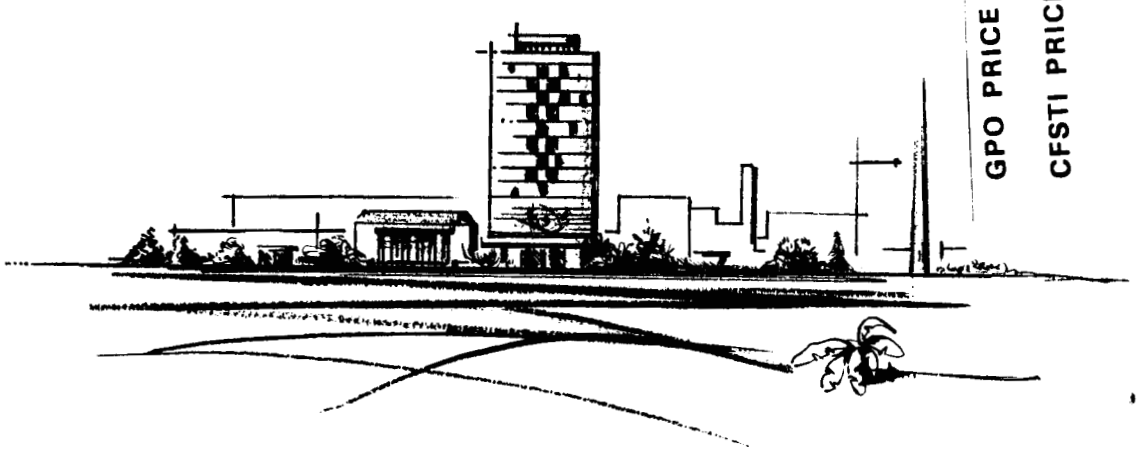
RESEARCH REPORT

[redacted]



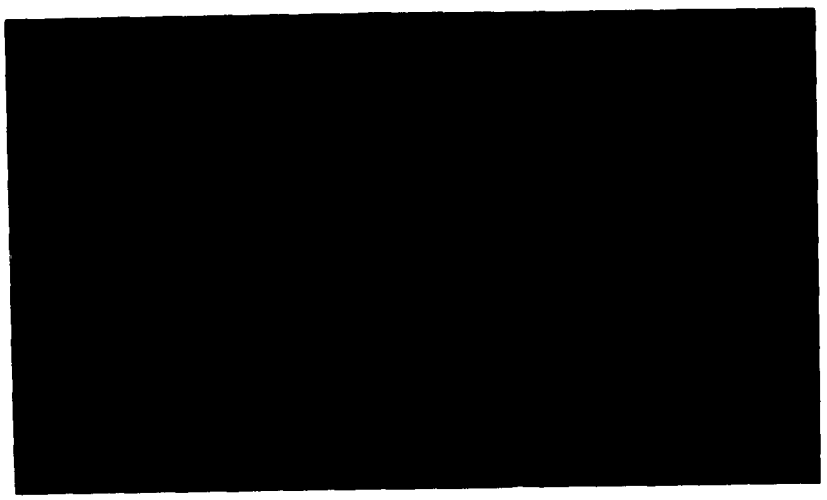
BATTELLE MEMORIAL INSTITUTE

COLUMBUS LABORATORIES

BATTELLE MEMORIAL INSTITUTE

COLUMBUS LABORATORIES • 505 KING AVENUE • COLUMBUS, OHIO 43201

DEDICATED TO THE ADVANCEMENT OF SCIENCE



FIELDS OF RESEARCH

Aeronautics — Astronautics
Agricultural Chemistry
Agricultural Economics
Alloy Development
Applied Mathematics
Area Economics
Biochemistry
Biophysics — Bionics
Catalysis — Surface Chemistry
Ceramics
Chemical Engineering
Chemical Processes
Communications Science
Computer Technology
Corrosion Technology
Earth — Atmospheric Sciences
Electrochemistry
Electronics
Energy Conversion
Engineering — Structural Materials
Environmental Systems
Extractive Metallurgy
Extreme-Temperature Technology
Ferrous Metallurgy
Food Technology
Foundry Practice
Fuels — Combustion
Glass Technology
Graphic Arts Technology
Immunology — Cancer Studies
Industrial Economics
Industrial Physics
Information Research
Inorganic Chemistry
Instrumentation
Light Alloys — Rare Metals
Lubricant Technology
Materials Separation — Concentration
Mechanical Engineering
Metal Fabrication Engineering
Metal Finishing
Metallurgical Processes
Microbiology
Microscopy — Mineralogy
Nondestructive Evaluation Technology
Nonferrous Metallurgy
Nucleonics
Ocean Engineering
Organic Chemistry
Organic Coatings
Packaging Research
Particle Dynamics
Petrochemicals
Petroleum Engineering
Pharmaceutical Chemistry
Physical Chemistry
Production Engineering
Psychological Sciences
Pulp — Paper Technology
Radioisotopes — Radiation
Reactor Technology
Refractories
Reliability Engineering
Rubber — Plastics
Semiconductors — Solid-State Devices
Sound — Vibration
Systems Engineering
Textiles — Fibers
Theoretical — Applied Mechanics
Thermodynamics
Transportation
Welding — Metals-Joining Technology
Wood — Forest Products

FOURTEENTH QUARTERLY STATUS REPORT

on

THE CULTIVATION OF HYDROGEN-FIXING BACTERIA

to

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

Contract NASr-100(03)

October 25, 1966

by

J. F. Foster

Period Covered: July 1, 1966 to September 30, 1966

BATTELLE MEMORIAL INSTITUTE
Columbus Laboratories
505 King Avenue
Columbus, Ohio 43201

Battelle Memorial Institute · COLUMBUS LABORATORIES

505 KING AVENUE COLUMBUS, OHIO 43201 · AREA CODE 614, TELEPHONE 299-3151 · CABLE ADDRESS: BATMIN

November 21, 1966

Office of Grants and Research Contracts
Code SC-NASr-100(03)/36-02-903
National Aeronautics and Space Administration
Washington, D. C. 20546

Gentlemen:

Task Order Contract NASr-100(03)/36-02-903
"Research on Techniques and Procedures for
the Cultivation of Hydrogen-Fixing Bacteria"

This is the fourteenth quarterly status report on the experimental study of the continuous culture of Hydrogenomonas eutropha. It describes and presents tentative interpretations of the experimental results from six runs in the continuous-culture apparatus, in which the organism was grown under a controlled environment for periods of 3 to 5 days in each run.

Yours very truly,

John F. Foster

John F. Foster
Principal Investigator

JFF:js
Enc: 25 copies

FOURTEENTH QUARTERLY STATUS REPORT

on

THE CULTIVATION OF HYDROGEN-FIXING BACTERIA

by

J. F. Foster

INTRODUCTION

Six runs were made in the continuous-culture apparatus during the quarter. Most of the runs extended for about 100 hours each, or the equivalent of 1 work week of 5 days and 4 nights. In general, alternate weeks were used in preventive and corrective maintenance of the many automatic components of the total system, in calibrations, and in studying the data collected from the preceding run. Interpretations of these data were used to develop hypotheses for mechanisms of growth and nutrient assimilation as a guide for the selection of environmental conditions in the following run. Data from the first four runs were analyzed in moderate detail, and the results led to interpretations that are supported by a more detailed examination of the data from the fifth run. These latter data are presented and discussed in this report, because the results show growth rates much higher than had been previously observed with this organism. The sixth run was made during the final week of the period. The results have not been interpreted yet, but qualitative examination indicates the optimum growth conditions were not approached as nearly as they were for the fifth experiment.

OBJECTIVE

The long-range objective is to select optimum environmental conditions for continuous cultures growing in steady state at high conversion efficiencies. This objective remains unchanged, as discussed and described in the preceding report. During this period, a short-range objective was to develop procedures for maintaining rapid growth and for reaching high cell densities during the batch-growth phase that must precede the establishment of active steady-state growth.

BACKGROUND OF CULTURE CHARACTERISTICS

As background for the description and discussion of experimental results in following parts of this report, this section reviews some characteristics of the growing cultures that have been observed in past experiments.

After inoculation, the culture passes through a lag period of slow growth, goes into logarithmic growth, and then growth decelerates. The sequence is similar to that reported for batch growth of many different organisms in the bacteriological literature. Deceleration in growth following the logarithmic phase has been attributed generally to a depletion of the nutrient composition so that deficiency of one component limits growth. Paralleling depletion, some bacteria might be discharging an inhibitory product which retards growth as its concentration increases with the rise in cell population.

The culture apparatus used in this research program has been designed and developed to maintain a constant environment in which the major nutrients are made available at preset concentration levels and are replaced as rapidly as they are consumed. Much effort was devoted to the exploratory study of various concentrations of urea and CO_2 maintained in the environment as the major sources of cellular nitrogen and carbon. There appeared to be no simple correlation between substrate concentration, rate of substrate conversion, pH range, and the growth rate of the organism. The culture characteristically would undergo unpredictably different growth rates in the logarithmic phase of successive cultures and would often approach a resting stage at a maximum cell concentration of about 2.5 grams (dry weight) per liter.

Upon some occasions, when growth was decelerating, an abrupt change in culture environment might renew growth at a logarithmic rate somewhat lower than was observed in the preceding logarithmic phase. The growth inducing change might be diluting the culture with an equal volume of fresh nutrient, changing the urea concentration, adding concentrated urea solution, or diluting continuously with fresh medium at a dilution rate lower than the growth rate. By such empirical means, it was possible to reach cell densities as high as about 5 grams (dry weight) per liter on occasion. However, reactivated growth did not always take place. Also, the growth rate was slow at this level, and the culture trended irreversibly toward a resting condition.

Additional observations of culture characteristics indicated that

(1) The pH could change spontaneously, and the change might be in either direction.

(2) The pH trended upward as the resting phase was approached, and it was necessary to add relatively large amounts of acid to maintain pH below 7.

(3) Urea consumption rates did not appear to be related to other culture characteristics, such as growth rates or cell density in the culture.

(4) When a known amount of urea was added to the culture, the measured increase in its concentration in the medium was usually lower than would be calculated from the amount added.

(5) An abrupt change in culture environment, as outlined above, was sometimes followed about 3 hours later by a spontaneous abrupt change in measurable characteristics of the culture.

(6) Gas consumption rates were proportional to cell density during the first part of the logarithmic phase, but a sharp decrease in rates almost always occurred before the limit of logarithmic growth of the cells was reached.

These observations combined with the data presented in following sections lead to a hypothetical mechanism of nutrient assimilation.

A decomposition product derived from urea (possibly ammonia) may be the primary nitrogen source utilized by the organism. Details are given in the discussion.

EXPERIMENTAL RESULTS

Four experimental runs are described briefly because each contributed some guidance for the next experiment, although none met the objective of a high cell density with a high growth rate.

Run 23035-51* was set up to test the possibility of maintaining optimum concentrations of urea and CO_2 in the culture by adding urea to compensate for low pH and by increasing CO_2 partial pressure to compensate for high pH. The pH limits were arbitrarily set at about 6.0 and 7.0.

The initial concentration of urea in this run was about 0.35 grams per liter. After inoculation the culture grew at a moderate rate equivalent to a logarithmic growth-rate constant of K = 0.16.**

The urea concentration was permitted to decrease as growth proceeded. Continuous dilution was started at a dilution rate D = 0.17** after 13 hours of batch growth, at which time the cell density was about 0.4 gram per liter. About 4 hours after the beginning of continuous dilution, the decreasing urea concentration reached the preset minimum of 0.1 gram per liter; thereafter urea was added periodically on demand to maintain urea concentration at this value.

The pH fluctuated slightly between 6.1 and 6.2, but never reached the extreme limits that would have caused corrective additions of urea or CO_2. The pH fluctuations, however, occurred in a regular time cycle that correlated with fluctuations in urea demand and gas consumption. Specifically, the pH would decrease spontaneously to 6.1 for a period of 2 to 3 hours and concurrently the urea demand and gas consumption would rise. Then the pH would rise to 6.2 for 6 to 8 hours and the input rates for urea and gas would then decrease. This fluctuation pattern was superposed on a continuous rise in average demand for all materials which correlated proportionately with a continuous slow rise of cell density in the culture. The cell density was increasing because the growth rate of about K = 0.20 was slightly higher than the dilution rate of D = 0.17.

These pH fluctuations were barely within the limits of detection of the pH recorder, but they were sufficiently regular and reproducible to be qualitatively significant. In retrospect, it is evident that the culture medium was too highly buffered to permit much pH fluctuation. Buffer concentration was decreased in later runs, and the periodic changes in culture composition were identified by larger pH changes.

*Experimental runs are referenced by the serial number and page of the notebook in which are recorded the primary data.
**See preceding report, pages 5 and 6, for definition of K and D.

In this experiment, grab samples of the culture were analyzed for protein content. The protein content in unit volume of culture was approximately proportional to the total cell weight in the same sample, as would be expected. However, the rate of rise in cell density was so slow that the experiment was terminated when the cell density reached about 2 grams per liter.

A few samples were used to obtain a count of viable cells per unit volume of culture. The viable cell count was approximately proportional to total cell weight in three out of four determinations. These were exploratory measurements that will have to be repeated to obtain quantitative results.

Run 23035-57 was conducted primarily to examine culture growth in a medium with low urea concentration. The initial concentration was 0.1 gram per liter, and the concentration was allowed to decrease as the organism consumed and/or converted the urea inventory.* Initial growth was relatively slow at K = 0.14. Measurable urea concentration in the culture fell at a constant rate to less than 0.01 gram per liter 13 hours after inoculation, at which time the cell density was 0.3 gram per liter. At the same time the gas-consumption-rate curve changed sharply from its initial rising trend and remained constant for about 6 hours. A similar break is usually observed whenever the culture medium becomes deficient in some requirement for vigorous growth, and the break is always followed after some hours by a sharp decrease in growth rate. In this culture, growth continued for 7 hours, and cell density reached 0.8 gram per liter before growth virtually ceased.

It was possible to estimate a material balance from these observations and with the assumption that all the nitrogen inventory originally supplied in the medium must have been incorporated in the cell structure by the time growth ceased. On the basis that there was originally 0.1 gram urea per liter of medium and that all of this was converted and assimilated in a series of chemical and biological steps to form 0.8 gram of cells per liter, cell nitrogen could not represent more than about $(0.1 \times 0.467/0.8) \times 100 = 5.8$ percent of the cellular material. This is about half of the (approximately) 12 percent nitrogen found by chemical analysis of cells harvested from an actively growing culture. This material balance supports the observations reported in the eighth quarterly report, in which it was concluded that there were three and possibly four modes of urea conversion in Hydrogenomonas cultures. The current experiment represents Mode 1/2 in which half as much urea is assimilated by a slowly growing culture in a urea-deficient medium as would be assimilated during active and vigorous growth.

This interpretation was further supported in the current culture studies by adding a single dose of urea equivalent to 0.18 gram urea per liter. Growth was resumed and gas consumption rose immediately. The cell density increased at a slow rate and reached about 2.8 grams per liter after about 30 hours. The converted urea nitrogen thus represented less than 5 percent of the incremental cell production during the conversion period. Intermittent dosages with urea continued to support slow growth with correspondingly increasing gas consumption, until the cell density reached 5 grams of cells per liter of culture before the experiment was terminated.

These observations indicate that there are probably no inhibitory by-products formed by the culture when growth is limited by nitrogen deficiency. The culture is characteristically whiter in color, and it tends to foam strongly while being stirred.

*Many of the changes described for this and following cultures are qualitatively similar to those illustrated graphically in Figure 1 and presented later with the discussion of Run 23035-75.

Run 23035-62 used a medium containing 0.75 gram urea per liter. Logarithmic growth proceeded without dilution at a rate of K = 0.16. As soon as the growth rate started to decrease, half the culture was discarded and was replaced by diluting the remainder 1:1 with fresh nutrient. This procedure tended to accelerate growth for a brief period, but overall growth rate still decreased with time. The 1:1 dilution was repeated twice at intervals of about 12 hours, and the cell density approached 1.5 grams per liter before continuous dilution was started. No marked effect on growth rate occurred after each 1:1 dilution or during continuous dilution.

These observations indicate that deficiencies in trace or major nutrients are probably not the cause of progressively slower culture growth, since these were renewed with each addition of fresh nutrient. The alternative possibility is that of a buildup or variation in the inventories of one or more intermediate products derived from cellular or enzyme activity directed toward the urea. Such an intermediate need not be a growth inhibitor, but it conceivably could affect the extracellular chemical equilibria that determine the nutrient species available at the cell wall for transport into the cell. The nutrient composition, including unidentified intermediates, presumably also influences metabolism and feedback-control mechanisms within the organism.

Run 23035-68 was set up and controlled as an exploratory experiment to test partially the concept that fluctuations in the inventory of some unspecified intermediate may influence growth. The nutrient medium was made up with less phosphate buffer, so that pH changes might reflect more sensitively the formation or depletion of a decomposition product derived from urea. Experience had shown that pH changes occurred spontaneously during changes in growth rate, indicating that the hypothetical intermediate influenced the pH of the culture. The pH control was preset to add urea at a minimum pH and CO_2 at a maximum pH by overriding the controls that maintain urea and CO_2 at preset minimum concentrations. Initially, the urea concentration in the medium was 0.5 gram per liter. The urea concentration control was preset to maintain at least 0.1 gram per liter, so that the initial concentration could decrease from 0.5 to 0.1 gram per liter during the first growth period. The growth rate was about K = 0.18. This is significantly higher than the growth rate of K = 0.16 in the preceding experiment, when the urea concentration was 0.75 gram per liter initially. It is also higher than the growth rate of K = 0.14 in Run 23035-57 when initial urea concentration was 0.1 gram per liter.

The pH of the lightly buffered medium reflected changes in the culture environment that correlated with other changes in material demands. These changes, as outlined below, supported the hypothesis that an intermediate is indeed formed from urea and that it may serve as the primary nutrient in some of the growth periods. The pH of the medium was 6.5, which was reduced to 6.1 when CO_2 was dissolved in the medium from the gas phase, containing 6 percent CO_2. When the cell density reached 0.3 gram per liter, the pH of the culture started to increase. Growth continued, but rising gas demand leveled off 2 hours later and pH stabilized at 6.4. Anticipating a decrease in growth rate, half the culture was removed when the cell density reached 1 gram per liter and was replaced with fresh medium. This abrupt change in culture environment induced several identifiable responses in the culture. A lag phase with a growth rate of K = 0.13 persisted for 1.7 hours after dilution, then a more rapid growth, with K = 0.19, started. Concurrently with the beginning of rapid growth, gas demand increased proportionately with increasing cell density. The pH decreased to a minimum of 6.1, then the trend spontaneously reversed and pH increased. When the cell density again reached 1 gram

per liter, both gas demand and growth rate levelled off, at which time the pH was 6.6. The charts showed an abrupt increase in urea-conversion rate 3.5 hours after dilution. This is equivalent to the doubling time of the culture when it was diluted. Similar changes correlated with doubling time have been observed repeatedly in other cultures. They are interpreted now as a change in the mode of urea conversion, induced by a feedback mechanism within the cell, which responds to environmental changes. It is hypothesized that the induced change in mode of urea conversion may be one in which ammonia is formed from urea by an extracellular enzyme released from the cell.

The pH continued to increase until it reached 7.0; at this preset maximum, further increase was prevented by compensating additions of CO_2. Growth rate had decreased to about K = 0.08. CO_2 additions to hold pH at 7.0 increased CO_2 partial pressure to 10 percent during 8 hours. Then pH-related CO_2 demand decreased and its partial pressure fell during the next 8 hours until the CO_2 control took over to maintain the CO_2 minimum concentration at about 6 percent partial pressure.

About an hour before the CO_2 partial pressure reached the minimum control point of 6 percent, the urea control was arbitrarily reset to raise the urea concentration from 0.1 to 0.3 gram per liter and to maintain the urea at this new concentration level. This change induced later changes in the culture activity that were qualitatively similar to those described above. Seven hours after the urea concentration was adjusted upward, the growth rate increased abruptly from K = 0.08 to K = 0.12 and gas demand showed an abrupt rise concurrently. The pH decreased slowly from 7.0 for an additional 4 hours until it reached 6.8. Then it decreased further in only 1 hour to 6.2, when the pH control added urea continuously for 1 hour to hold pH at this preset minimum. The pH trend abruptly reversed at this point, and pH increased in 1.3 hours to 7.0, where the rise was stopped by automatic CO_2 addition. Growth rate held steady at about K = 0.11 during this period, but gas demand increased at a rate greater than the increase in cell density while pH was falling from 6.8 to 6.2. Exactly at the time pH reached its minimum of 6.2 gas consumption levelled off. When the pH was controlled at 7.0 maximum by adding CO_2, there was a continuous rise in partial pressure up to 35 percent CO_2 in the gas phase, which then was held at this level for many hours. Growth did not appear to be affected and continued until the cell density reached 3.9 grams per liter. Finally, CO_2 partial pressure started to decrease and had reached 30 percent in the gas phase when the experiment was terminated. During the last day of the experiment the overall gas demand passed through a high maximum, then through a minimum that was still at a high rate compared with that in past experience. Finally, gas consumption rose from the minimum during the last 6 hours. During the last 6 hours growth rate dropped to K = 0.09 but presumably would have increased again (based on the final period of increasing gas rates) had there been time to continue.

The foregoing observations on the whole support the speculative hypothesis that there is an intermediate in the culture system which influences growth rate and pH as well. It is indicated that the rate of growth not only reflects the buildup or depletion of an inventory of some by-product or intermediate in the culture but also reflects the past environment during a period equal to the average generation or doubling time. One or more feedback mechanisms may be operating to determine culture activity at a selected time, and these mechanisms may have different time constants. Since the details of such mechanisms and their time constants are not yet understood, the next experiment was set up as an attempt at further clarification.

Run 23035-75 consisted of two consecutive batch cultures of about 30 hours each. Figures 1 and 2 show the plots of pH, cell density, gas rates, urea concentration, and CO_2 partial pressure as functions of time. Both cultures grew rapidly after inoculation. The pH rose slowly, passed through a broad maximum, then through a sharp minimum, and finally increased rapidly to the maximum permitted by the automatic control. A sharp break in gas consumption occurred when the pH reached its second maximum, and growth decreased. Urea conversion increased abruptly when pH was rising rapidly toward the second maximum, indicating that urea was decomposing more rapidly than it could be assimilated.

In order to estimate the amounts of unassimilated nitrogen and carbon accumulating in the culture medium that are not represented by dissolved urea and CO_2 (which are measured by continuous analyses) a material balance was calculated for each batch culture. Figure 3 shows the results of these calculations. It was assumed that the increments of cells produced in consecutive 2-hour periods contained 12.2 percent nitrogen and 53 percent carbon as products of the conversion of urea and CO_2. Urea converted during the same period, containing 46.7 percent nitrogen, was the total nitrogen input. Carbon input was the sum of urea carbon and CO_2 carbon, 20 percent and 27.3 percent, respectively, of urea and CO_2 converted during the same period. The cumulative differences between inputs and cell products equalled the inventories of aqueous nitrogen and aqueous carbon dissolved in the medium or adsorbed on the cell walls as unidentified intermediates.

Figure 3 shows that carbon and nitrogen inventories increased steadily in Batch 1, then started to decrease as rapid growth occurred. Then the culture apparently changed its mode of growth, with increased urea and CO_2 conversion and without a corresponding increase in assimilation. Batch 2 repeated the major features of Batch 1, but initial accumulation of inventories was slower, and the maximum in the nitrogen inventory was lower. Figure 3 also shows for convenient reference the variation in pH and the varying growth rates as K, calculated from the slopes of segments of the cell density curves of Figures 1 and 2.

CONCLUSIONS

(1) The initial growth period is critical in determining the course of active growth in denser cultures that are produced later.

(2) Urea is converted to one or more intermediate compounds, but their influence on future growth is not understood.

(3) Once the culture changes its mode of growth it is not yet known how it can be induced to revert rapidly to the initial mode, in which efficiency of conversion is higher and growth is more active.

(4) The change in mode of growth is identified by a sharp pH minimum, followed by a rapid rise. Presumably, this is caused by release of ammonia as an extracellular decomposition product from urea.

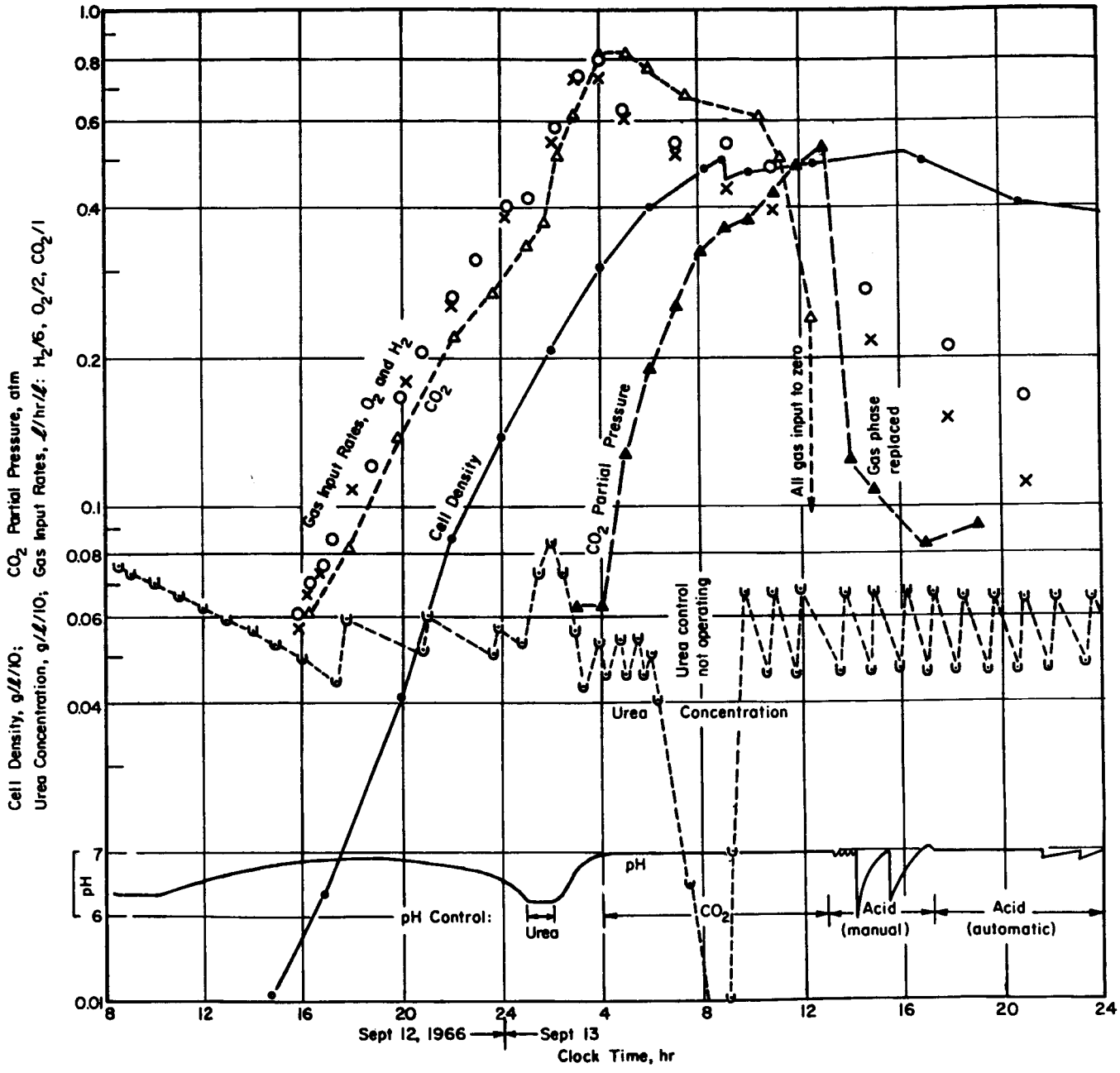


FIGURE 1. FIRST BATCH CULTURE OF HYDROGENOMONAS EUTROPHA, RUN 23035-75

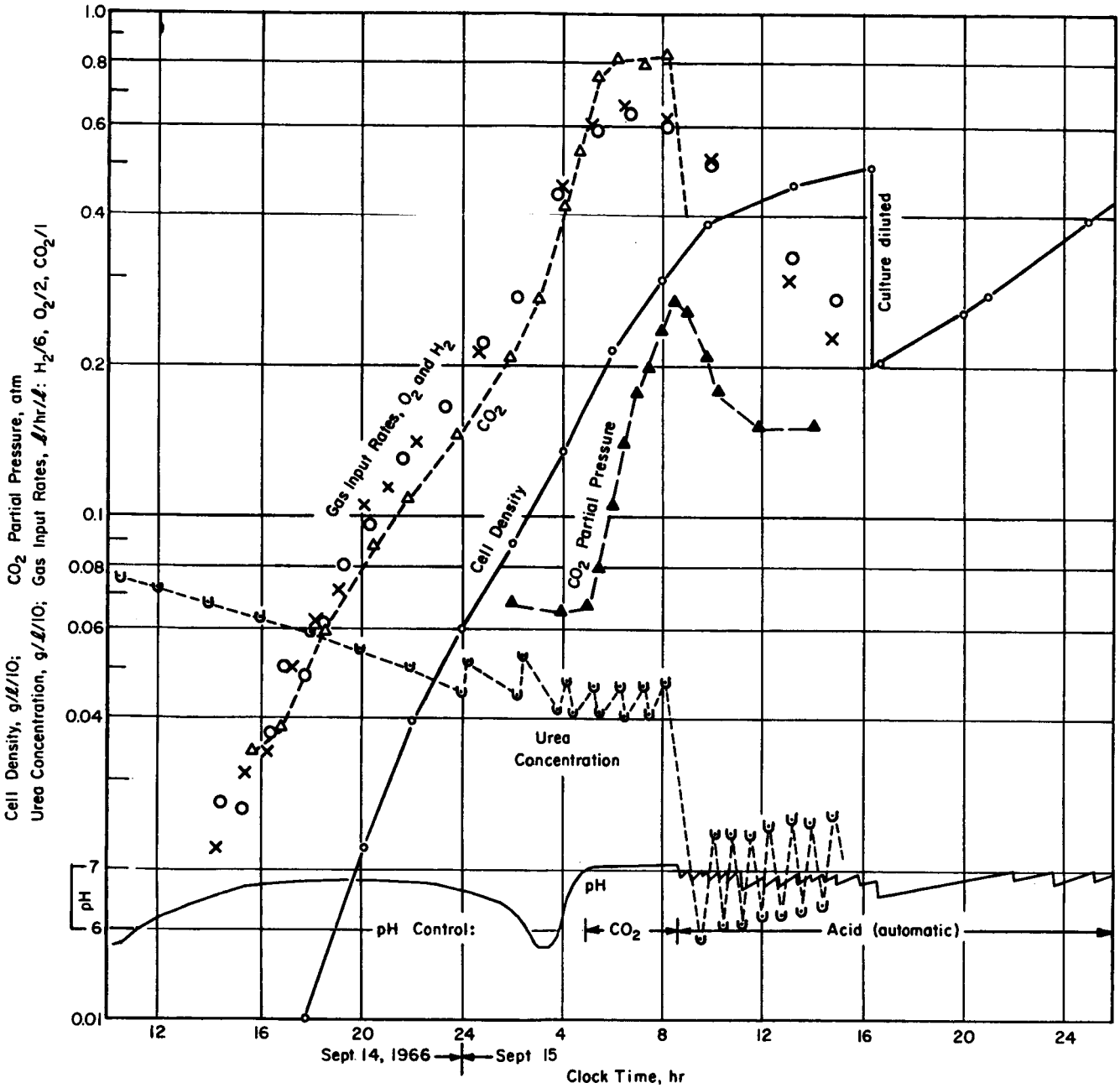


FIGURE 2. SECOND BATCH CULTURE OF HYDROGENOMONAS EUTROPHA, RUN 23035-75

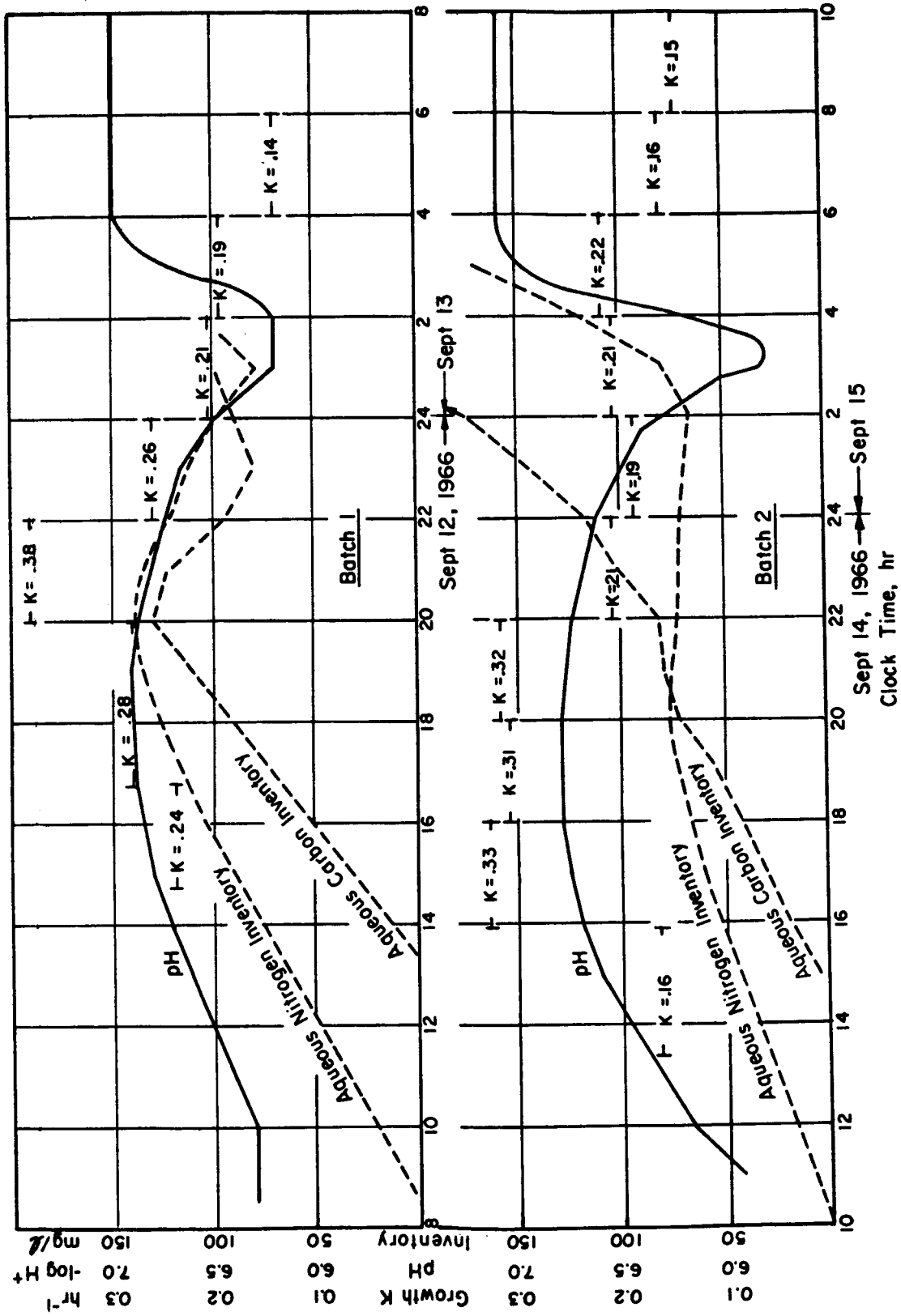


FIGURE 3. MATERIAL BALANCES FOR BATCH CULTURES OF HYDROGENOMONAS EUTROPHA, RUN 23035-75

(5) The growth rate at any time reflects the environment in the culture over a preceding period no longer than one generation of the organism.

(6) Changes in the growth of the culture extending over longer than one generation reflect long-term changes of inventories of intermediate compounds derived from the primary nutrients.

(7) Encouraging progress has been made in understanding conditions for efficient culture of the organism. A cell density of 5 grams per liter was attained in batch culture in about 24 hours, using a dilute inoculum. This period is shorter than has been possible in other experiments.

(8) Growth rates during a part of the culture periods exceeded any that have been observed previously. The maximum observed in Batch 1 was $K = 0.38$ for a short time. This equals a doubling time of 1.8 hours. In Batch 2 the maximum growth rate was about $K = 0.32$ for 6 hours, or a doubling time of about 2.2 hours. It is concluded that there is no genetic or morphological limitation on growth of the organism with a doubling time of about 2 hours, although side reactions and enzymatic feedback mechanisms may have to be controlled by methods presently not known.

FUTURE WORK

Work will continue on the identification of an optimum environment for efficient continuous culture, using the concept of extracellular inventories of intermediates that influence long-term changes, and intracellular reactions that adjust to the external environment within one generation. Continuous determination of ammonia in the culture will be used to extend our understanding of the culture environment.

The primary data used as the basis for this report are recorded in Research Notebook No. 23035, pp 51-79, and on supplementary charts and forms referenced therein.

JFF:js