded in photosynthetic investigations. However, growth activity is a reliable indicator of the direction and rate of metabolic turnover. Catabolic activity is much stronger and anabolic processes are comparatively weak in nongrowing cells (36). Enzyme proteins in nongrowing cells undergo fast degradation, products of degradation accumulate, and the size of the active part of the metabolic apparatus is reduced to a minimum.

The duration of the period of intensive degradation under unfavorable growth conditions is expected to depend on the physiological state of cells and on external factors. Eventually, with little left to undergo degradation,

catabolic processes also decline to a minimum. In an impoverished cell, metabolic activity is more or less stabilized at a low level. However, the fact that the cell cannot be maintained in this state indefinitely indicates that even under these conditions degradation proceeds at a slow rate until the death of the cell.

Thus, in a batch of nonsynchronized cells undergoing the so-called starvation (2, 37), three groups of complex processes take place: the depletion of reserve materials in the process of respiration, a change in the age composition of cells under observation, and progressive degradation of metabolic mechanisms due to absence of growth. Both moieties of the photosynthetic apparatus, the one responsible for dark reactions and the other constituting photochemical part of photosynthetic machinery, are affected. An indirect evidence for the enzymatic part to be affected by age changes and metabolic turnover comes from studies on the degradation of enzyme proteins in bacteria and yeasts (36) as well as from observations on the decline in the respiratory and growth activity with the age of the cells (18, 19). A more direct proof is supplied by the fact that photosynthetic activity in older cells is much lower than in younger cells under conditions of light saturation where the rate of the photosynthetic process is affected by enzyme activity (6).

Indications for the photochemical part of the photosynthetic apparatus to be affected by changes bound with cell development and metabolic turnover are as follows:

1. Studies on chlorophyll turnover in higher plants indicated that chlorophyll undergoes a rapid degradation in older tissues (38-40).

2. Observations on synchronized algal suspensions demonstrated that photosynthetic activity in older cells is inferior to that of cells of

intermediate age also at intensities below light saturation where the rate of photosynthesis is assumed to be dependent on photochemical reactions (41).

3. Fluorescence intensity for older cells was shown to exceed that of younger cells (42). With the inverse ratio existing between fluorescence yield and photosynthetic activity, observations on the increase in fluorescence with the age of the cells provide most direct proof for changes in the distribution of energy in the primary photochemical act, changes bound with cell development.

Thus, a cell never stands still. It is in a constant flux. An interplay of biological factors may explain the inconsistency of observations on photosynthetic activity of cells. Of these factors, shifts in the age composition in a batch of cells under investigation and changes in the size of the active part of metabolic mechanism in each particular cell, as affected by metabolic turnover, deserve thoughtful consideration.

Preparation of this article was supported by funds from the National Aeronautics and Space Administration.

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Figure 1

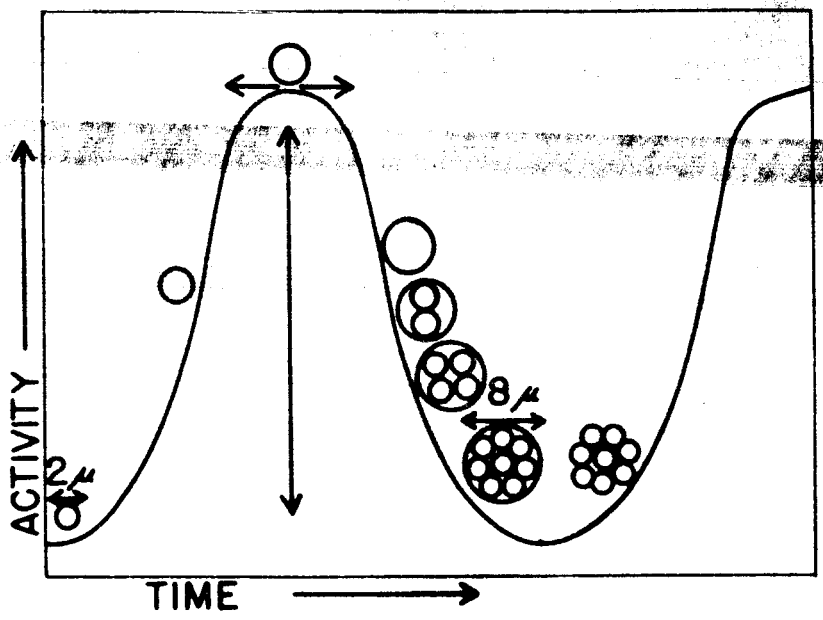


Figure 2

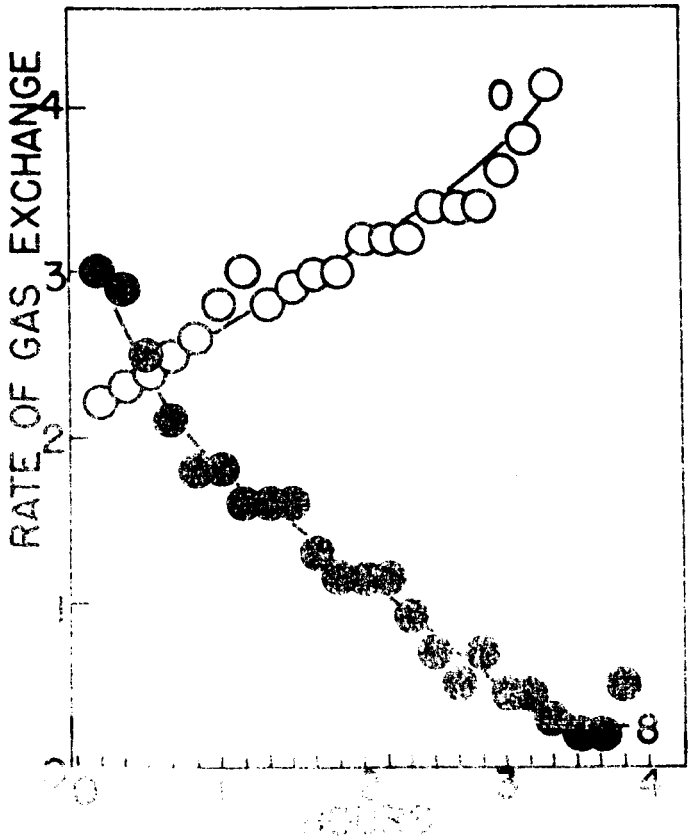
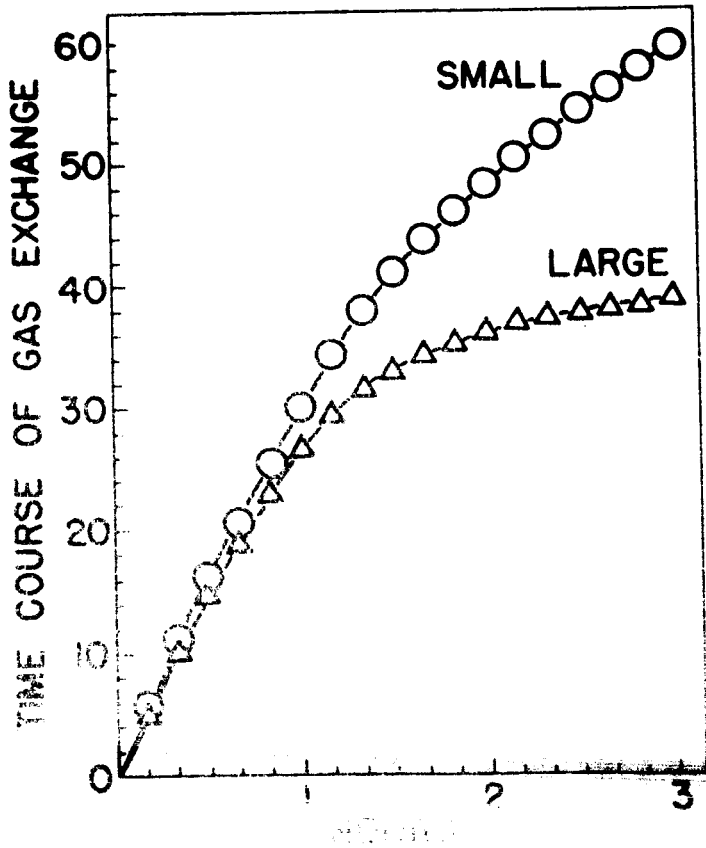


Figure 3



Buffering activity of algal cells and its effect on cell division [1, 2]

Constantine Sorokin

Department of Botany, University of Maryland

College Park, Maryland, U.S.A.

(Received)

Carbon dioxide, as well as other acids, has been reported to exert an inhibitory effect on cell division (10, 11, 12). The inhibitory concentration, though readily determinable for a given cell suspension, varied from one experiment to another in an extended series of experiments, indicating that other factors were also involved. Of these factors, concentrations of other ions, particularly of bicarbonate ion, proved to be of paramount importance (11).

In the course of investigations, the attention was drawn to a property of cells to render with time an originally unfitted suspending fluid more suitable for cell division. In a medium with a high initial concentration of hydrogen ions, this cell activity is presumably due to the immobilization of hydrogen ions as the result of the secretion by

the cells of substances of high buffering capacity. This report describes the effect of cell secretions on the pH of the suspending fluid and the importance of this activity to cell division.

Material and Methods

Observations were made on synchronized cells of green, high-temperature alga, Chlorella 7-11-05 (9). Cells were synchronized by a light:dark regime (8, 14) and then routinely grown in a complete inorganic medium (11, 13) for 7 hours at 38° and at a light intensity of 2,000 foot-candles. During the light period, cell suspensions were supplied through bubbling tubes with a 5 per cent carbon dioxide-in air mixture. Microscopic examination indicated that, under these conditions, 7-hour cells had no internal subdivision into daughter cells. However, potentially these cells were capable of cell division since, after being transferred in the same medium into dark, they readily divided into daughter cells.

For the purpose of observation, cells at this stage were centrifuged and resuspended in double distilled water or in dilute sulfuric acid. Acid concentrations were expressed in millimoles per liter of the suspending fluid. Cell suspensions were placed in dark at 35° and supplied through bubbling tubes with atmospheric air purified and moistened by being passed through norite, ascarite, and water. The progress in cell division was followed by counting undivided mother cells. This number was subtracted from the initial number of cells in a given volume of the suspension and the difference, describing the number of divided cells, was expressed in per cent of the initial number of cells. pH measurements were made with glass electrode on Beckman Zeromatic pH

meter. Dry weight measurements were made by drying aliquots of cell suspension to a constant weight at 100°.

Results

Typical observations on the effect of acid concentration on cell division are presented in Figure 1. Concentrations of sulfuric acid in the suspending fluid ranged, in this experiment, from 1×10^{-1} to 1 mM/l with corresponding pH from 3.5 to 2.5. After 16 hours, the pH in some cell suspensions drastically changed, depending on the initial concentration of sulfuric acid. At 1×10^{-1} mM and 2×10^{-1} mM H_2SO_4, the pH increased from 3.5 and 3.3 to 8.8 and 8.5, respectively. Cell division was close to 100 per cent. At 3×10^{-1} mM H_2SO_4, the pH changed from 3.1 to 7.2, and only a negligible number of cells (less than 10 per cent) divided after 16 hours. At acid concentrations of from 4×10^{-1} mM to 1 mM, changes in pH were small and no division was recorded.

Fig. 1

A change in the pH of the medium was not caused by an instantaneous leak from cells of substances already present in the cells at the time of their transfer into a given suspending fluid. If this were so, then washing of cells in H_2O would remove these substances and lower or nullify the subsequent ability of cells to adjust the pH of a suspending fluid. In an experiment shown in Figure 2, cells, before being resuspended in an acid-containing medium, were washed with distilled water and centrifuged between washings. The number of washings had no effect on the subsequent ability of cells to change the pH of the suspending fluid. The change in the pH generally depended on the original acid concentration. Cell division was observed at 5×10^{-2} mM

Fig. 2

H_2SO_4 and was not affected by the washing of cells. At 6×10^{-2} mM H_2SO_4, cell division took place only in suspensions in which the pH, after 16 hours, was close to or above 8. No cell division was observed at 7×10^{-2} mM or at higher concentrations of H_2SO_4.

Fig. 3 The effect of cell activity on the pH of the surrounding medium is a gradual and, therefore, time-dependent phenomenon. Figure 3 shows the time course of changes in pH of suspending fluids containing different concentrations of H_2SO_4. At 1×10^{-1} mM H_2SO_4, the pH rose in 3 hours from 5.7 to 7.2. Then, after 48 hours from the beginning of the experiment, it attained the final value of 9.0 and remained at this level. At 1 mM, the pH increased slowly with time and, even after 96 hours, it was well below that attained by the cell suspension in 1×10^{-1} mM H_2SO_4. At higher acid concentrations of 10 and 100mM, the rise in pH was very slow and almost negligible. Cell division was observed at 1×10^{-1} mM, but not at higher acid concentrations. At 1 mM H_2SO_4, cells were probably damaged before the pH reached a physiologically tolerable level.

The ability of cells to affect the pH of the medium is not specifically bound with the division stage. In another experiment, 0-hour cells, in which cell division was completed, were transferred into 1×10^{-1}, into 5×10^{-1} mM H_2SO_4, and into distilled water. A change in

Fig. 4 pH with time was observed in all three media (Figure 4).

The critical inhibitory acid concentration recorded in experiments shown in Figures 1 and 2 differed: in the experiment shown in Figure 1, it was 3×10^{-1} mM, and in that shown in Figure 2 it was 7×10^{-2} mM. This might be due to the difference in the size of the inoculum. In these and previously reported experiments (10, 11), the amount of cells per volume of the suspension was the same only within the experiment.

In different experiments it varied and was, therefore, not comparable.

Fig. 5 In the experiment depicted in Figure 5, two series of acid concentrations were studied with inoculum in one series 8 times larger than that in the other. In the series with lighter inoculum, the dependence of final pH and of cell division on the initial acid concentration was clearly seen. At 9×10^{-2} mM H_2SO_4, the pH, after 16 hours, was still below 7; and at 1.1×10^{-1} mM, it did not reach 5. No cell division was recorded at these two acid concentrations. The pH reached a value of about 8, and the cell division took place only at 7×10^{-2} mM H_2SO_4 and at lower acid concentrations. Acid concentrations (9×10^{-2} and 1.1×10^{-1} mM), proved to be inhibitory to cell division in the series with lighter inoculum, had no inhibitory effect in experiments with heavy inoculum. The activity of a larger amount of cells affected the suspending medium in such a way that the pH, after 16 hours, increased to 9; and cell division was observed in all tested acid concentrations.

Fig. 6 In a number of later experiments, the inhibitory acid concentration was determined at different levels of inocula expressed in grams of dry weight of cells per liter of suspension. The results are plotted on a semi-logarithmic scale shown in Figure 6. An acid concentration (abscissa) found to be critical at a given size of the inoculum (ordinates) was marked and the points were connected to give three curves. The two upper curves with shaded area between them are plotted on an expanded scale (indicated on the left ordinate) to demonstrate the dependence of cell division on acid concentrations at low levels of the inoculum. The higher of these two curves (filled triangles) is for acid concentrations still non-inhibitory to cell division; and the other curve (filled circles) is for concentrations proved to be inhibitory. The lower curve to the right

(open circles) plots inhibitory acid concentrations over a wider range of inocula on a telescoped scale indicated on the right ordinate.

If data presented in Figure 6 were plotted on a non-logarithmic scale, then the dependence of acid concentration critical to cell division on the size of the inoculum would be, within limits, linear, and the slope of the straight line would indicate how much the size of the inoculum must be increased to cancel a given increase in acid concentration. Calculations presented in Table 1 indicate that, to counteract an increase in acid concentration from 5×10^{-2} to 9×10^{-1} mM, dry weight of cells must be increased from 0.1465 to 4.462 gm/l. Thus, 18-fold increase in acidity involves a 30.5-fold increase in dry weight. The ratio between number of doublings in acidity and that in dry weight is 1:1.2. This ratio gives a relative rate of change in these two variables and is a generalized indicator of the relationship between the acidity of the medium and the size of the inoculum as two parameters affecting cell division under the conditions of these experiments.

Table 1

Discussion

The relationship between a living cell and its liquid environment is complex. Both influx and efflux of ions and undissociated molecules take place simultaneously across the cell membranes. The transport of substances into and out of cells has been considered in a number of reviews (4, 5). Algae have been a favorite subject in studies on the permeability of cell membranes (1). However, an active secretion by algal cells, thought to be responsible for their buffering capacity, is a neglected field barely touched upon in reviews on algal physiology (2, 3).

The buffering capacity of cells greatly depends on the environmental factors prior to and during observations, particularly on the composition

and concentration of the medium. The physiological condition of cells and the level of their metabolic activity are also of primary importance. These "inner" factors depend to a great extent on the environmental conditions. An interplay of the external and internal factors takes place against the genetic background. The hereditary constitution of the organism determines the range of variations and the level of the response of the organism to the conditions of its outer and inner environment.

As a vital phenomenon, the secretion of substances with buffering properties extends over a period of time. During this period it can be enhanced by favorable conditions or impeded and even stopped by unfavorable factors. The effects of the concentration of bicarbonate ions and of temperature were reported elsewhere (11).

Under given experimental conditions, the capacity of cells to buffer a certain volume of the suspending fluid depends on their number. Previously reported pH values critical for cell division in marine eggs (6) and acid concentrations affecting cell division in algae (12), as well as carbon dioxide concentrations inhibitory to the division in marine eggs (7) and in algae (10, 11) are, therefore, particular cases of a more generalized picture involving the buffering capacity of cells, which enables them to counterbalance the inhibitory effects of these agents.

A ratio of the amount of cells in a given volume of suspending fluid to the amount of acid that cells are capable of buffering would be a convenient index descriptive of the buffering capacity of cells. However, this ratio depends on the acidity of the medium. With the increase in acidity, the ratio of the amount of cells necessary to buffer a new level of acidity becomes disproportionally higher. If concentrations of cells and of the acid are expressed in terms of $\log_2$, then an increase by one

unit, that is a doubling, in acid concentration requires 1.2 units of the increase in cell concentration to buffer a new level of acidity.

Two factors may cause a deviation of this proportion from 1:1 ratio. One is a time factor. At an acid concentration high enough to suppress the secretive activity of the cells, the slow-down in secretion will expose cells to a harmful acid concentration for a longer time. The ensuing injury to the secretive capacity will result in disproportionally more cells required to buffer the new level of acidity. Another factor is metabolically produced carbon dioxide. With the increase in population density, the effect of carbon dioxide evolved by the cells will add to the effect of the initial acid concentration and, under certain conditions, may become by itself a decisive factor limiting cell activity. With further increase in acid concentration each of these factors will cause the amount of cells necessary to buffer the acidity to be increasingly higher and eventually will stop division at any cell concentration.

The calculated ratio, 1:1.2, has no universal significance since it depends on the external factors, physiological condition of cells, and hereditary constitution of the organism. But the concept of referring the inhibitory acid concentration to the amount of cells may prove to be useful in this and related fields. The slope of the curve relating these two variables is characteristic of the buffering capacity of cells. The concept can also be useful in relating other cell activities (growth, respiration, and, in general, enzyme activity) to the changes in acidity. It can possibly be broadened to include external factors other than acidity, e.g., poisoning. In studies of the effects of the concentration of hydrogen ions and of other external factors, the activity of cells that enables them to counterbalance and to withstand the adverse conditions is of primary biological importance.

Summary

Buffering capacity of cells was evaluated in terms of its effects on changes in pH of the suspending fluid and on cell division. Synchronized 7-hour cells of the green, high-temperature alga, Chlorella 7-11-05, were centrifuged out of a complete nutrient medium, resuspended in different concentrations of sulfuric acid, and supplied in darkness with atmospheric air. It was observed that the effect of buffering activity of cells on pH of the surrounding medium was a gradual, time-dependent phenomenon. Changes in pH and the degree of the completion of cell division depended on the initial acidity of the medium and on the amount of cells per volume of suspension. With the increase in acidity, the amount of cells required to buffer it increased disproportionally faster. Thus, within certain range of acid concentrations, a doubling in acidity required 2.2 times increase in the amount of cells necessary to buffer the new level of acidity. Factors affecting buffering activity of cells were discussed.

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Table 1. Calculations of the ratio of acid concentration critical for cell division to size of inoculum required to counterbalance it.

Characteristic	Unit	Absolute values from	Absolute values to	Increase fold	Log_2 of the increase	Ratio
Acid concentration	mM/l	0.05	0.9	18	4.16	1
Inoculum dry weight of cells	gm/l	0.1465	4.462	30.5	4.93	1.2

Legends

1. Cell division and pH in suspensions of synchronized 7-hour cells of Chlorella 7-11-05 after 16 hours in darkness in different concentrations of H_2SO_4. Circles, pH of the suspending fluids at the beginning of the experiment; triangles, pH of cell suspensions after 16 hours in darkness; crosses, cell division after 16 hours in darkness in per cent of the original number of cells.

2. Cell division and pH in suspensions of synchronized 7-hour cells of Chlorella 7-11-05 after 16 hours in darkness in different concentrations of H_2SO_4. After harvest and before being placed in darkness, cells were washed in distilled water. Circles, no washing; triangles, one washing; squares, two washings; crosses, three washings. The arrow perpendicular to the curves separates variants in which cell division took place (upper left portion of the curves) from those in which there was no cell division (lower right portion of the curves).

3. Time course of changes in pH in suspensions of synchronized 7-hour cells of Chlorella 7-11-05. Concentrations in mmoles/liter of H_2SO_4 are indicated on the curves.

4. Time course of changes in pH in suspensions of synchronized 0-hour cells of Chlorella 7-11-05. Concentrations in mmoles/liter of the H_2SO_4 are indicated on the curves.

5. Effect of the size of the inoculum on cell division and pH in suspensions of synchronized 7-hour cells of Chlorella 7-11-05 after 16 hours in darkness in different concentrations of H_2SO_4. Circles, cell division; triangles, pH; open symbols, light inoculum; filled symbols, heavy inoculum.

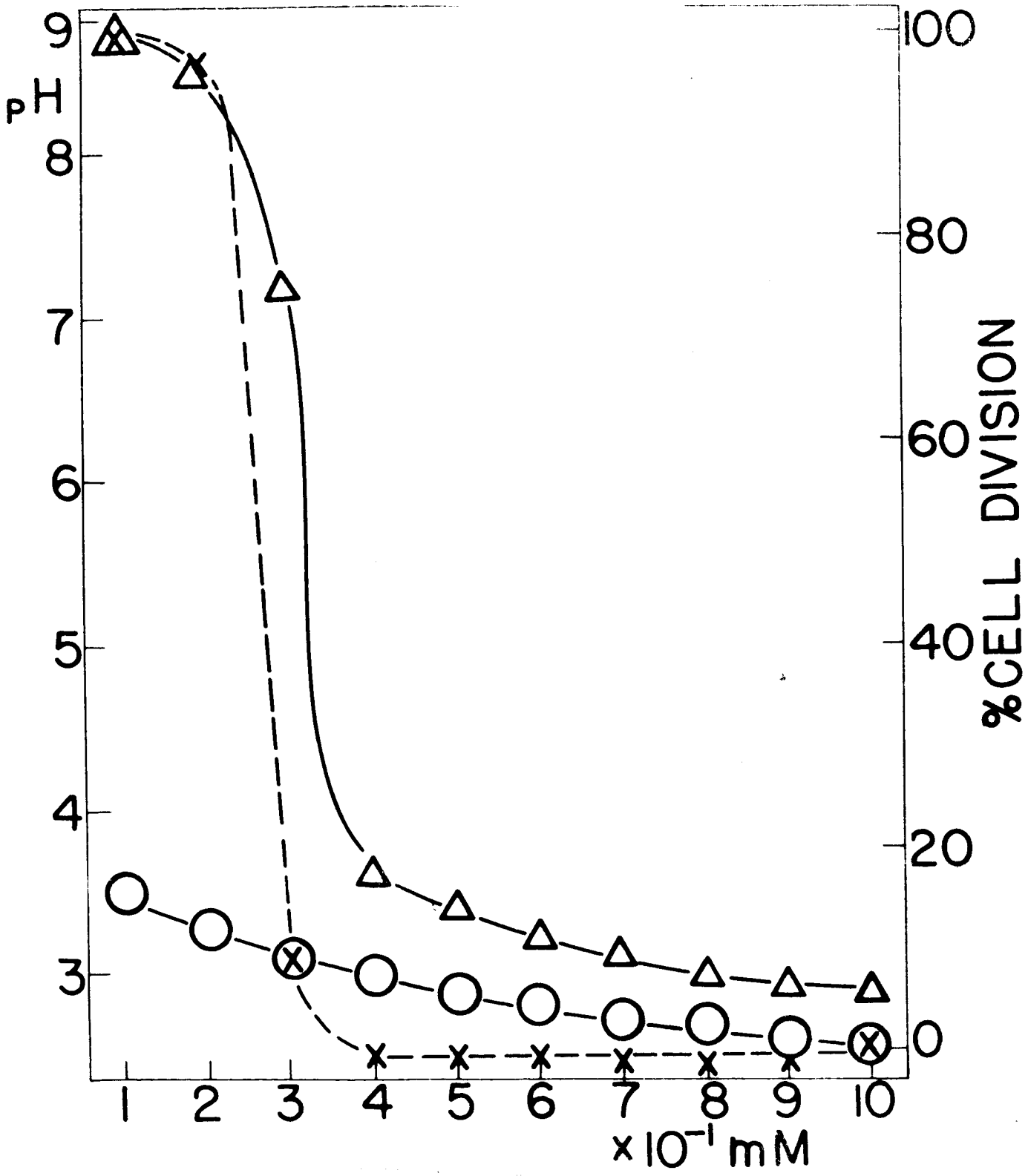
6. Critical concentrations of H_2SO_4 affecting cell division in synchronized 7-hour cells of Chlorella 7-11-05 at different levels of inoculum. Circles, inhibitory H_2SO_4 concentrations; triangles non-inhibitory concentrations. Open symbols plotted on the telescoped scale (right ordinate); filled symbols plotted on the expanded scale (left ordinate).

Footnotes - Page 1

1. The work was supported by funds from the National Aeronautics and Space Administration.

2. This is a scientific Article A 1016, Contribution No. 3405 of the University of Maryland Agricultural Experiment Station.

Figure 1


pH
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8
7
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20
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%CELL DIVISION
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4
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10
x10⁻¹ mM

Figure 2

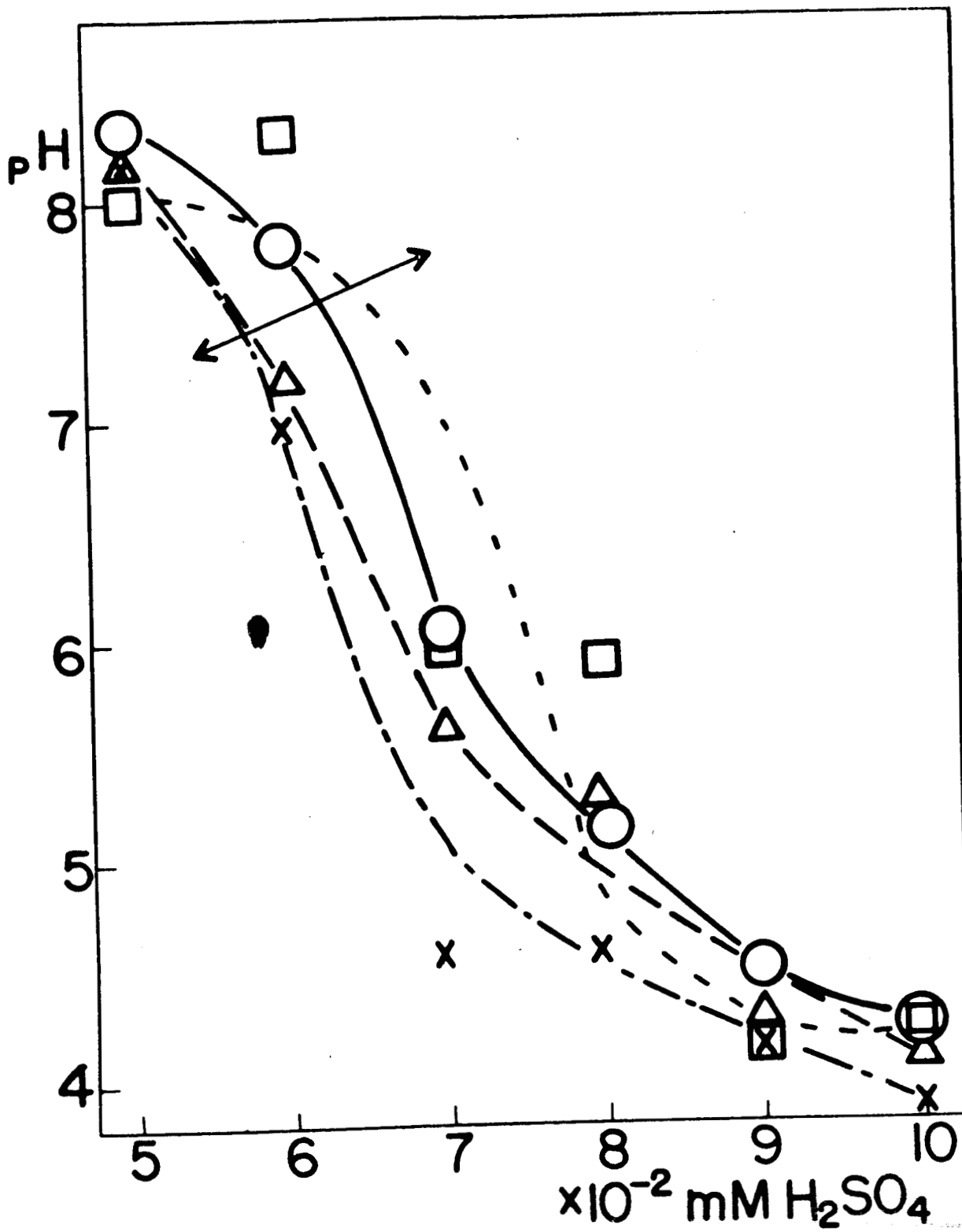
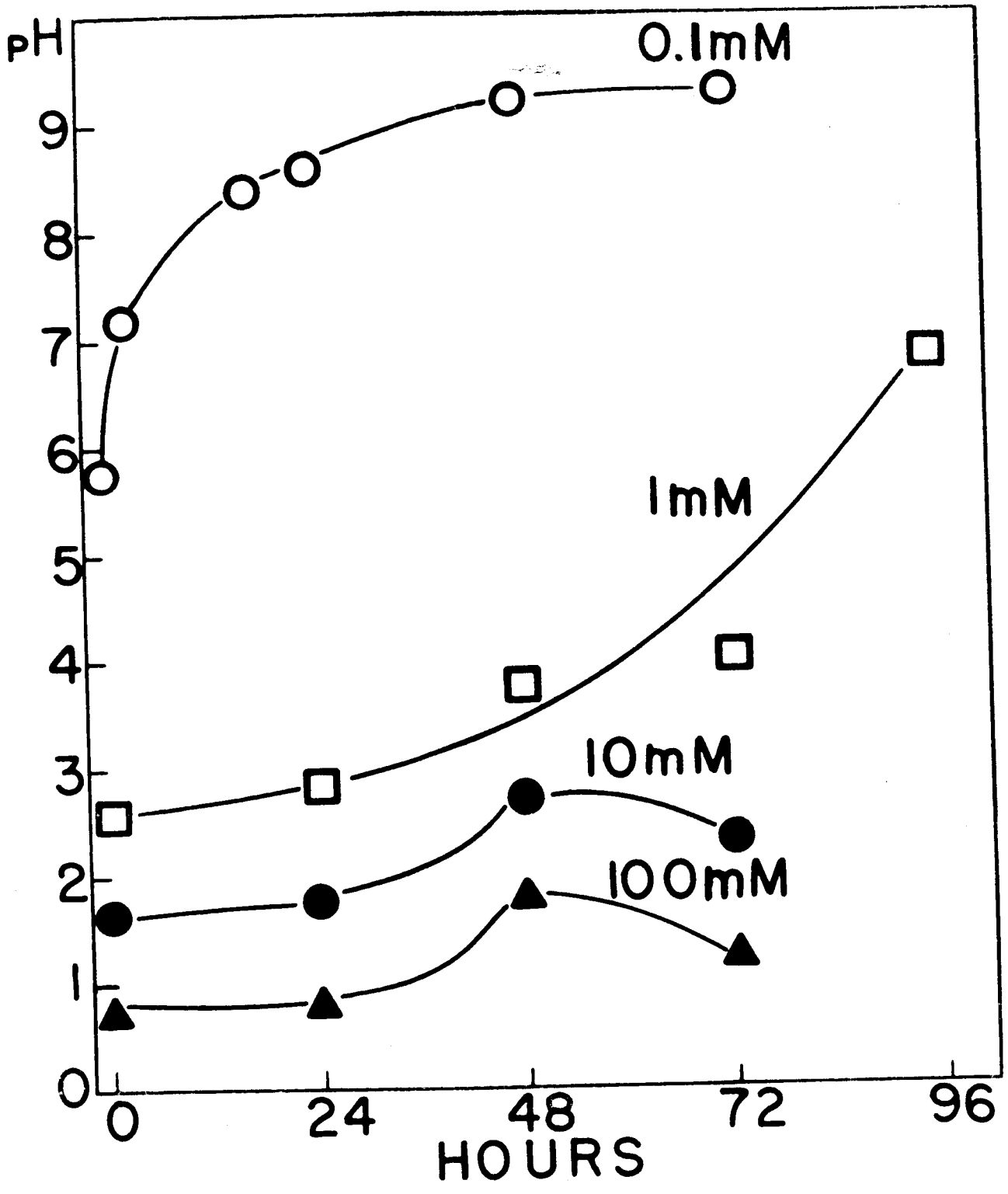
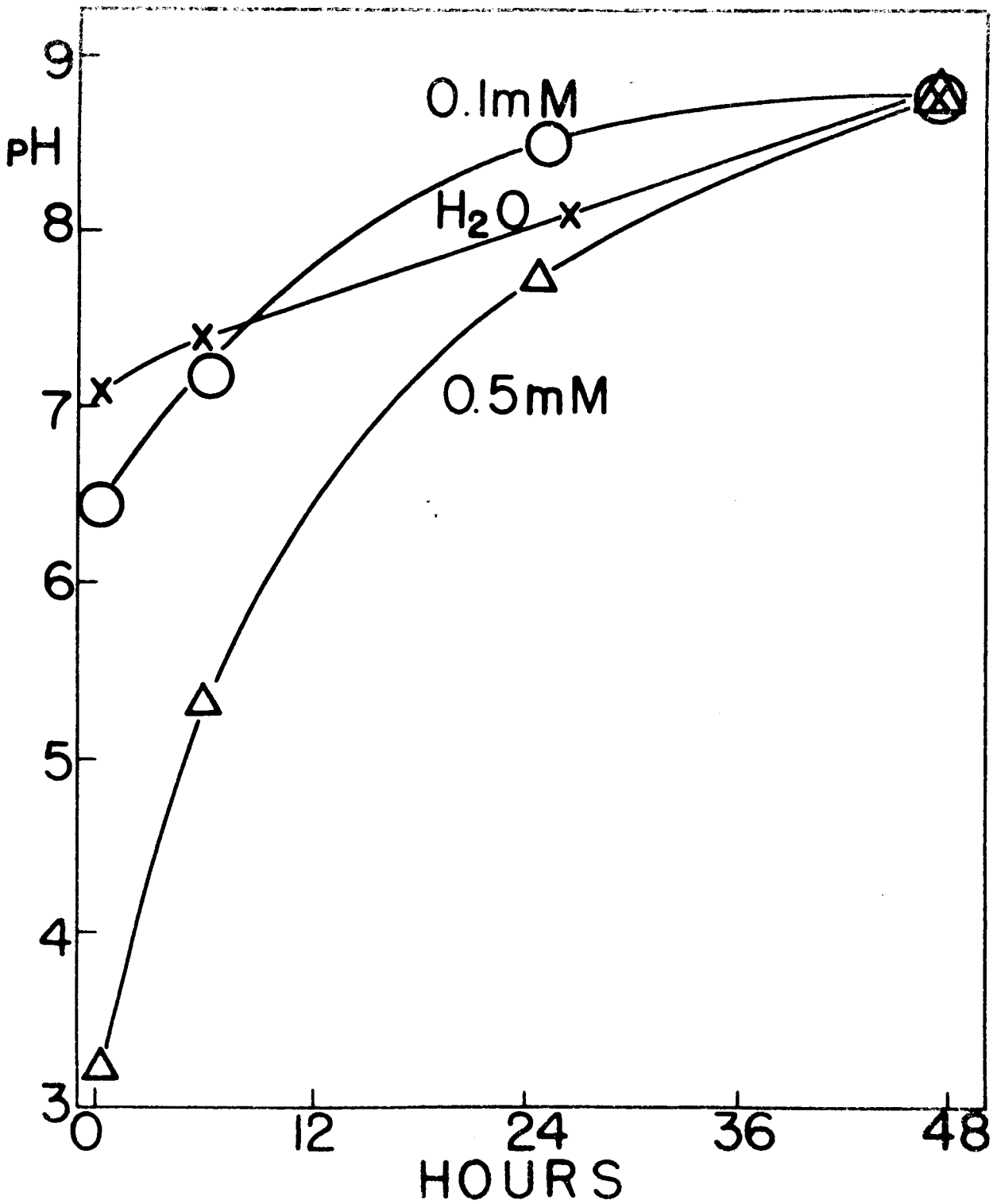


Figure 3




9
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6
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3
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H_2O
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12
24
36
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HOURS

pH
% CELL DIVISION
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80
60
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7
6
5
1
3
5
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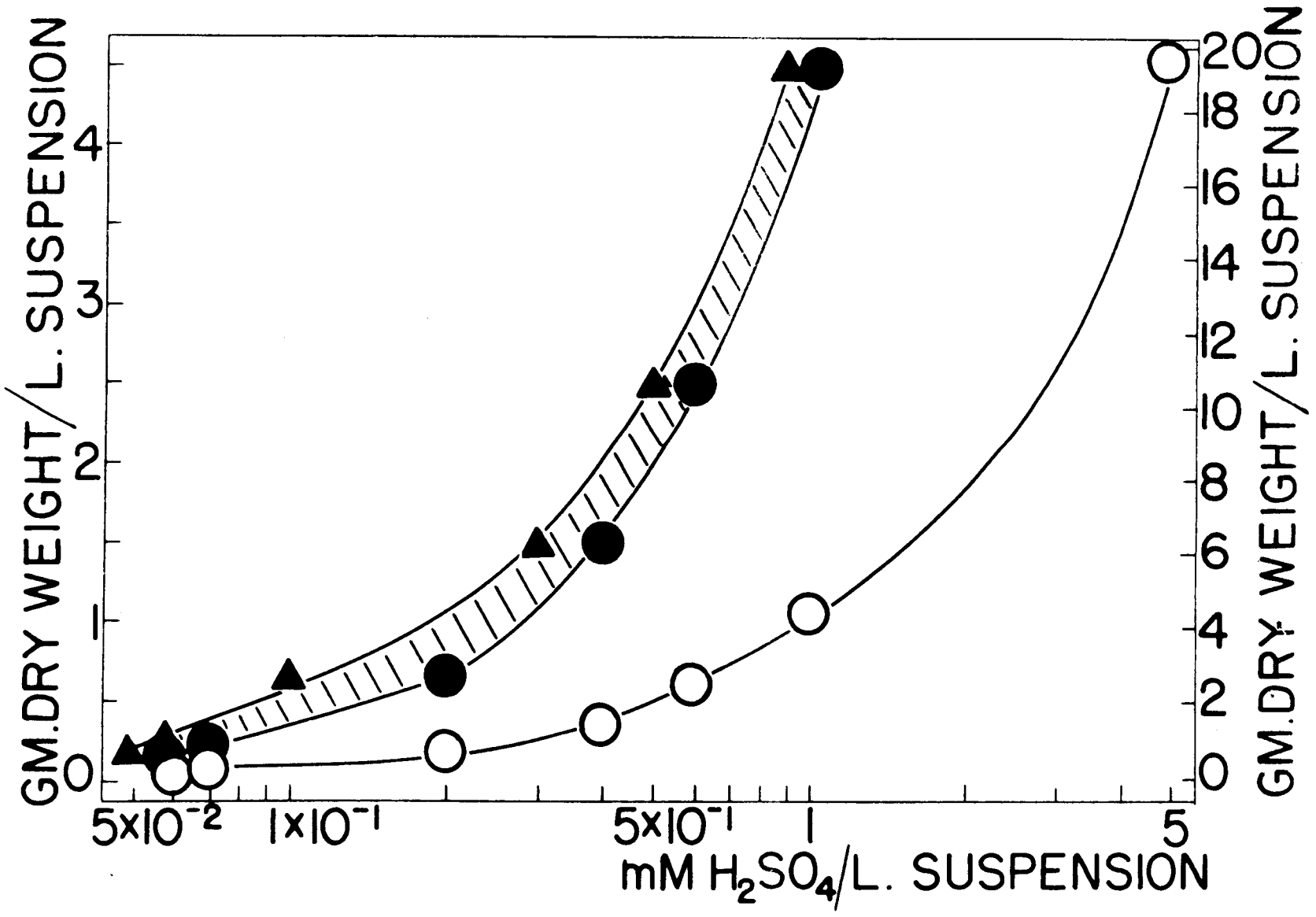
9
11
× 10⁻² mM

Figure 6


GM.DRY WEIGHT/L. SUSPENSION
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1
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5x10⁻²
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8
10
12
14
16
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20
GM.DRY WEIGHT/L. SUSPENSION