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SYSTEMS APPROACH TO EVALUATING HYDROGENOMONAS CULTURES

by John F. Foster and John H. Litchfield

Prepared by
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SUMMARY

Cultures of Hydrogenomonas eutropha are confirmed as a stable process operating at room temperature for waste conversion, to consume the metabolic wastes CO_2 and urea; and the hydrogen by-product from water electrolysis. Environmental measurements with feedback controls can balance consumption of these wastes by individual make-ups at the same rates as consumption. In this manner, a stable culture with constant conversion rates can be maintained in steady-state growth for long periods and is suitable for consideration as a continuous waste conversion process.

The growth rate indicated by careful experimental measurements is a fractional increase of 0.33 per hour of the weight of bacterial cells in the culture, which is harvested continuously from a cellular culture maintained at a constant concentration of 6.1 g (dry weight) per liter of liquid culture. These values can be used for calculating the maximum production rate of cellular material in evaluating potential practical applications.

INTRODUCTION

Measurements of the growth rates of Hydrogenomonas eutropha in both continuous and batch cultures were undertaken to provide data on their possible use for waste

utilization in aerospace life support systems. The growing bacteria convert carbon dioxide and urea, waste products of human metabolism, plus waste hydrogen from electrolysis of water to obtain breathing oxygen, into cell material containing a substantial proportion of protein. The harvested cells are thus a candidate material for supplementing the food supply in a life support system. The second major product of an actively growing culture is water, which would be recycled to the electrolysis cell to close the oxygen cycle.

Bongers and Kok (1) and Jenkins (2) have discussed comparisons with competitive systems for waste conversion, and have shown significant superiority for the Hydrogenomonas cultures, based on reasonable estimates of optimum performance. Our assignment has been to develop a quantitative experimental base for detailed estimates of process efficiencies, equipment requirements, and for adequate control principles. An unusual combination of the types of information required, as described in the following section, directed us to a semiempirical view of the culture apparatus, its operation and calibration procedures, and the overall response of the growing organism among the component variables. The results thus obtained have had predictive value for estimating the best possible performance and the probable production capacity of the culture. The estimated predictions have been supported in part by results from other laboratories, based on more conventional and probably more precise experimental procedures.

ELEMENTS OF THE PROBLEM

Any evaluation of the use of a bacterial culture in aerospace life support includes the implicit requirements of all aerospace functions for minimum weight, volume, material, and power consumption. An exploratory evaluation (such as this one) seldom, if ever, benefits from the guidance of additional specified requirements

characteristic of a particular mission. Conversely, an aerospace functional process is finally selected because it rates high in trade-off studies based on specific mission requirements, and in overall point-by-point comparisons with competitive alternatives. An appropriate exploratory evaluation should therefore attempt the dual approach of (1) optimizing all conversion and production rates simultaneously to meet a generalized objective, such as "high efficiency," (which may be variously defined), and (2) exploring the limits of controllable variation of individual conversion rates for possible special uses of the conversion system.

Because of these multiple requirements, the proven experimental and interpretive procedures (1) of chemical engineering for process evaluation, (2) of microbiology for characterizing nutritional requirements of bacteria, and (3) of biotechnology for defining production capacity of continuous cultures, were adapted in combination to the purposes of the study. It was advisable to combine methods because this waste conversion process differs from most processes being subjected to analysis and evaluation in two ways: (1) a preferred product or reaction is not initially identified, and any one of the waste conversions, or the protein product, may later be judged the critical part of the process, and (2) aerospace costs are greatly influenced by weight and efficiency, so that even small incremental improvements in rates gained by exhaustive optimization studies may eventually be critical for acceptance of the process.

These considerations favored a systems approach to the problem, which was used in apparatus design and calibration, in carrying out cultures in the apparatus with either batch or continuous operation, and in interpreting the data in a manner predictive of process capability. The information and discussion presented in this paper illustrate a design and experimental effort using the systems approach which assumed that multiple interactions would occur among many of the environmental variables and responses to the environment by the growing organism. Therefore,

it did not seem experimentally or economically feasible to use the alternative of individual study of each variable with all others held constant.

GENERAL OBJECTIVES

The elements of the problem described above are met by the following general objectives:

1. The experimental apparatus should be as similar as possible in form and in principles of operation to the ultimate prototype that might eventually be used in long-term testing for stable operation.

2. The experiments should seek to establish the highest rates of conversion of waste materials that can be attained by control of the environment. There should be negligible or minimum by-products. The only limit on growth of the culture should ultimately be the genetically determined enzyme reactions and growth processes in the cells operating under an ideal external environment.

3. The experimental apparatus should be capable of handling sufficient throughput to provide enough product for evaluating its nutritional quality.

4. The experimental program should involve enough operating experience to permit a practical appraisal of the safety, reliability, and appropriate maintenance procedures of the total apparatus and its critical components.

WORKING HYPOTHESIS FOR EXPERIMENTS

It was necessary, then, to search for maximum cell growth, when several different growth-limiting factors in the environment in various combinations might be the critical ones under various sets of environmental conditions. Experiments were set up under the hypothesis that growth-limiting factors could be recognized

by identifying cause-and-effect relationships among environmental variables, growth rates, and conversion rates and the environment changed. The method selected was precise measurement of inputs, outputs, and inventories of the growing-culture system under controlled environments, to the fullest extent technically feasible. By deducing reasonably accurate material balances from such data, it would presumably be possible to relate improved results to sets of imposed environmental conditions. A precision of about two percent in each measurement was sought, and overall, about 5 percent agreement between inputs and outputs was believed feasible and necessary for interpretation of the data.

GUIDELINES FOR APPARATUS DESIGN

The apparatus was designed and built only to study Hydrogenomonas cultures, and not the total conversion system. Its integration with other components of the total system needs to be considered in detail only after the optimum environment for the bacterial cultures is better understood. Therefore the waste gases and urea were injected from stored supplies of pure materials.

The explosive range of hydrogen-oxygen mixtures is so wide (roughly 5 to 95 percent hydrogen) that substantial amounts of CO_2 in a ternary H_2-O_2-CO_2 gas phase would not be expected to inhibit explosion at any composition reasonably close to the estimated optimum mixture containing 10 to 20 percent oxygen. Therefore, the apparatus was designed to handle explosive gas mixtures safely, rather than to avoid their use.

A conventional liquid-phase medium, vigorously stirred in contact with the gas phase by shaft-mounted impellers in a completely closed and pressurized cylindrical vessel, was chosen for study of the growth characteristics of Hydrogenomonas and the apparatus was designed for this purpose. The harvesting

and sampling systems for continuous removal of a measured portion of the liquid phase were gravity dependent, and would have to be replaced by centrifugal separators in a zero-gravity environment. The other subsystems were gravity insensitive in principle, in that gases and liquids were transferred by flow down a pressure gradient, and gas-liquid equilibration was accomplished primarily by mechanical mixing.

Many considerations of safety, convenience, range of controllable variables, and accuracy of material balances, favored a design in which materials were added to the closed system only on demands registered by individual detectors for each major reactant, or to replace the liquid nutrient solution removed in harvesting surplus cell product. Specifically, there was no flow-through of gases with discard of an unused surplus of the gas mixture. None of the gas phase was removed from the pressurized system, but increments of the makeup gases, H_2, O_2, and CO_2, were added as required during extended experiments.

In the terminology of continuous-culture technology, the apparatus could be operated either as a chemostat or a turbidostat, or with some features of each. It seemed probable during the design stage that its major use would be as a turbidostat, as this operational mode appeared to be most economical of materials in space applications.

EXPERIMENTAL PROCEDURES

Bacteriological Methods

A strain of Hydrogenomonas eutropha obtained through the courtesy of Dr. R. Repaske of National Institutes of Health was used in these studies (3). Stock cultures were maintained in a liquid medium developed by Bongers or on a 2 percent

agar medium of the same composition (Ref. 4, Table 1). The same liquid medium was used in the continuous culture system.

The pH of this medium is 6.8 and its ionic strength is 0.06. All of the components of the liquid medium except the ferrous ammonium sulfate are dissolved in water and sterilized by filtration. The ferrous ammonium sulfate is filter-sterilized separately and then added to the remainder of the medium. This procedure prevents the formation of a precipitate that occurs when all of the ingredients are added together.

In the case of the agar medium, double strength mineral salts-urea and ferrous ammonium sulfate solutions are filter-sterilized and then added aseptically to an equal volume of sterile 4 percent agar to give a final 2 percent agar medium. The cultures are incubated in an atmosphere of 80 percent H_2, 10 percent O_2, and 10 percent CO_2 for 7 to 14 days at 25 C.

The liquid medium described above is prepared in the same manner for continuous culture studies. A 30 ml sample of inoculum from a liquid culture is injected into the culture vessel with a hypodermic syringe.

Continuous Cultures

The purity of the culture is maintained by operating the apparatus as a closed system, which includes a stored supply of sterile nutrient, and a reservoir to contain the harvested material. Thus nutrient can be added in increments to the culture vessel, and an equivalent volume of the bacterial suspension can be removed to the harvest reservoir without interrupting operation. The culture is thereby essentially kept at a constant cell density in a constant total volume. Additional supplies of nutrient may be added to the stored supply as necessary, and surplus harvest may be removed without contaminating the system.

The apparatus was completely assembled, then washed, rinsed, treated with hypochlorite solutions, and ethylene oxide-Freon mixture and rinsed again. Such a procedure is not absolute protection against some resistant organisms. However, the time and cost advantage was considerable over the alternative of using heat-resistant components and absolute sterilization. The results showed that the culture was uncontaminated with other organisms. To start a run, the culture vessel is charged with nutrient and inoculated with a sterile syringe and needle through a rubber diaphragm.

EXPERIMENTAL APPARATUS

Foster and Litchfield (4) described the first working model of the apparatus constructed according to the above guidelines. Since then a better understanding of the environmental requirements and responses of the organism, Hydrogenomonas eutropha (5,6) has indicated means for improving apparatus convenience and reliability. In the course of experimentation the apparatus has evolved through five models, each based on recognition of additional requirements for better control of environmental factors and easier interpretation of the data records.

The latest models of the apparatus monitor and control the composition of the ternary gas phase by individual sensors for the partial pressures of oxygen and carbon dioxide. The partial pressure of hydrogen is measured indirectly by difference, and makeup hydrogen is supplied in response to a low-pressure signal from the pressure control.

In another publication (7) Foster and Litchfield reported operation of Model III with added features for liquid-phase composition control (Figure 1 and 2). The liquid phase is monitored and adjusted to maintain preset values of temperature, liquid volume, pH range (maximum and minimum limits independently set),

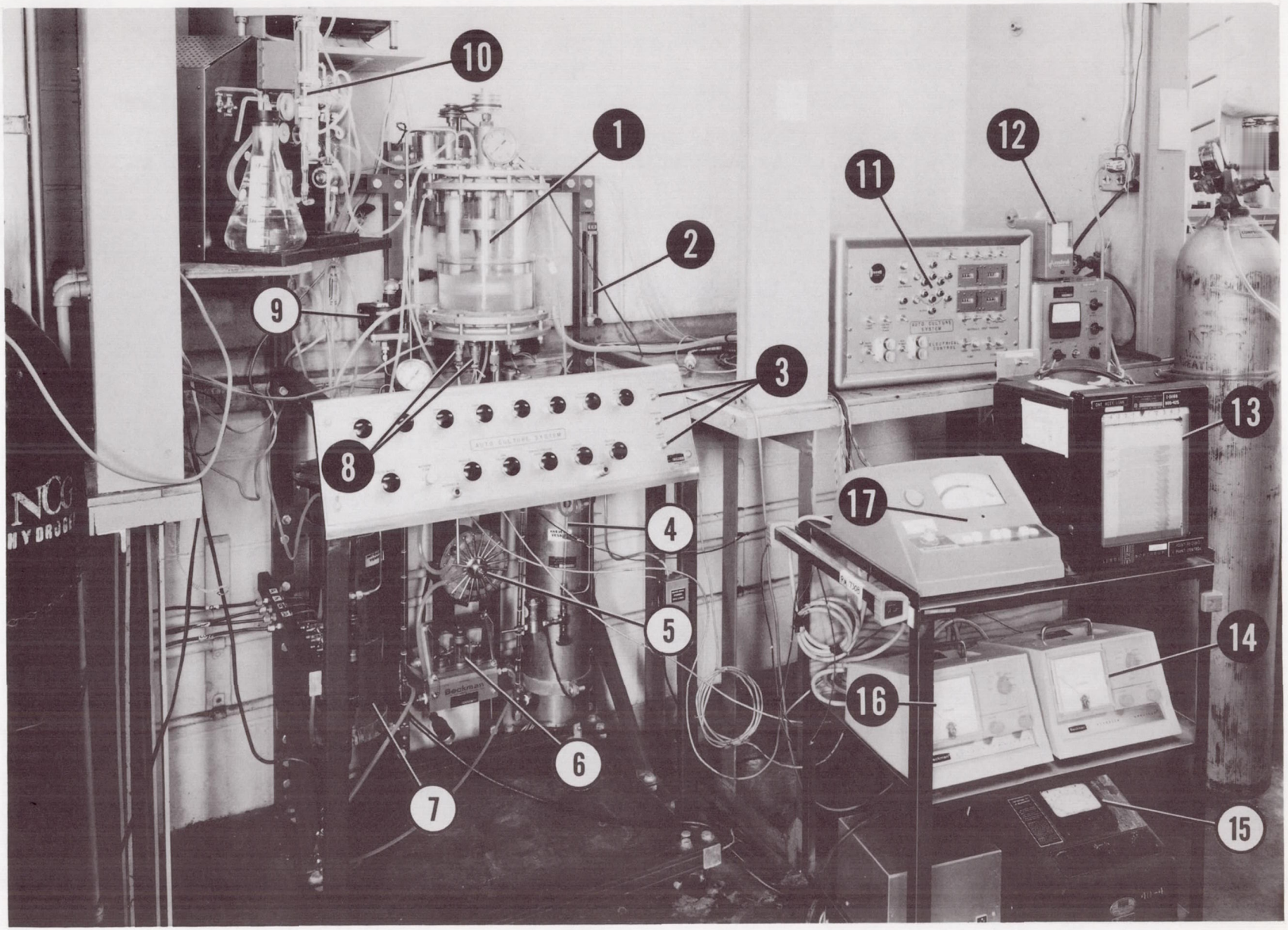

FIGURE 1. HYDROGENOMONAS CULTURE SYSTEM

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KEY TO FIGURE 1.

1. Pressurized, Agitated, Baffled, Temperature Regulated, Growth Vessel, 6 L Total Volume
2. Gas Flow Calibrating Rotameter
3. Metering Valves for O_2, H_2, and CO_2
4. Harvest Vessel
5. Peristaltic Pump for Liquid Sampling Loop
6. Flow Chamber for pH Electrodes
7. Nutrient Storage Vessel
8. Gas Sensors for O_2, and CO_2 (may also be placed in gas phase, see Fig. 2)
9. HI-LO Pressure Switch
10. Urea- and/or NH_3 Injector Assembly
11. Electrical Controls, Timer-Relay Complex
12. Event Recorder
13. Recorder-Controller
14. H_2 Concentration Meter
15. pH Meter
16. O_2 Concentration Meter
17. CO_2 Concentration Meter

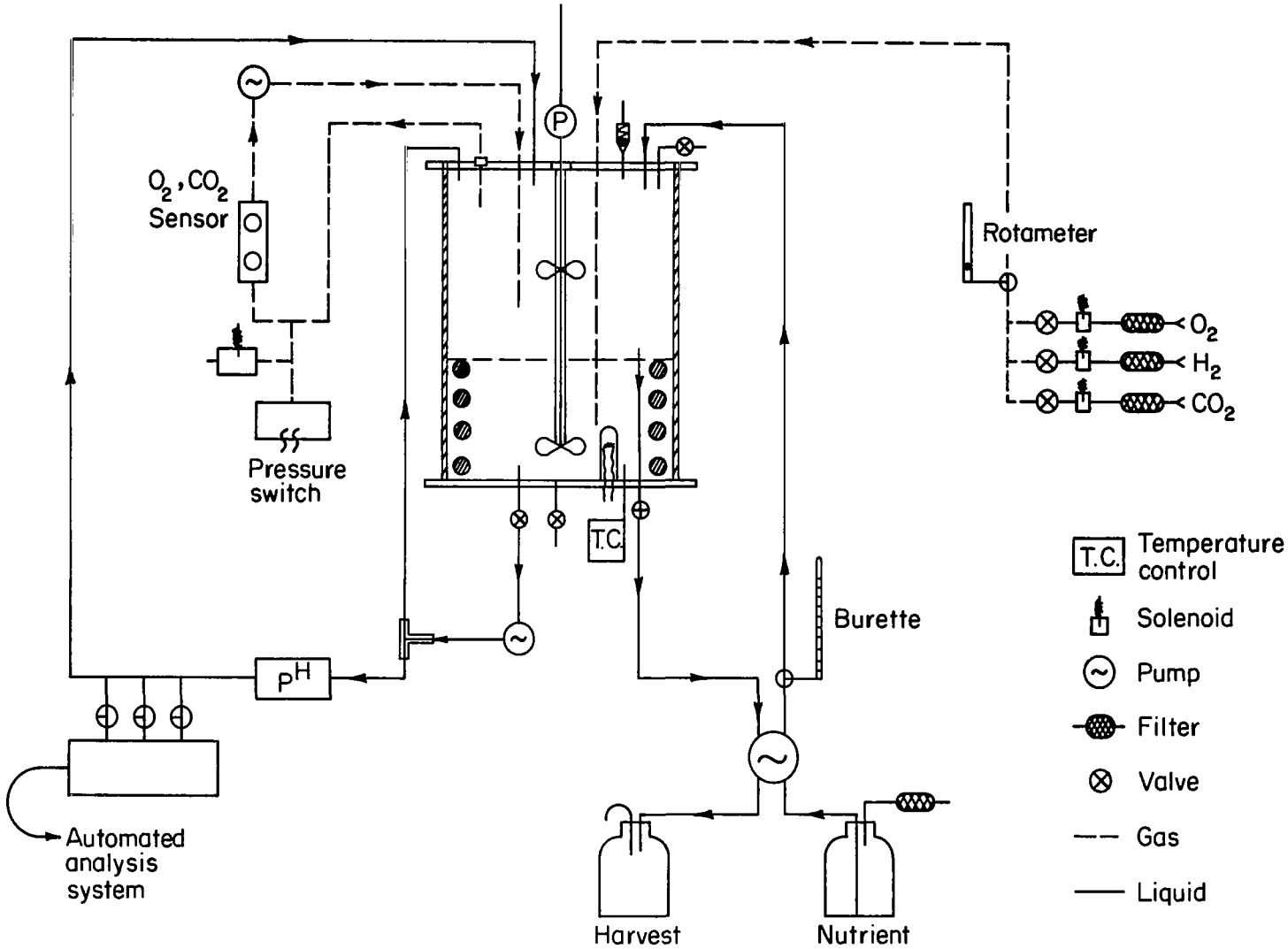

FIGURE 2. SCHEMATIC DIAGRAM OF HYDROGENOMONAS CULTURE SYSTEM

turbidity (cell population density), and the concentrations of selected solutes, such as urea, ammonia, phosphate, or any other for which a direct or indirect colorimetric analysis can be devised. The cell density is controlled by withdrawing a measured portion of the liquid-phase cell suspension in response to a high-turbidity signal, and simultaneously diluting with an equal amount of liquid nutrient solution.

Eventually, for long-term steady-state continuous cultures, it may be possible to dispense with many of the individual composition controls. The nutrients consumed would be replaced by adjusting the composition of the make-up nutrient solution to balance consumption, as deduced from the quantity of cells removed in response to the turbidity measurement. This was the original design concept for a turbidostat, but it was modified as individual sensors-controllers for dissolved nutrients were added piecemeal to support studies of constantly varying environments. With a varying environment in transition between steady states of operation, the culture never remains at a steady state condition long enough to respond to control by an overall change in makeup nutrient composition.

Usually modifications in analysis and control were made as expedients for immediate use, and without integrating them into the total system for permanence and convenience. Finally a redesign of the instrumentation and controls were imperative, because the data were being recorded on five separate strip charts with three different time scales. The discordant records required a prohibitive amount of time and effort to correlate the various scales and to arrive at material balances with a resolution of less than three minutes on the time scale, which was entirely within the precision of the recorded data.

The redesigned control system of the current Model V (Figure 3) was built around a newly available recorder-controller with a 12-point numbered recording sequence, and six individual control circuits (Leeds & Northrup Speedomax Model H). Most of the environmental parameters and the input-output rate data were thus

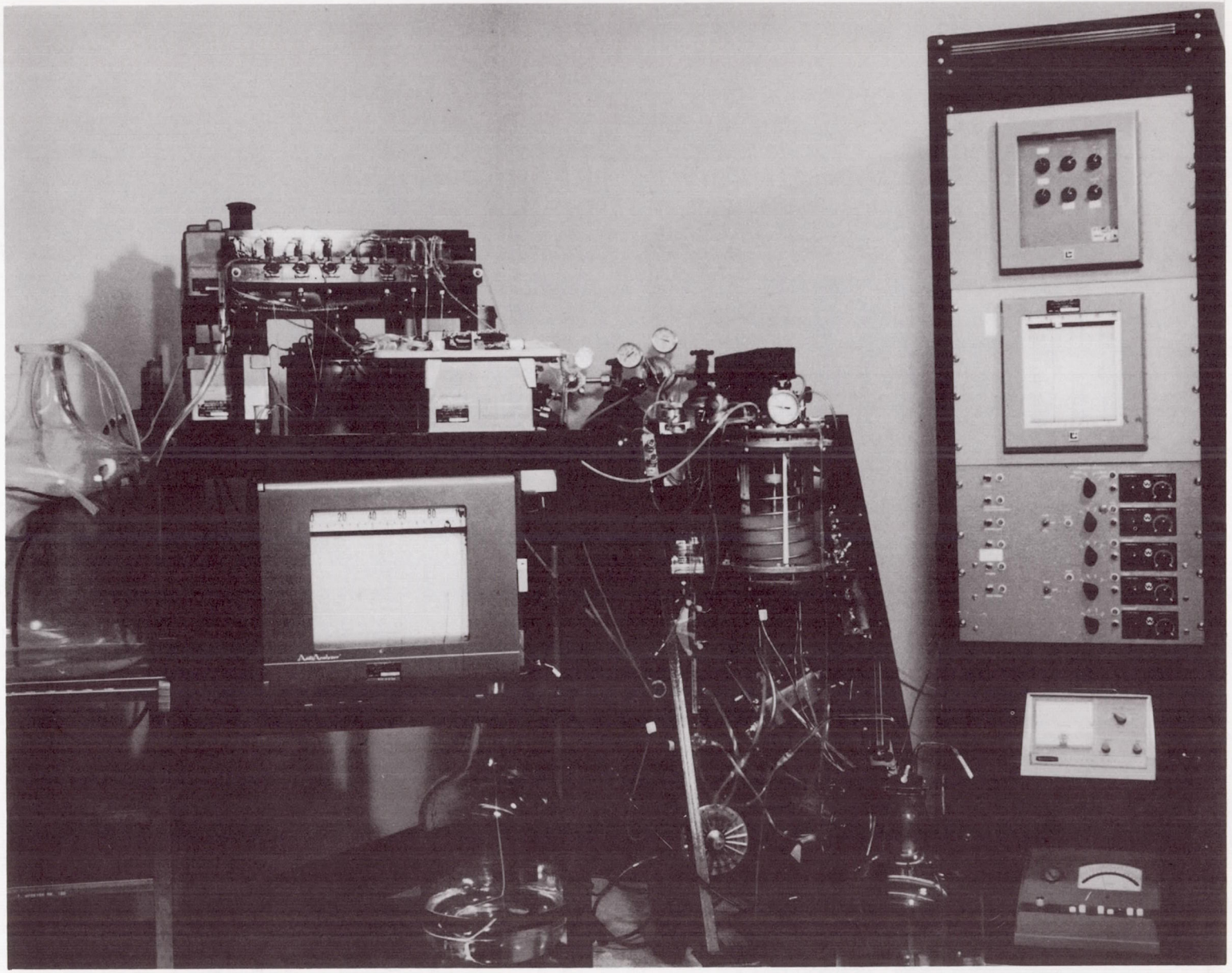

FIGURE 3. CURRENT MODEL OF HYDROGENOMONAS CULTURE SYSTEM

printed on a single chart as a 12-point record which repeated 50 cycles per hour. The six controls were electrical switches mechanically operated by the movement of the recorder as it indexed the individual signals from each sensor. It was found advisable to depart from the ideal of a single integrated system and a comprehensive data record in three respects, which improved reliability without sacrificing precision and convenience:

1. Two colorimetric systems (Technicon AutoAnalyzer) for cell density (optical density) and urea (or ammonia) concentrations analysis were incorporated intact with their own two-pen recorder-controller using a separate strip chart. This retained the subsystem circuits with electrical compatibility between the detector and null-balance circuits of the colorimetric analyzers. The master 12-point recorder was not electrically compatible with the colorimetric subsystem. The 12-point recorder was used, however, to register a "high" or "low" indication of each concentration on the master chart. There was the added advantage that using the two control circuits in the satellite recorder-controller released two of the six control circuits of the master unit for other purposes.

2. The master recorder-controller was designed by the manufacturer to monitor and control the pH of up to six separate systems with inputs from individual glass and reference electrode pairs. Thus, there was no problem in using one glass electrode input and two control circuits for measuring pH of the culture and controlling between upper and lower pH limits.

There were problems encountered with circuit or sensor instabilities and transients when primary signals from Clark and Severinghaus electrodes (for oxygen and carbon dioxide measurements, respectively) were intermittently fed directly to the recorder amplifier input through the recorder switching sequence. Much better reliability and stability were achieved after each primary signal was fed into its own amplifier with continuous readout on separate oxygen and carbon dioxide

meters. The secondary signal from the recorder terminals on each meter was then transferred to the master recorder. Earlier problems were thus obviated without identifying the exact source. In addition the meter readouts were useful in the systems approach to calibration discussed in the next section.

3. Close temperature control was achieved by using a solid-state temperature detector-controller completely independent of the master control system. This released another control circuit in the master system for other use. A second and independent sensor also immersed in the culture recorded temperature on the master chart.

Key to Recorder Charts

This section summarizes and interprets the types of data recorded on the strip charts of the two recorders incorporated as part of the Auto-Culture System (Figure 4, Leeds & Northrup 12-point recorder; Figure 5, Technicon AutoAnalyzer 2-pen recorder).

Both chart drives operate at a speed of two inches per hour, so that they have equal linear-time scales. Clock time is written manually on both charts occasionally for reference and to guard against the remote possibility of chart slippage. The Technicon chart (Figure 5) produces two continuous records of cell density and urea concentration in the culture. These recorded curves are converted into gravimetric concentration units by the equation

$$\log_{10} (100/T) = KC \quad ,$$

where T = transmission, percent

K = conversion constant

C = concentration, g/1.

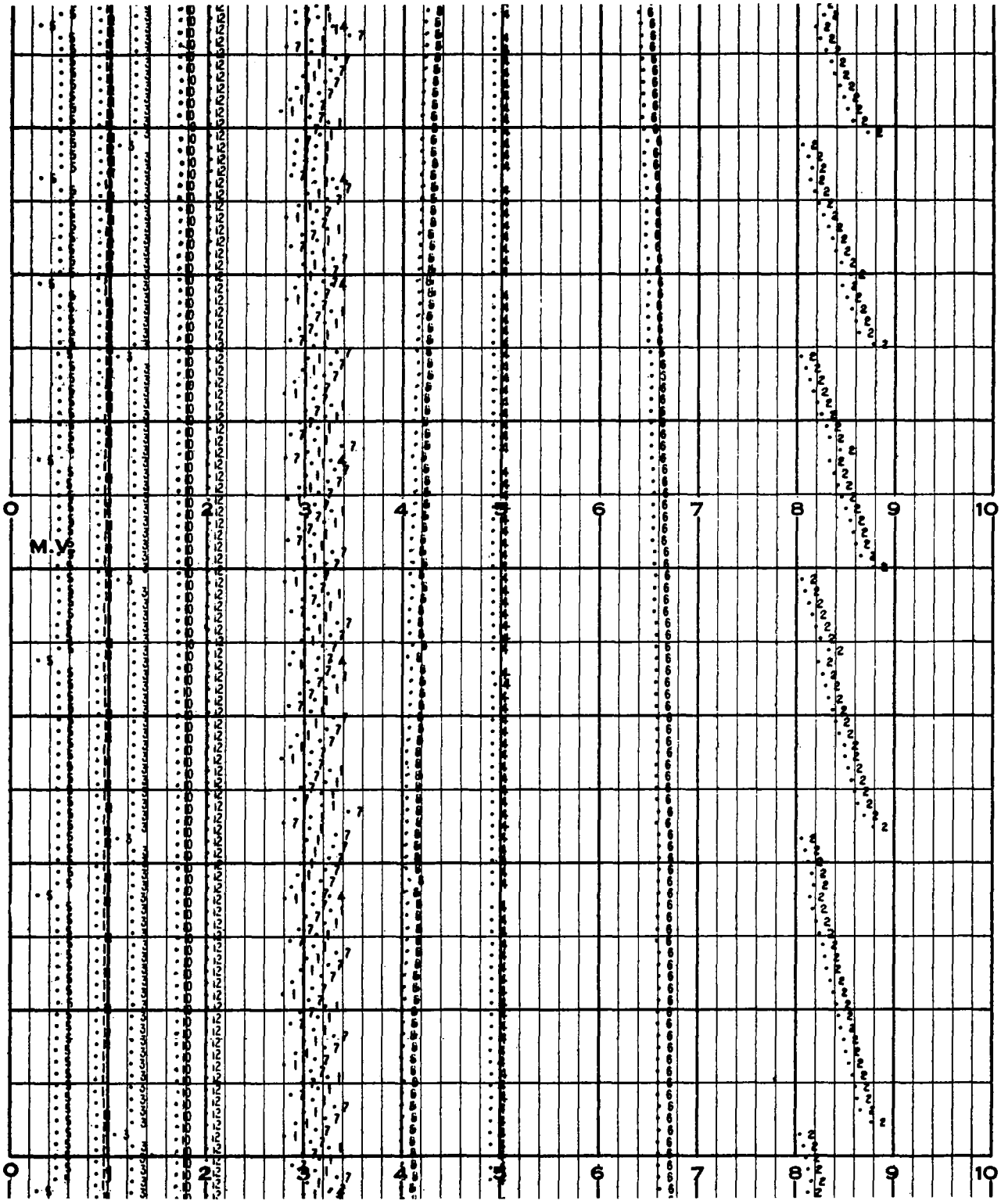

FIGURE 4. TYPICAL CONTROL CHART SHOWING pH, OXYGEN, CARBON DIOXIDE, AND TEMPERATURE RECORDED DURING GROWTH

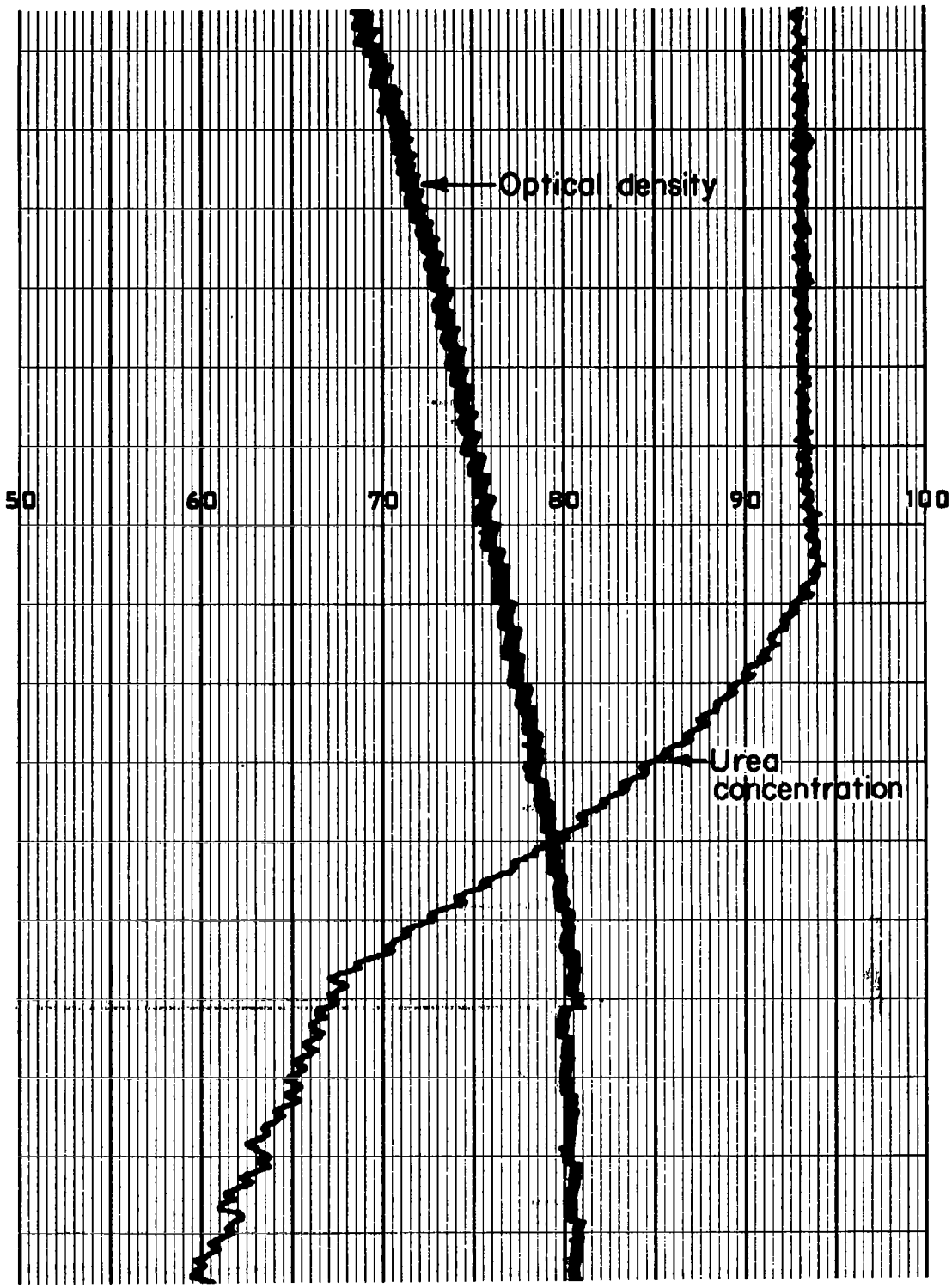

FIGURE 5. TYPICAL CONTROL CHART SHOWING UREA CONCENTRATION AND CELL DENSITY DURING GROWTH

The conversion constant, K, for each curve is determined periodically during an experiment by measuring cell density of a grab sample in a separate spectrophotometer calibrated for gravimetric determinations, and by measuring transmission of a standard urea solution of known concentration in the analytical system. Control circuits for nutrient and for urea additions are keyed to cell density and urea concentration measurements.

The Leeds and Northrup 12-point recorder prints 12 different curves on the chart in six colors, one color for each of two curves (Figure 4). Each curve is composed of discrete dots and is identified by a curve number printed beside each dot, as described in the manufacturer's manual. The switching mechanism in the recorder selects the 12 input signals in sequence, allows 6 seconds for balancing and printing each signal, and then switches to the next signal. Thus, the complete measuring system repeats every 72 seconds, and the dots on one curve are separated by 72 seconds on the time axis of the chart. Some of the curves record the values of environmental variables over a continuous range, and are associated with one of the six control circuits. These circuits are used to respond with material inputs at some demand level. Other curves record only responses to a particular demand and thus count incremental inputs. The 12 numbered curves are assigned to the following functions, which are discussed below:

1. Temperature record
2. Oxygen partial pressure; control circuit
3. Oxygen input record
4. Low pressure; hydrogen control circuit
5. Hydrogen input record
6. CO_2 partial pressure; control circuit
7. Temperature record, same as (1)
8. pH; high pH control circuit
9. pH; low pH control circuit

10. Urea input record (Technicon control circuit)

11. CO_2 input record

12. Nutrient input record (Technicon control circuit)

Temperature of the culture is recorded on Curve 1 and also on Curve 7. Thus, the temperature is measured every half cycle of 36 seconds, and Points 1 and 7 alternate to form a single temperature curve.

Hydrogen is added in response to a control signal represented by closing of a low-pressure switch. Curve 4 registers one arbitrary reading when the switch is closed, and another arbitrary reading when the switch is open because of high pressure. Thus Curve 4 is neither proportional to pressure nor to hydrogen partial pressure which is unlike other concentration-recording curves discussed below. Curve 5 is a counting circuit which registers a one-point arbitrary reading to the left of its arbitrary baseline for each increment of hydrogen added. If there is no hydrogen demand, Curve 5 registers on its baseline. When hydrogen is being added only on low-pressure demand, Curves 4 and 5 are duplicates, but are not superposed. However, there is an optional dial position on the control panel under which one increment is added during each measurement cycle of 72 seconds. Curve 5 will register such time-dependent additions, even though Curve 4 shows high pressure.

Carbon dioxide concentration is recorded on Curve 6. The chart reading is related to CO_2 concentration by the equation

$$\log_{10} (p_2/p_1) = K_{CO_2} (S_2 - S_1) \quad ,$$

where p_2 is the partial pressure of CO_2 corresponding to the reading S_2 on the recorder scale

p_1 is partial pressure of CO_2 corresponding to S_1 on the recorder scale

K is a system constant determined by calibration measurements on two CO_2 concentrations at two points on the recorder scale.

The value of K ordinarily does not change. Once it has been measured, the system can be calibrated by Orsat analysis of the CO_2 concentration in a gas sample corresponding to one reading on the recorder scale.

Curve 11 is a counting circuit which registers one CO_2 addition by printing one point at a characteristic position off its arbitrary baseline.

Oxygen concentration is recorded on Curve 2. The chart reading is related to oxygen by the equation

$$p_2 - p_1 = K_{O_2} (S_2 - S_1) \quad ,$$

where p_2 and p_1 are partial pressures of oxygen corresponding to readings S_2 and S_1, respectively, on the chart scale, and K is the system constant. Calibration is performed by measuring the oxygen concentration with Orsat analyses of gas samples corresponding to known scale readings. Curve 3 is the counting circuit for oxygen increments, which is the same in counting method as the other counting circuits.

pH is recorded by both Curves 8 and 9, for controlling both high and low pH, as explained previously. Neither the recorder scale nor the chart scale read directly in pH, and calibration must be made with samples drawn from the culture when the scale reading is taken. The chart scale is linear with pH, according to the equation

$$pH_2 - pH_1 = K_{pH} (S_2 - S_1) \quad ,$$

where the pH of two samples, pH_1 and pH_2, corresponds to two scale readings, S_1 and S_2; and K_{pH} is the system constant.

Urea is recorded on the Technicon chart, as noted above, and urea increments are counted on Curve 10 in a manner similar to the other counting circuits, described above.

Nutrient medium additions (in response to cell-density control) are counted similarly on Curve 12.

Graded Calibrations for System Evaluation

Three grades of calibrations monitor both the reliability of the sensors which control environment of the automatic culture system, and the reliability of the measuring devices, which collect data on inputs, outputs, and inventories. Primary and secondary calibrations use direct measurements on system properties or quantities, and comparisons with secondary standards, respectively. The tertiary calibrations are system-oriented, time-coordinated comparisons of two or more variables, which correlate through constant ratios and the like, provided the measuring devices involved are stable. Tertiary calibrations can be adapted to many integrated systems, provided the instrumentation is appropriate, the mode of operation is compatible with measurable fluctuations of system parameters, and timing is precisely correlated. Two advantages of tertiary calibrations are that they do not disturb the operating culture system, and they offer substantial reassurance of valid data. The following sections give individual illustrative examples of primary and secondary calibrations. Following, in a combined section, somewhat more detail is presented on the principle methods of tertiary calibrations, because these are a useful part of the systems approach to evaluating Hydrogenomonas cultures.

Gas Composition. - The primary measurements of the concentrations of CO_2 or oxygen have been by absorption in a reagent specific for each gas according to the procedures of Orsat analysis. Such measurements serve as primary calibrations of the sensors and give concentrations as mole fractions in the gas mixture. Orsat analysis could also have been used for direct determination of hydrogen, except that no primary calibration was required, since no sensor was adequately specific for hydrogen in the ternary mixture of gases.

Two types of Orsat pipettes have been used, which differ in the method of isolating the gas sample for measurement. The conventional Orsat withdraws one small measured sample for both analyses from the gas phase by liquid displacement, and thus maintains inventory measurement and does not deplete the gas phase seriously. The industrial-type Orsat has a fixed-volume sample chamber which needs thorough flushing with gas phase to collect a valid sample; a separate sample is used for each analysis. Manipulations are simpler and less time-consuming, but the gas phase is substantially depleted for each analysis, which disturbs the gas-liquid equilibria of a continuous culture.

It should be emphasized that the sensor signal is proportional to the partial pressure of its gas, and partial pressure changes each time total pressure is reduced when a sample is withdrawn. On the other hand, the mole fraction measured by either Orsat analysis apparatus does not change with total pressure. Care must be exercised to correct for these differences for high calibration accuracy.

A monochromatic infrared spectrophotometer with calibrated scale reading in percentage CO_2 was tried as a secondary standard. It was not suitable for analyzing grab samples of gas phase, because too much preliminary flushing was required through the optical path before a stable reading was reached. The infrared detection of CO_2 should be suitable for systems from which a continuous discharge of gas phase through the instrument could be tolerated.

Gas Input. - Gases are injected into the system in increments of known amounts, in response to demands initiated by the individual sensors. Primary calibrations of increment size were made by diverting a series of gas increments from the input line to a calibration line of similar flow characteristics, and collecting the gas by water displacement, with a volume or weight measurement of the water displaced by each increment. The gas volumes were corrected to standard temperature and pressure, and for solubility and water vapor content, so that they could be converted to gas quantity in units of mass. Flow rate during injection is a function of the

selected area of a variable orifice in the gas supply line and the selected constant upstream pressure, maintained by the adjustable pressure regulator on the gas-supply cylinder. One available secondary calibration of flow rate is therefore the pressure registered by the gauge on the input line in combination with the reproducible setting of the area of the variable orifice. A direct secondary calibration of flow rate (which checks the reliability of both pressure and orifice settings) is obtained by diverting the gas stream through a calibrated flow meter whose scale readings have been related to the primary calibrations of volume of each gas collected during a known time interval.

The size of a gas increment is directly proportional to time of flow at constant flow rate. Time is measured directly with a stop watch against a signal light connected in parallel with the solenoid of the master gas valve upstream from the variable orifice. The variable-interval time control, which controlled each master gas valve, incorporated two calibrated dials for coarse and fine adjustment of the time interval. Primary calibrations also included checking of reproducibility of the time interval with repeated settings of the interval dials at the same readings, and confirmation of the accuracy of the proportional relationship between open-valve time and volume of gas increment collected. Once these were established, the most efficient procedure for changing the size of any gas increment was by variation of the time interval, which did not interrupt operation. The flow rate could also be recalibrated with a stop watch during operation. The flow rate could also be recalibrated without disturbing the system by passing gas through the flow meter long enough for a reading between periods of gas demand initiated by the sensors.

Cell Concentration. - Primary calibration for determination of the concentration of bacterial cells in the culture are made by (1) withdrawal of a well-mixed sample of liquid cell suspension, (2) separation into replicate samples of known

volume, alternate (3) centrifugal separation and (4) washing by resuspension of the precipitated cells, and finally (5) drying the replicates to constant weight under vacuum.

Other parts of the same uniform sample are diluted in various measured proportions and the optical densities of these dilutions are measured in a double-beam spectrophotometer at the wavelength for maximum differential absorption by cellular material, 650 nanometers in the case of H. eutropha. This procedure defines a secondary calibration range in which optical density is proportional to dry weight concentration of cells. The dry-weight conversion factor (4.25) is thus used with only occasional gravimetric calibrations to convert optical densities to dry weight concentration of cellular material. The optical density measurement on a diluted grab sample is a secondary standard for calibration and adjustment of the cell-density control, that adds diluent on demand, on the basis of relative optical density measured continuously in a flow-through optical system (Technicon AutoAnalyzer) attached to the culture vessel.

In principle, a single sample is sufficient when diluted in proportions to give an optical density in the range of linear variation of optical density with dry weight (where the conversion factor applies). Experience has shown that a modified procedure employing three different dilutions in the linear range provides better accuracy of calibration. The optical density of each dilution sample is plotted against its corresponding dilution fraction (e.g., dilution of 1:2 = 0.5; 1:5 = 0.2) on a rectilinear graph. The three points should fall along a straight line which extrapolates to the origin. The procedure is rapid enough that a failure to meet the criterion can be interpreted as to source of error and the measurement can be repeated on the original sample by using other dilution samples for additional measurements, as required.

Dilution Rate. - The primary calibration of dilution rate requires a measurement of the volume of each increment of the liquid nutrient added in response to

dilution demand, a count of the number of additions per unit time, and a measurement of the total volume of the liquid culture. All of these measurements can be made without disrupting the continuous-culture process. The increment-volume measurement substitutes a calibrated burette temporarily as the nutrient reservoir by valve changes; the volume of an increment is determined directly as the difference in burette readings before and after one addition. Culture volume is read from a calibrated scale on the glass wall of the culture vessel, while stirring is momentarily stopped. With a stainless steel vessel, a calibrated sight glass was used. The increment addition rate is recorded electrically as an individual-count record of each increment added on the calibrated time scale of the strip chart.

Secondary calibrations result from liquid-volume balances. The nutrient reservoir is a volume-calibrated vessel, so that total volume pumped equals the product of single-increment volume and the number of increments. It was this secondary calibration that revealed a defect in the electrical counters with mechanical totalizing register dials in an earlier model, in that they occasionally registered two counts per increment because of switching transients. The difficulty was corrected by substituting the recording of counts on the time chart, which can register no more than one counter per cycle of the controller.

Liquid-Phase Composition. - The primary calibration for urea and/or ammonia concentration in the culture (based upon the diacetyl monoxime and alkaline phenol-hypochlorite procedures respectively) was preparation of a standard solution of known concentration by gravimetric or volumetric methods. These standards could be introduced into the sampling line of the analytical system by a valve change. No other parts of the automatic analytical sequence were altered. Therefore, an interpolated standard sample culminated in a colorimetric measurement at an appropriate monochromatic wavelength for specific indication of the known component in the standard (8,9). Thus the reading on the recorder chart was determined as a function of concentration at one point for each standard solution used.

In the calibration procedure, water is supplied to the sample intake before and after each standard solution. This represents a reference analysis at zero concentration, which checks the zero point of the recorder and the cleanliness of the flow cell in the colorimetric system. One other secondary calibration was used optionally to check the consistency of the data obtained. The optical density of the colored reagent developed in the analysis of each standard solution was calculated from the recorder reading and plotted against concentration. These plotted points for various concentrations should fall in a straight line passing through the origin. Otherwise either the zero of the recorder amplifier was shifted electrically or the optical windows of the absorption cell needed cleaning. The shape and location of the plotted curve ordinarily identifies the source and magnitude of error so that the system can be adjusted and a correction applied to the data recorded prior to the adjustment.

Tertiary Calibrations. - Tertiary calibrations are time-referenced comparisons among environmental variables of the system which fluctuate over a significant range during normal automatic operation of the culture system. The concept of such calibrations was developed because they are essential to the operation of a steady-state culture over long periods with quantitative measurements of inputs and outputs. Confirmation of the validity of the data, and adjustment of controls as necessary to maintain the desired conditions of the steady state, permits evaluation with confidence of the conversion rates for waste products that can be achieved by Hydrogenomonas cultures.

Tertiary calibrations, as discussed below, require in general a more sensitive recording system than would be required for control only of system parameters within tolerable limits of variation. The recorder circuits were accordingly modified to give larger deflections corresponding to changes in partial pressures of carbon dioxide and oxygen. This expanded the concentration scale on the recorder chart, so that auxiliary zero compensation or zero suppression circuits were

used to bring the recorder-controller readings on-scale in the range of the gas concentration set on the automatic control.

At each gas addition, both the total pressure and the recorded gas concentration (partial pressure) increased by a measurable amount, which depended on the quantity of gas injected in one increment, the volume of the gas phase, the temperature and the solubility of the gas in the liquid, and the liquid volume. All of these variables are related to each other by the basic ideal gas law, $pV = nRT$, and by the law of gas solubility. Contemporary values of each variable were also available separately in quantitative terms determined by primary and secondary calibrations (except for handbook values of gas solubility). The tertiary calibration thus was a test of the correlation of simultaneous values by congruence in the gas equation. In effect, then, the addition of one gas increment produced changes Δp in partial pressure of the gas recorded by the sensor, and Δp in total pressure on the pressure gauge, corresponding to Δn the quantity of gas in the increment added, if all calibrations were valid. Once this correspondence had been confirmed numerically, it was reassuring over considerable periods to read the chart qualitatively, using as evidence of system stability a constant difference between maxima and minima on the recorded curve of partial pressure variations.

Such capabilities for detailed examination of system conditions also could reveal other effects, predicted or novel, that might be helpful or pertinent to interpretation of results. Thus, a change in CO_2 concentration would change the performance of the oxygen sensor, as reported in the literature, but the magnitude of the effect and the possible necessity for repeated primary calibration could be assessed immediately.

The recorded partial pressure difference, Δp, for both oxygen and CO_2 decreased as the rate of gas consumption increased, reflecting the time lag in response in each sensor, and the consumption of an appreciable amount of gas while the sensor was

responding. True values of partial pressure could be deduced by correcting for lag if such data were needed. However, time lag observations were primarily useful in predicting occasionally the deterioration and need for recharging of a sensor before its control function suffered.

Qualitative examination of the form of the time response curves of the CO_2 sensor demonstrated also that a significant part of the CO_2 increment apparently was transferred, as expected, by successive equilibria to CO_2 in solution and then to hydrated CO_2 (H_2CO_3, carbonic acid) and to bicarbonate ion by dissociation of the acid. It appeared from increment material-balance estimates, as described above, and from highly correlated pH variations that a part of these species derived from CO_2 were not accessible to the immersed CO_2 sensor through its semipermeable membrane, and thus were not registered as part of the concentration signal. One wonders by analogy whether one form or another of dissolved carbon dioxide might be the primary or sole carbon carrier in transfer across the semipermeable cell wall during autotrophic growth with CO_2 as the carbon input. Using the systems approach to cell culture, such questions of growth mechanisms and extracellular nutritional paths could be studied experimentally and theoretically. Detailed quantitative measurements of this type were not justified within the scope of these culture studies.

The tertiary calibration of cell density measurements was not entirely satisfactory because there was no easily applied procedure which would provide an independent check of the instrument indicating cell density in the operating culture and would give its answer within a few minutes. There were no suitable synthetic or natural samples of cell suspensions of known concentration and optical density which could be interpolated into the continuous sampling system as standards. Fresh samples must be used, because optical density tends to change with aging. And the only available fresh samples were those of unknown absolute cell density being produced in the culture.

Two types of tertiary calibration for cell density in the operating culture did give results that were reassuring as to the validity of the secondary calibration of the control instrument by means of a sample withdrawn from the culture, which was described earlier. On a number of occasions the experimental protocol required withdrawal of a major part of the liquid-phase culture, and diluting the remaining portion in known proportion to give a lower cell density. This operation required only a short time, and none of the other control and analytical operations were disturbed. Thus the ratio of cell densities before and after dilution was indicated by the change in the recorder reading, and could be predicted from the dilution ratio. Prediction was often confirmed by measurement. There were sometimes indications of an optical density component not related to dilution ratio, which might have been a change in quality of light scattered by the diluted suspension or a slight clouding by a precipitate formed as a secondary effect of pH or composition change during dilution.

A less direct procedure for tertiary calibration of cell density measurements was confirmation of the expectation that cell density should increase logarithmically with time over a considerable range of cell concentrations during periods of active growth of batch cultures. These growth curves were checked by secondary calibrations of the culture with diluted samples at particular times during the active growth phase. The comparable data were internally consistent, so that interpolation by readings from the recorded cell density on the strip chart of the recorder were justified.

Overall, the most significant of tertiary calibrations is the deducing of partial or comprehensive material balances, representing the compilation and correlation of many different measurements into a consistent characterization of the dynamic system as a whole. This was accomplished to an extent which permitted useful evaluation, as summarized in the next section on the experimental results and their interpretation.

EXPERIMENTAL RESULTS AND DISCUSSION

The significant experimental results, which contribute to evaluation of the culture system from an engineering viewpoint, are those which define (1) the normal range of conditions or average environment under which the culture can be operated, (2) essential control and structural features of a prototype apparatus, as indicated by experimental operation, (3) scaleup experience, (4) conversion rates attainable for each waste material handled by the process, and (5) quantity and quality of product recovered.

Research on the growth characteristics and properties of Hydrogenomonas eutropha have been carried on in parallel in several other laboratories (10-14) which have been pursuing somewhat different paths toward similar assigned objectives, all of which bear on the total system of culture for waste conversion. In presenting and discussing the significant results in the five categories listed it is essential to recognize and indicate the advances by other laboratories. In particular, definitive work by Bongers and Medici (15) from the RIAS Laboratory demonstrated relatively rapid growth in a dense culture under steady-state conditions for a period of at least 7 days. These best results should be used as noted below in evaluating potential applications of the system. Other pertinent results from the RIAS Laboratory and from contemporary work by Repaske at NIAID-NIH are also described qualitatively in the appropriate context with the credit to the originator of the experiments.

Optimum Environmental Conditions

The microenvironment of dissolved materials at the interface between the liquid and the bacterial cell wall is a function both of the average composition of the bulk liquid phase and the rate of transfer of materials from the bulk liquid by

diffusion across the static layer of liquid surrounding each cell. In rapidly growing dense cultures, material transfer rates must be supported by increasing concentration gradients across the static layer. Thus, higher bulk concentrations of the critical solutes are required to maintain optimum supplies of nutrients available for assimilation into the cell surface. After near optimum conditions have been reached, with rapid growth taking place, individual deficiencies are identified and corrected by raising the bulk concentration of single components, and noting any corresponding growth acceleration.

Temperature. - The temperature of the culture system was maintained at 31 C by means of a cooling water jacket around the vessel in the earlier design. In subsequent designs, cooling water coils were placed in the vessel in contact with the medium. The temperature was controlled at 33 C in later experiments based on information furnished by Bongers.

Gas-Phase Composition. - The culture system was agitated strongly by an immersed impeller with the result that the liquid phase was presumably saturated by dissolved gases, which were thus available to the growing cells. So long as both the mixing of the liquid to entrain and disperse a maximum of gas phase bubbles was adequate, and the partial pressure of each gas was maintained by controlled input of make-up gas, the composition of the gas phase was not critical over a considerable range.

Partial pressure of oxygen in the gas phase during our experiments was commonly about 0.12 ± 0.02 atm (~ 10 percent). The criterion for adequate gas supply under the system concept was whether the rate of gas consumption and the rate of growth were both affected or not by a change in gas partial pressure. No effect of changes in partial pressure of oxygen moderately above and below this range indicated that oxygen was neither toxic nor deficient at the level of 0.12 atm.

The same criterion applied to the hydrogen supply indicated that 1.0 ± 0.1 atm partial pressure was acceptable. A wider and lower range of 0.07 to 0.7 atm was reported suitable by Bongers and Medici (15).

Experiments under many combinations of environmental conditions indicated that cultures could be more easily stablized in a reasonably rapid growth phase at partial pressures of carbon dioxide in the range 0.02 to 0.07 atm. This is a lower range than the 10 to 12 percent CO_2 used by most other investigators. That percentage was normally chosen because they were sparging with an excess of gas mixture in the proportions of 6:2:1 for H_2:O_2:CO_2 to balance approximately the well established gas consumption rates in the same proportionate ratios. Our preference for relatively low CO_2 partial pressures arises from a concept that this is one means of limiting possible secondary interactions among the various dissolved CO_2 species and counterpart alkaline species from ammonia and buffer.

Carbon dioxide as a primary carbon source can apparently be tolerated by the organism at partial pressures up to 0.2 atm without marked detriment, according to exploratory cultures in this laboratory.

Liquid-Phase Composition. - Foster and Litchfield (7) reported the inorganic salts contained in their basal medium, which were present in solution at inoculation and were added in the makeup solution used to replace the liquid cell suspension removed in the harvest. Bongers has subsequently assayed the minimum required mineral salts and nutrient concentrations by measuring the growth response in continuous cultures. At the same time he reported percentage utilization of salts by analysis of cell ash composition. The nutrient defined by Bongers' studies is qualitatively similar to the composition published earlier by us, which has been used with only minor variations throughout our experiments, and which was originally suggested for our use by Bongers. We therefore present in Table 1 our estimate of a composition suitable for supporting active growth in a dense culture

at 33 C, based on our own experience and a summary of our information from other laboratories.

TABLE 1. ESTIMATED MEDIUM COMPOSITION FOR DENSE ACTIVE CULTURES OF HYDROGENOMONAS BACTERIA (Adapted from L. Bongers)

Gas Phase		Liquid Phase					
		Established Requirements, mM/Liter				Other Additions μM/Liter	
Gas	Partial Pressure, atm.	Major Nutrient	Conc.	Metal Ion	Conc.	Ion	Conc.
CO_2	.05	N	60-100	Mg	1	Cl	**
O_2	.12	P*	25	Fe	0.05	SO_4	**
H_2	1.0	K*	33	Zn	0.002	Mn	0.3
		Na*	0-10	Ni	0.0008	Cu	0.007
		Dissolved Gases	Equilibrium	Ca	0.004	Al	Impurity
						Cr	Impurity

* Buffer to control pH at 6.7

** Counterion added with metal ions

Different investigators have used as a nitrogen source in the medium either ammonium ion or urea, or combinations, depending upon the immediate objective of the experiment. Foster and Litchfield in their progress report (7) on the experimental work in this laboratory discussed the use of urea as the sole nitrogen source in the nutrient. This sole source was decided upon because it fulfilled, in accord with the system approach, the concept of using the major nitrogen source that would be available in the primary waste material, urine, in the application of the process being developed. The use of urea only has continued in this laboratory in the culture

studies, because one of the recent intermediate objectives has been production of up to 50 lb. (dry weight) of harvested cells for nutritional quality evaluation. The possibility of better and more easily controlled nitrogen sources has not been examined experimentally.

In this recent cell-production effort the operational difficulties of maintaining a constant supply of nutrient nitrogen in the medium suitable for rapid growth of the organism have been confirmed. The various supplementary studies accompanying production have included repeated experiments in which, alternatively, urea was added as a feedback response to a signal indicating (a) low urea concentration, (b) low ammonium ion concentration, or (c) low pH. None of these control options consistently could achieve a steady-state of high growth rate with a dense culture in a large scale equipment (56-liter volume). The use of pH control was a direct equivalent of control by continuous measurement specifically of ammonium ion in the medium, because pH varied directly with ammonium ion concentration when other parameters were not changing. With any of these controls, the growing culture could assume one of two modes of growth. In one mode it would appear to convert urea directly, while generating only a low concentration of detectable ammonium ion. After a time it would often change over to a mode in which apparently extracellular urease was released and urea was converted rapidly to ammonium ion in the medium. When this occurred no method of control of urea additions was found consistently to prevent a slow-down of growth activity and a much reduced rate of assimilation of the ammonium ion produced. When this change occurred with too high an inventory of urea present, the pH and/or ammonium concentration rose beyond limits tolerated by the organism. No satisfactory procedure was found to reverse the process, although some success was achieved in delaying or minimizing the effects.

The two other laboratories with related interests in the culture of Hydrogenomonas have used different approaches in supplying the nitrogen requirements of the organism.

Bongers at RIAS first supplied nitrogen as a combination of ammonium ion and urea in approximately 1:2 molar ratio. In this combination, according to Konig and Schlegel (16) the ammonium ion inhibits the generation of urease and thus slows down the converstion of urea into ammonia. Presumably, if urea is not converted to ammonia too rapidly, the organism could assimilate both of these nitrogen compounds concurrently. With some of Bongers' cultures, urea could not be detected by analysis even though it was added as two-thirds of the nitrogen supply. It would thus be concluded that the urea can be hydrolyzed almost immediately to ammonia, and that urease generation and release to the liquid-phase nutrient solution may occur even in the presence of ammonia. We inferred from our results, as noted above, that urease was not generated and released for a considerable time, and that urea was assimilated with only a small concurrent conversion to ammonia. However, we were unable to stablize this mode consistently, whereas the cultures supplied with a urea-ammonium ion mixture remain at steady state with fairly rapid growth over periods of days. Our information on Bongers' more recent cultures does not include the nature of the nitrogen supply. It appears that the organism can be adapted to the use of ammonium ion so that it grows equally well when urea is hydrolyzed and ammonia is assimilated, as it did for us under our best conditions when converting mostly urea without the intermediate ammonia being much in evidence.

Repaske, who was a pioneer in Hydrogenomonas research in this country, has relayed two suggestions based on his recent use of large-scale batch cultures of 25-liter volume in a new fermenter. First, he has identified a magnesium deficiency as the cause of the interruption in log phase growth in batch cultures which he was attempting to carry up to high cell density. This break point is similar to the interruption we have observed repeatedly, and which we found to be a major difficulty in reaching a suitable cell density for study of steady-state operation. His

suggestion is that rapid growth can be maintained with a relatively high initial magnesium ion concentration and by supplementing with added magnesium during the phase of increasing cell density preceding steady-state operation.

Second, his suggestion is to use ammonium ion as the nitrogen source in Hydrogenomonas culture, and to hydrolyze urea before it is added to the culture. As noted above, some rapidly growing dense cultures have been observed in which hydrolysis apparently occurs spontaneously as the urea is added. With the use of m agnesium ion supplements to reach a dense culture, the isolated urea hydrolysis step may not be necessary.

Thus it appears that there is available now the information required to specify a suitable liquid medium composition and protocol for inoculating a culture, bringing it directly to a high population density through a batch growth procedure, then entering the transition into a steady-state continuous culture by diluting at an appropriate rate determined by the turbidity control.

Controls and Structural Features

Both the controls and the construction of the experimental apparatus serve as a guide to a prototype system, which could be less complex than the present unit that was designed for many quantitative measurements. The major input control to maintain steady-state rapid growth is that for the nitrogen-containing nutrient. This could be achieved by controlling pH with ammonium hydroxide additions, or with urea in a culture that would hydolyze it rapidly, as discussed above.

It appears likely that an oxygen detector and feedback control of oxygen concentration is also indispensable. Controls of the CO_2 partial pressure, and of the cell population density by dilution in response to an optical density determination are probably necessary. These would safeguard the steady state over long periods.

However, there is a possibility with further work that CO_2 requirements could be inferred from hydrogen consumption, and both gases could be added in response to a minimum pressure control. Furthermore, the active cell population and its rate of growth is a direct function of nutrient requirements, as indicated by gas and ammonium consumption. It is therefore possible that dilution rate could be tied in as a secondary factor with these controlled parameters.

It seems probable that stirring rate and power requirements could be reduced in a prototype. A high agitation rate (1725 rpm) was used in the experimental apparatus. Cooling is necessary because of mechanical heat generated by stirring. A gas-phase sampling loop could take gas from the head space past composition controls and back into a gas distributor immersed in the liquid phase below a smaller impeller, which would stir the liquid and distribute the bubbles for equilibration with the liquid, thus minimizing cooling requirements. Other features of the apparatus are reasonably close to the present conception of a prototype system, and could be considered individually in a systematic redesign of the integrated apparatus.

The major requirements of volumetric capacity and material transfer rates have now been quantitatively identified, as outlined in this report, to guide such a redesign.

Scale-up Requirements

There is no apparent scale-up problem in reaching high conversion rates for this process to accommodate the waste conversion requirements equivalent to several men or more. The volume of liquid culture required to convert continuously the CO_2 effluent from one man is about 12 liters. Two different culture vessels have been used with the same controls for experimental studies and for production of cell material. The larger unit was designed from the results

obtained with the smaller one, and operated satisfactorily with cultures of up to 35 liters. The smaller vessel used 2 to 3 liters of culture, so a 10-fold scale-up to a capacity equivalent to a 3-man load was not difficult. It is concluded that further scale-up could be accomplished according to known principles without serious problems.

Conversion Rates

Conversion rates of individual waste materials by the culture system are tied together by the proportions in which they are used as expressed in material balances, and by the specific growth rate of the organism. Growth with the attendant production of cellular material is the major if not the only conversion process. It is convenient to express growth rate as the constant k in the relationship $dC/dt = (k - D) C$, where C is the concentration of cells in the convenient unit of grams (dry weight) per liter of culture, dC/dt is the differential rate of change of concentration per hour, D is the dilution rate expressed as the ratio of the volume of diluent added per hour to the total liquid volume of the culture. Thus, in a steady-state continuous culture $k = D$ and the concentration change dC/dt is zero. In a batch culture, $D = 0$ and the fractional increase in concentration each hour is expressed by k.

In either case, the production rate of new cellular material is kC in units of grams (dry weight) per liter of active culture per hour. Bongers has been helpful in relaying to us the best results obtained for continuous cultures in a continuation of the studies described earlier (10). These latest growth parameters should be used in estimating conversion rates and attainable capacities for designing and operating a prototype apparatus: $k = 0.33\ hr^{-1}$ at $C = 6.1$ g (dry weight)/liter. These values were held in continuous culture at steady-state for periods of at

least 7 days, and contributory data will be awaited in the next formal publication. Our own experimental results of $k = 0.3\ hr^{-1}$ at C = 4 g/1 for 4 days, substantially support these values, but ours alone were insufficient to be used with confidence for process appraisal.

The elemental composition of cellular material is quite constant in active cultures, and it can be predicted on the basis of periodic analyses that the percentages of carbon and nitrogen will be about 50 ± 2 percent C and 12.5 ± 0.3 percent N. Nitrogen percentages may range more widely with poor growth, either because of nitrogen deficiency, or for other reasons with an excess of nitrogen available. If it is assumed that cellular material is the principle product with no major by-products, then the steady-state growth parameters given above indicate a simple material balance in which new cell material is formed from waste products at 0.33 x 6.1 = 2.0 g/liter-hr. This represents formation of 1.0 g carbon/liter-hr and 0.25 g nitrogen in the new cell material alone. By stoichiometric equivalence, these production rates represent conversions of urea at 12.9 g/liter-day to supply all of the nitrogen and part of the carbon, and 78.5 g of CO_2/liter-day to supply the rest of the cellular carbon.

Such calculations of conversion rates are usually combined with mean values of CO_2 output by man (1 kg per man-day) and urea excretion in urine (30 g per man-day) (17) to estimate the volume of culture required to process continuously the wastes from one man. The culture volumes so calculated are 12.7 liters per man for CO_2 and 2.3 liters per man for urea. Furthermore, 12.7 liters of culture operating to convert CO_2 produces 2.0 x 24 x 12.7 = 610 g of cellular material per man-day, which contains about 70 percent protein and is about four-fold the normal daily protein intake per man. These discrepancies in the use of a single system to process two wastes directly require consideration in planning an integrated life support system. Also the values given will be modified in closed-cycle operation

because some of the carbon and nitrogen inputs are not incorporated into the cellular material.

In accord with the system approach to examining the waste conversion processes, CO_2 input rates have been measured concurrently with measurements of cellular growth rates. During reasonably stable periods of growth at k = 0.23, covering periods of up to 9 hours, the carbon in the measured CO_2 input exceeded the carbon content of the measured new cellular material by 50 ± 5 percent. We have considered the possibility of (a) systematic errors in instruments or apparatus to cause an experimental artifact, (b) carbon inventory in solution as dissolved CO_2, $CO_3^=$, HCO_3^- in equilibrium with gas-phase CO_2, (c) organic carbon compounds, (d) other carbonaceous materials requiring specific analytical methods. No certain cause of this discrepancy has been identified. It is speculative but possible that a part of the carbon is in process of fixation and may be in the centrifuged cellular fraction, but is later removed in washing the cells after centrifuging. According to calculations based on the Henry's law constant for solubility of CO_2, the carbon inventory in solution is relatively small. For example, 6 volume-percent of gas-phase CO_2 equilibrates at 3 psig, 31 C, pH = 6.8 with about 0.1 g/liter of liquid-phase dissolved carbon. This calculation disregards the possibility of substantial concentrations of undissociated ammonium carbonate, for which no data were found in the literature.

If the carbon dioxide cycle includes a by-product in substantial proportions as indicated above but not identified, it will be necessary to reprocess the by-product to close the oxygen cycle. Should the reprocessing recover CO_2 for reuse, the required volume of the operating culture would be increased proportionately, to about 12.7 x 1.5 = 19 liters per man.

For closing the nitrogen cycle, the results have indicated that about half the available dissolved nitrogen (added as urea or ammonia) can be removed from

solution and converted by the growing cells in each pass through the culture. Discrepancies in a contemporary material balance for nitrogen are similar qualitatively to those described above for carbon. The implications are also similar. There appears to be a considerable inventory of nitrogen "in transit" that is not detected by conventional analyses for ammonia or urea in a contemporary material balance. If less than two times the production rate of cellular nitrogen is supplied to the growing culture, growth seems to be retarded. Thus the reprocessing or recycling of nitrogen must be considered, to recover that removed with the cells in the suspending medium during the harvesting step.

The conversion rates for hydrogen and oxygen are closely proportionate to the rate of CO_2 input, as supplied to maintain a constant partial pressure of CO_2 in the system. The proportions are 6:2:1 for H_2:O_2:CO_2 and are held closely during active growth, over a considerable range of partial pressure variation for any of the gases.

Quantity and Quality of Product

Assume that 1000 g of CO_2 are to be converted as the waste input to the process per man-day. Assume also that all the carbon input is to be converted into cellular carbon as a part of the over-all system for reducing and eventually releasing the contained oxygen by electrolysis of water. Then the quality of cellular material is fixed by the composition of the cells--particularly the fact that they are 50 percent carbon and 12.5 percent nitrogen. About 5 percent of the contained carbon is assimilated from urea, and the remaining 45 percent from CO_2 which fixes cell production at about 610 g per man-day.

A definite measure of the quality of the product has not been possible during the culture studies and the subsequent production phase for preparation and recovery

of pound quantities of cellular material. It has been assumed from empirical experience with a number of bacterial systems that cellular material produced during periods of active growth of the organism in the range $k = 0.20$ to $0.35\ hr^{-1}$ would be representative of the average composition available from a steady-state continuous culture.

In order to supply such material of representative quality for composition and nutrition studies, it is necessary to separate the cells from their suspension in the nutrient medium and wash them three times to remove the components of the medium and possibly adsorbed materials. The separation of cells from suspension in a liquid phase has only been possible so far by means of a centrifuge operating with an applied force of about 60,000 g. This is the normal process for preparation of small analytical samples to assay the dry-weight concentration of cells in a culture. The same procedure of four successive centrifugal separations interspersed with resuspension in pure water for washing becomes time-consuming and expensive on a larger scale because of limitations placed on the physical size and capacity of the separation vessels by the high centrifugal force that must be applied. Thus the cost of separation and the quality standards for the recovered and washed cellular material must represent a major consideration in an engineering appraisal of the competitive status of this waste conversion system.

CONCLUSIONS AND RECOMMENDATIONS

1. Cultures of Hydrogenomonas eutropha are confirmed as a stable process operating at room temperature for waste conversion, to consume the metabolic wastes, CO_2 and urea, and the hydrogen by-product from water electrolysis.

2. Environmental measurements with feedback controls can balance consumption of these wastes by individual make-ups at the same rates as consumption.

In this manner, a stable culture with constant conversion rates can be maintained in steady-state growth for long periods, which is suitable for consideration as a continuous waste conversion process.

3. The growth rate indicated by careful experimental measurements is a fractional increase of 0.33 per hour of the weight of bacterial cells in the culture, which is harvested continuously from a cellular culture maintained at a constant concentration of 6.1 g (dry weight) per liter of liquid culture. These values can be used for calculating the maximum production rate of cellular material in evaluating potential practical applications.

4. Calculations of applicable conversion rates for the waste CO_2 and urea-nitrogen should allow for recycle of about one-third of the carbon added as CO_2 and about one-half of the nitrogen added as urea. This is based on higher intake rates (in these proportions) by the system, to maintain a constant concentration, than are detectable as carbon and nitrogen in resulting cellular product. The by-products of carbon and nitrogen compounds that are removed with the cells in the continuous harvest have not been identified. It is recommended that future studies might seek to identify major by-products or other reasons for imperfect carbon and nitrogen material balances.

5. This investigation has pointed out one deficiency of the system approach used for evaluating interacting system variables. This approach does not easily identify responses to a changing environment that involve a substantial time period between input and response, when other interacting responses to the same change are out of phase. The remedy is to introduce an additional measurement, if possible, that anticipates the lagging response. This comment refers to endogenous cell processes, such as urease release, that follow by about one generation time a concurrent increase in urea and decrease in ammonia in the external environment. The remedy in this case might have been a measurement of urease concentration,

used to control the rate of urea addition. Other possible procedures would be adapting the cell processes themselves to the external environment.

6. The major remaining engineering problems are development of a more efficient process for separating cells from the medium and from wash water, so that a cellular material may be recovered more easily, and so that it may be suitable for a useful application, such as a protein supplement in nutrient.

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